PRODUCTION OF LYSINE BY LACTOBACILLI OR

ASPERGILLUS FICUUM

A Thesis

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By

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Dedication

I dedicate this Thesis to my mother Đurđica and my brother Dubravko whose love and generosity have followed me at every step of this journey.

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GENERAL ABSTRACT

In the animal feed industries, there is a global need for adding certain nutritional ingredients to augment deficits usually associated with plant-based materials. As a result, the industrial practices require direct addition of ingredients such as amino acids and vitamins. One of the key ingredients in this context is lysine. Alternately, the same goal can be achieved indirectly through *in situ* co-culturing of microorgan-isms. The focus of this thesis was genetic improvement of bacterial and /or fungal mutants, which could over-produce lysine. The accumulation of free lysine during microbial growth serves this end based on de-regulation of the lysine biosynthetic pathway. Microorganisms used in this thesis were nine species of lactobacilli and *Aspergillus ficuum.* Having in mind the highly complex nutritional requirements of lacto-bacilli, the assessment of possible lysine auxotrophy was performed. No lysine auxotrophs were found and the choice of *Lactobacillus plantarum* as the working species among nine others was based on its higher growth rate in minimal medium. Selection of mutants that overproduced lysine was carried out in the minimal medium supplemented with the following lysine analogs: *S*-aminoethyl-L-cysteine (AEC), DL-aspartic acid-β-hydroxamate (DL-ASP), β -fluoropyruvic-acid (FPA), L-lysine hydroxamate (LHX) and diaminopimelic acid (DAP). In *L. plantarum*, LHX was shown to be the most potent inhibitor; although, the bacterium demonstrated high resistance to all the analogs tested. The inhibition by LHX was obtained

only after significant alteration of the minimal medium M3. Furthermore, the mutant # 34, resistant to 2 mM of LHX, secreted only 4.52 μM of lysine in M3. To address the question of low lysine yield obtained by *L. plantarum*, thorough study of the regulation of aspartokinase (AK) was performed. It was found that AK exists as four isozymes, threonine sensitive, methionine sensitive and two lysine sensitive isozymes. Activity differed with respect to the growth stage of *L. plantarum*. Beside lysine, threonine and methionine have influenced the repression of AK isozymes, which suggested that effective lysine over-production could be obtained only if AK is simultaneously resistant to threonine and methionine analogs. In the case of *A. ficuum*, mutant #5-10 secreted 29.25 μM of lysine in the minimal medium, which was approximately 30 % higher than that of the wild type. DL-ASP was found as the most potent inhibitor only after the conidia were soaked for 8 h in 0.03 % Tween 80. Ammonium phosphate as a nitrogen source enhanced lysine secretion in *A. ficuum* compared to five other nitrogen sources tested.

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Finally, most of all I am thankful to my family – my mother, brother and sister-in-law. I thank my sister-in-law for her assistance with English and simply for being a good sister-in-law. I am truly thankful to my brother for his help with the computer work and graphs. I especially

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ABBREVIATIONS

AAA	aminoadipic acid pathway
AEC	*S*-aminoethyl-L-cysteine
AEC^R	mutants resistant to AEC
AK	aspartokinase
DAP	diaminopimelic acid pathway
DL-ASP	D, L- aspartic acid- β-hydroxamate
$DL\text{-}ASP^R$	mutants resistant to DL-ASP
DL-DAP	D, L- diaminopimelic acid
DTT	dithiothreitol
FPA	β-fluoropyruvic acid
GI	gastrointestinal tract
GRAS	generally regarded as safe
HSV	herpes simplex virus
IS	insertion sequence
LAB	lactic acid bacteria
LAM	lysine assay medium
LHX	L-lysine- β-hydroxamate
LHX^R	mutants resistant to LHX
MIC	minimal inhibition concentration
MMA	minimal medium for Aspergilli
MRS	deMan, Rogosa and Sharpe medium
PDA	potato dextrose agar
SMMA	minimal medium for sporulation of Aspergilli
TEMED	tetramethyl-ethylenediamine
TCA	tricarboxylic acid cycle
WT	wild type

1. INTRODUCTION

Lysine, due to inability of humans and animals to synthesize it, is designated as an essential amino acid. It must be obtained through the diet. However, lysine is known as a first limiting amino acid in feed. Importance of lysine-rich diet is connected with its biological role during animal growth and development, especially in young animals. Most crops are low in lysine content, which decrease their performance as a feed and ultimately influence their price. Currently, crops are enriched with lysine by adding the crystalline lysine, exclusively produced by fermentation with *Corynebacterium glutamicum*. This thesis postulates that microbial biotransformation can add higher lysine content and can contribute to better overall characteristics of the meal. Due to the applied aspect of this thesis, it was of high importance to use microorganisms with the GRAS (Generally Regarded As Safe) status. The choice of microorganisms used here was not accidental; lactobacilli have a reputation as 'friendly' bacteria that contribute to the nutritional value of feed and food. *Aspergillus ficuum* has a commercial value as source of phytase that is important for decreasing the phytic acid content, the undesirable component of canola meal. The reasoning behind employing both prokaryote and eukaryote was that lysine biosynthesis (and regulation) in prokaryote and eukaryote occurs via two distinct pathways that share no commonalities. While prokaryotes synthesize lysine in a complex pathway with a complex regulation, they are reasonably fast acting and easy to manipulate. Eukaryotes, on the other hand, synthesize lysine in a simple pathway with simple regulation, but they are more complex and slow acting in comparison to prokaryotes.

The approach of obtaining the spontaneous mutants that would rather be considered as a variety of the strains was chosen. The genetically engineering approach, although more promising in terms of high yields, was not suitable due to the negative connotations it brings to the public. It is worth mentioning that, prior to this thesis, the basic parameters of culturing lactobacilli and aspergilli in canola meal have been established in this laboratory. It was found that canola meal supports the growth of lactobacilli and aspergilli in a pure culture as well as in co-culture. Solid-state fermentation parameters were established. This thesis was a continuation in the sense that it provided the answer about lactobacilli/fungi lysine over-producers that can be used in solid-state fermentation of canola meal.

Here is shown that we can obtain lactobacilli (*Lactobacillus plantarum*) and Aspergilli (*Aspergillus ficuum*) lysine over-producers using spontaneous mutation and selection based on resistance to the lysine analogs. Some successes have been reported with members of lactobacilli genera lysine over-producers; yet, considerably few. The big challenges met in this thesis were the complex nutritional requirements and remarkable adaptive abilities of lactobacilli that led to the resistance to the lysine analogs. From the practical and applied aspect, this was an obstacle for obtaining high yielding lysine over-producers. However, from the scientific perspective, this was certainly intriguing enough to pursue the study of nutritional requirements, the regulation of aspartokinase and possible connection of the two. Use of *Aspergillus ficuum* was shown to be a more favorable system for lysine-overproduction than lactobacilli. One can speculate that this is due to the simplicity of the lysine biosynthetic pathway and its regulation in eukaryotes as opposed to prokaryotes. Surprisingly, there are no precedents in literature on this subject. Finally, this thesis provides some insights related to

basic physiology of lactobacilli as well as possible employment of *L. plantarum* and *A. ficuum* as a lysine over-producers.

1.1 Objective and hypothesis

The objective of this thesis was to obtain regulatory mutants of lactobacilli and/or *Aspergillus* that over-produce lysine. These mutants can be used for the enrichment of lysine content in feed. The longer-term focus of our laboratory's research is enhancing lysine content of canola meal or canola plants. However, lysine is the first limiting amino acid in most crops, which undoubtedly gives broader scope of practical application of this thesis beyond canola.

The main hypothesis was that the bacterial and/or fungal mutants that overproduce lysine could be obtained. In order to achieve this, study of nutritional requirements of lactobacilli and study on regulation of lysine biosynthesis in *L. plantarum* were performed.

2. LITERATURE REVIEW

2.1. Lysine: History, chemistry and biological activity

Lysine is first mentioned in late 19th century by the German chemist Drechsel (Greenstein 1961). Lysine was firstly obtained from casein, but Drechsel, assuming that the compound is urea obtained from unknown source, gave the name 'lysitine' (greek word for loosing). A few years later was established that 'lysitine' is a mixture of lysine and arginine. In order to distinguish pure compounds obtained in the mixture, the name 'lysine' was given by Drechsel in 1891 to diaminocsproic acid, the next analog to ornithine.

Lysine (Lys, K) is a basic and branched amino acid with six carbons and two amino groups on terminal ends as shown on Figure 2.1.

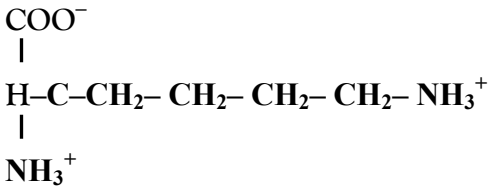

Figure 2.1. Chemical structure of lysine (Voet and Voet 1990).

The systematic name for lysine is (S)-2, 6-diaminohexanoic acid with no tautomers known and brutto formula $C_6H_{14}N_2O_2$. Low pK_a value of the carboxylic group (2.16) and high pK_a of the ε amino group (10.79) suggests that its primary amine (4-aminobutyl) functional group is protonated at physiological pH (≈ 7.4) allowing it to act as a donor in hydrogen bonding and/or as a general base in catalysis. Lysine is a charged, polar amino acid and its aqueous solution never assumes neutral charge. Its asymmetrical structure suggests optical activity; lysine has two enantiomers: leve (L, left) and dexter (D, right). Note that only leve form (L-lysine) is biologically active, i.e. for protein synthesis. Lysine is typically involved in the following reactions: amine acylation, the ninhydrin reaction, carboxylic acid esterification, and specific oxidation. One can emphasize ninhydrin reaction and amine acetylation as an important feature from the prospective of this thesis and the biological function of lysine. The ninhydrin reaction is a basis for the majority of chemical assays used for determination of lysine; the chemical assay used in this thesis is one of them (Vogel and Shimura 1971). Acetylation of the lysine amino groups is chemically analogous to the acetylation of the N-terminus. Functionally, the acetylation of lysine residues is used to regulate the binding of proteins to nucleic acids. The cancellation of the positive charge on the lysine weakens the electrostatic attraction for the (negatively charged) nucleic acids (Voet and Voet 1990).

According to the chemical properties, the charge and basic character of a side chain (or changes on it) are responsible for the biological activity of lysine. An important role of lysine plays in metabolism is in collagen formation. It has been known that collagen biosynthesis involves post-translational modifications of the initial polypeptide chain. Intracellular modification consists of hydroxylation of lysine and proline residues followed by glycosylation

of hydroxylysine residues to galactohydroxylysine, chain association, disulphide bonding and formation of the triple helix (Oikarinen et al. 1976). Procollagen-lysine-5-dioxygenase (lysyl hydroxylase) catalyzes hydroxylation of lysine to hydroxylysine and it is the key enzyme in collagen formation requiring vitamin C as a cofactor. Having influence on collagen formation, lysine, proline and vitamin C indirectly affect function of various tissues, and therefore, lack of these ingredients in the diet can cause serious damage and dysfunction in metabolism (Saha et al. 2005).

Apart from its role in collagen formation, much of the research on the role of lysine has been directed to its effect in the treatment of herpes simplex virus (HSV). Having an essential role in HSV replication, it was found that arginine promotes viral infection (Inglis 1968) and biosynthesis of ICP 8, a DNA-binding protein in HSV (Ruyechan and Olson 1992). The biochemical basis for successful treatment of HSV I and II with lysine is that the similar structures of lysine and arginine make them each other's antagonists. Indeed, lysine inhibits the replication of HSV (Loh and Oie 1969). To date, numerous reports have shown that lysine positively affects treatment of HSV I and II and therefore, diet enriched in lysine is highly recommended. However, high concentration of lysine is effective only if concentration of arginine is low in the medium (Maggs et al. 2000) implying that successful treatment (and prevention) against HSV depends on lysine–arginine ratio rather than on solely lysine rich diet.

Finally, the most general biological activity of lysine is its incorporation into proteins that serve as hormone, enzymes and antibodies in higher animals and humans.

22. Biosynthesis of lysine and its regulation

Lysine biosynthesis occurs in bacteria, fungi, algae and higher plants. Higher animals and humans have lost the ability to synthesize lysine. However, the biosynthetic pathways for lysine synthesis in the above mentioned species differ. It is worth mentioning that biosynthesis of other amino acids occurs via the same pathways in all species, making lysine biosynthesis rather unique. Lysine biosynthesis in microorganisms and plants occur via two distinct pathways: the diaminopimelic (DAP) pathway and α-aminoadipic acid (AAA) pathway (Vogel 1964). It was firstly suggested that the consistency of their distribution over a broad range of biological species implied that this dichotomy was probably not the result of a gene transfer between the species. Since that diaminopimelic acid is a bacterial cell wall constituent, most bacteria synthesize lysine via DAP. On the other hand, fungi that typically contain chitin in their cell wall, synthesize lysine via AAA. The simple and straight forward rule that is applied here does not explain what was the basis for lysine biosynthesis via DAP in plants suggesting that this dichotomy is more complex. It is known that both pathways show variations to some extent depending on the species, but simultaneous presence of both pathways in any of these species has never been reported.

2.2.1. Diaminopimelic (DAP) pathway

The DAP pathway is characteristic of bacteria, algae, Oomycetes, Myxomycetes, Hyphochytrids and higher plants. It is a branched pathway where lysine has been synthesized along with threonine, methionine and isoleucine, which are designated as aspartate family amino acids.

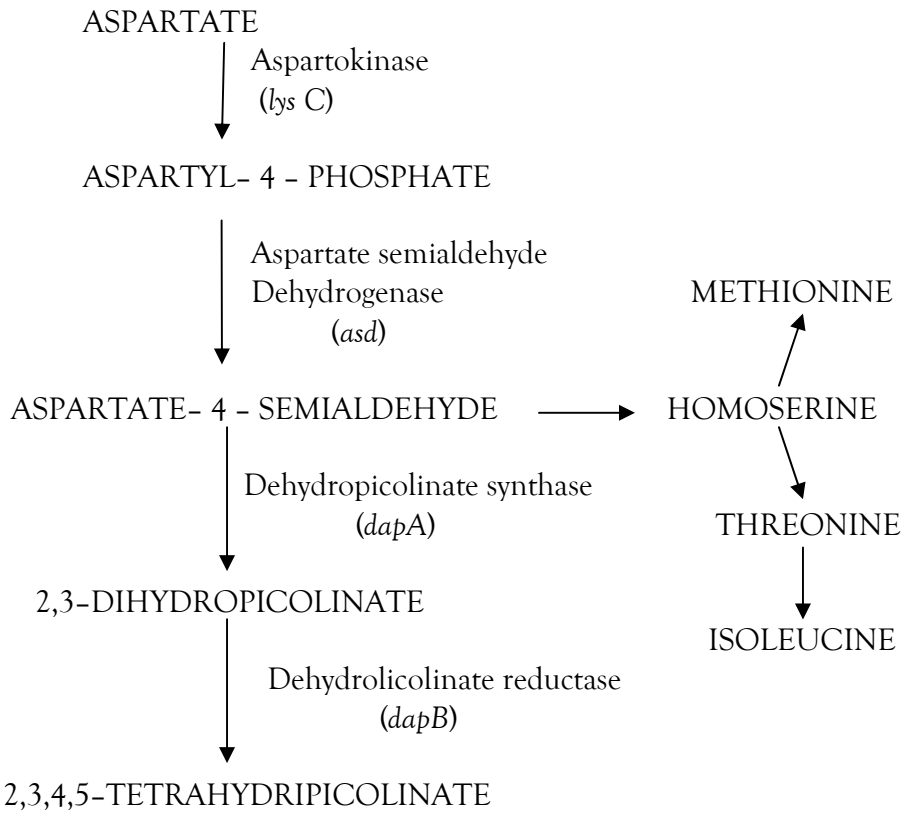

Figure 2.2. The first four steps in DAP biosynthetic pathway. These steps are common in all bacteria, plants, algae, Oomycetes, Myxomycetes and Hyphochytrids (Caspi et al. 2008). Enzymes and corresponding genes are designated as in *Corynebacterium glutamicum*.

Diaminopimelic acid is the direct precursor of lysine both of which are synthesized in the branch that solely leads to lysine. Due to the industrial importance of lysine, DAP has been studied in detailed. It has been found that there are four variations of DAP known. It seems that most species share biosynthesis from aspartate to tetrahydropicolinate (Figure 2.2) and the last step of biosynthesis, but not the steps between tetrahydropicolinate and *meso*–diaminopimelate (Figures 2.3-2.5).

Lysine biosynthesis I is most commonly found in bacteria (Figure 2.3). It is characteristic for *E. coli*, some species of *Atcinomyces, Bifidobacterium, Chlamydia, Chlostridia, Deinococcus, Gluconobacter, Helicobacter, Streptococcus, Zymomonas, Salmonella, Shigela, Mycobacter* etc. Lysine biosynthesis II (Figure 2.4) is only reported in some species of *Bacillus, Staphylococcus, Streptococcus* and *Chlostridia.* The lysine biosynthesis III and VI (Figure 2.5) are not as complex as former variants because lysine is synthesized in three steps instead of five. In the dihydrogenase variant L–α–amino–ε–keto pimelate is obtained spontaneously from 2,3,4,5–tetrahydropicolinate. This pathway is used by only few bacterial strains: *Coynebacterium glutamicum* ATCC 13032, *Bacteroides thetaiotamicum* VPI-5482 and *Actinomyces neaslundi* MG1(Caspi et al. 2008). Note that in *C. glutamicum* both lysine biosynthesis I and III coexist. Finally, the diaminopimelate–aminotransferase variant or lysine biosynthesis VI is reported only in plants probably because that plants alone possess L, L–diaminopimelate–aminotransferase.

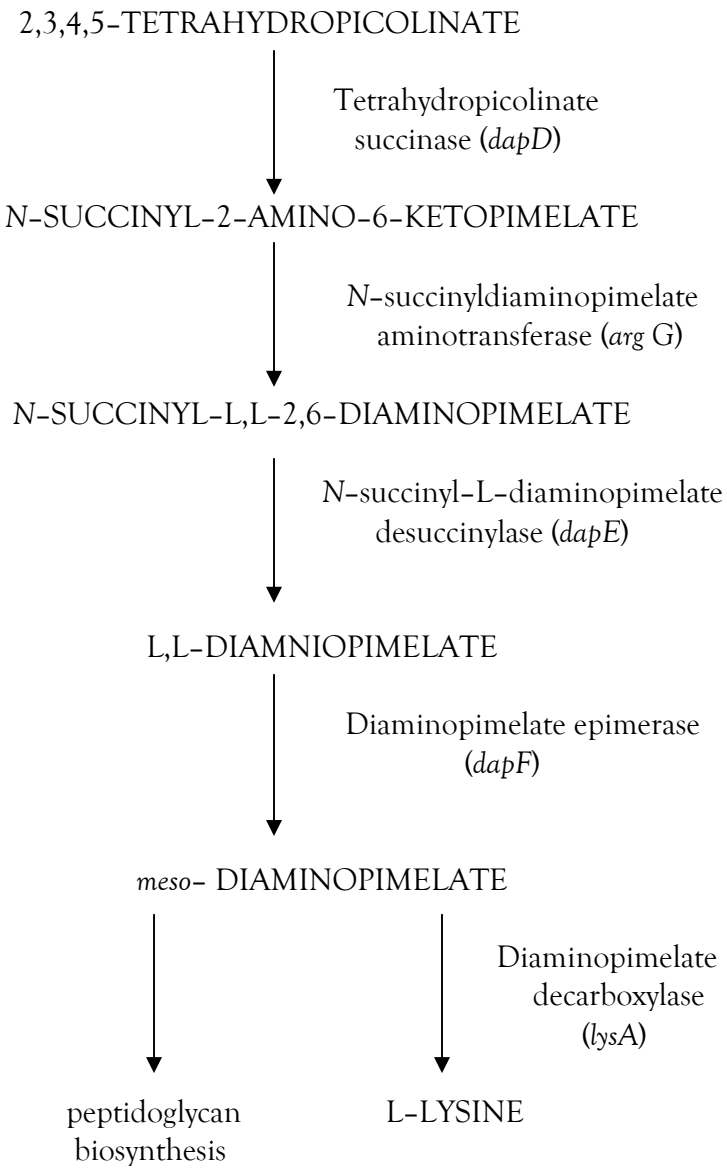

Figure 2.3. Lysine biosynthesis I, the succinylase variant. Enzymes with corresponding genes are shown in brackets according to *Escherichia coli* K 12 (Caspi et al. 2008).

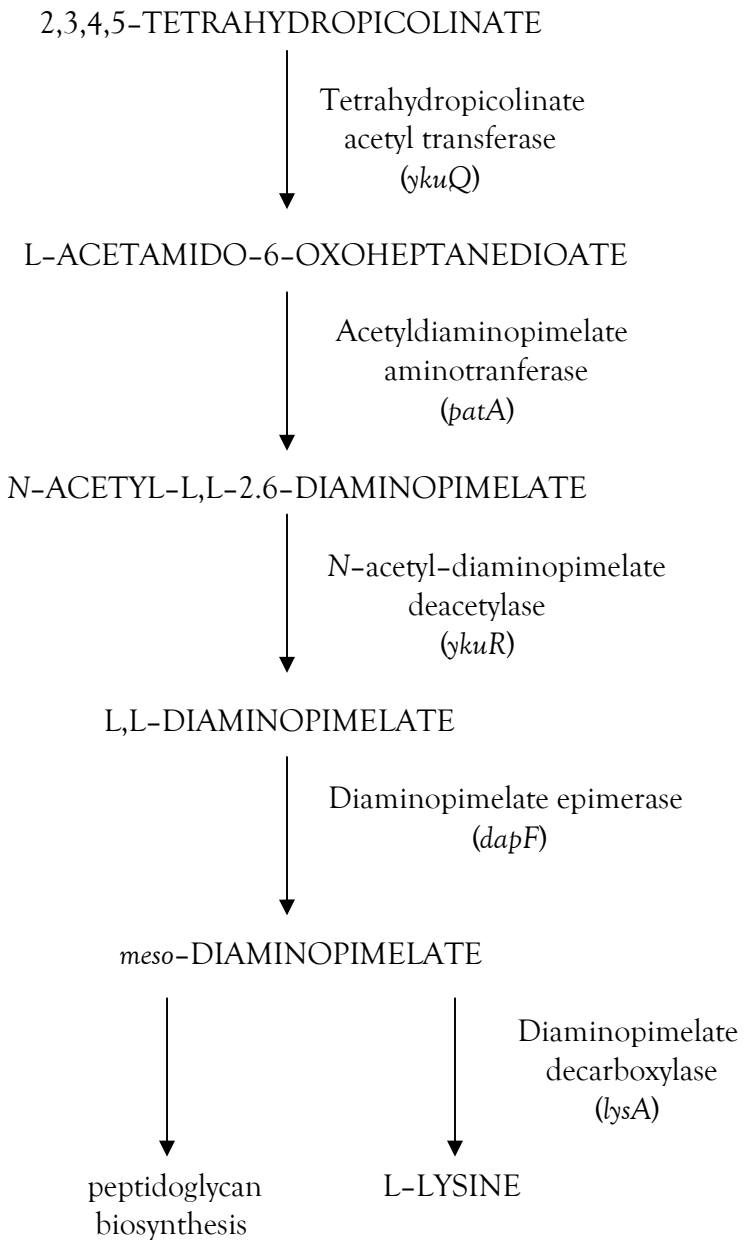

Figure 2.4. Lysine biosynthesis II, the acetylase variant. The enzymes with corresponding genes are shown in brackets according to *Bacillus subtilis* (Caspi et al. 2008).

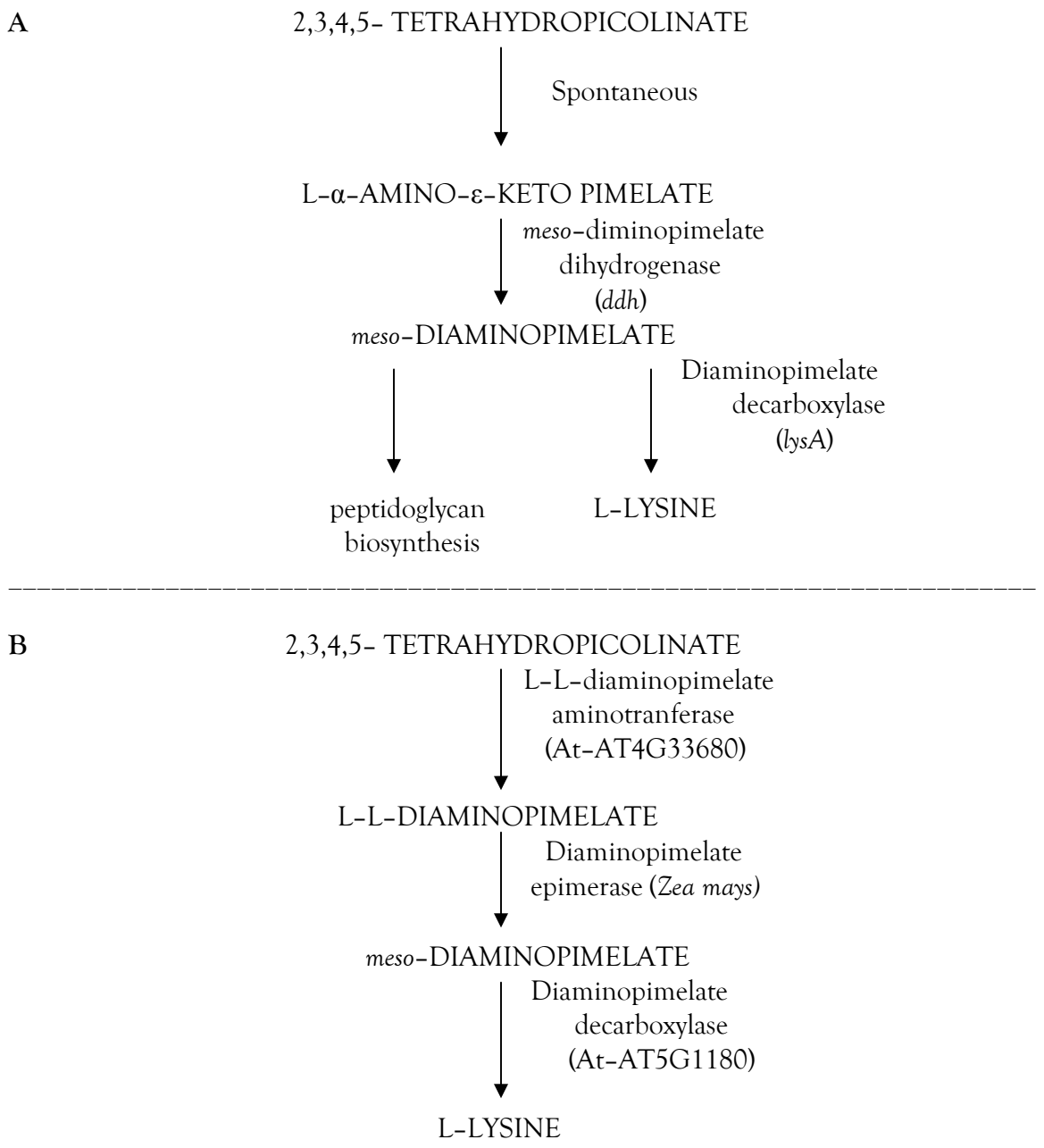

Figure 2.5. Lysine biosynthesis III, the dehydrogense variant and biosynthesis VI, the diaminopimelate–aminotransferase variant. The enzymes with corresponding genes on panel (A), lysine biosynthesis III, are shown according to *Corynebacterium glutamicum*, while enzymes and genes (in brackets) on panel (B), biosynthesis VI, are shown according to *Arabidopsis thaliana* (Caspi et al. 2008).

According to the genome sequence information that is provided for several species of lactobacilli, *Lactobacillus acidophilus* NCFM, *Lactobacillus plantarum* WCFS1, *Lactobacillus sakei* 23K, *Lactobacillus salivarius* UCC118 and *Lactococcus lactis* II 1403 have the potential to synthesize lysine via the succinylase variant (Figure 2.3). The pathway is complete only in *L. plantarum* WCFS1 (Caspi et al. 2008). The degree of pathway degeneration in other species differs from missing two genes in *L. salivarius*, *L. acidophilus* and *L. lactis* to missing six genes as in *L. sakei* 23K. On the other hand are *L. delbrueckii* ATCC 11842, having only the first two genes of DAP pathway, and *L. johnsonii* NCC 533 in which the pathway is completely absent. Interestingly, among the lactobacilli sequenced, *L. lactis* II 1403 alone has the spontaneous reaction from 2,3,4,5–tetrahydropicolinate to L–α–amino–ε–keto pimelate similar to *C. glutamicum* (Figure 2.5.A). On the other hand, *meso*–diaminopimelate dehydrogenase is not found in *L. lactis*. *Lactococcus lactis* seems to be the only species that has the potential to synthesize lysine via simple biosynthesis III and whose genome is sequenced (Bolotin et al. 2001).

As mentioned above, the DAP pathway is shared by lysine, threonine, methionine and isoleucine. Similar to lysine biosynthesis, the biosynthesis of the rest of aspartate family amino acids of amino acids has several variations known for each amino acid, but due to focus of this thesis being on lysine biosynthesis, they will not be discussed here.

The DAP pathway is typically regulated by feed-back inhibition, where products inhibit and/or repress the key enzyme in the pathway. In this case, aspartokinase is subjected to multiple feed-back inhibition and/or repression. Due to complexity of the pathway, aspartokinase usually exists in the form of isozymes, which are inhibited and/or repressed by

lysine, threonine, methionine or subjected to concerted inhibition of two or more aspartate family amino acids. The number of isozymes and manner of inhibition/repression vary among the species and it will be discussed in more detail in Chapter 4. Isoleucine seldom has influence on aspartokinase, in spite of the fact that it is the final product of the biosynthesis; it rather influences threonine deaminase, which is the first step in the branch of the pathway that leads to isoleucine solely.

It is known that lysine biosynthesis genes are scattered along the chromosome in *E. coli*, enterobacteria, members of Pasteureallacea and Vibrionaceae and *Shewanella oneidensis*. In contrast, in Gram-positive bacteria belonging to the *Bacillus/Clostridium* group, lysine biosynthesis genes were found within clusters potentially forming an operon (Rodinov et al. 2003). Moreover, the authors, using comparative genomics, hypothesized that lysine biosynthesis and transport is regulated by lysine-specific riboswitch, similar to purine and methionine-specific riboswitches (McDaniel et al. 2003, Mandal et al. 2003). This highly conserved metabolite-binding RNA domain designated as *LYS* element predominantly regulates either single *lys*C (encoding aspartokinase) or *lys*A (encoding diaminopimelate decarboxylase) genes or a composite lysine operon. Therefore, over-expression of these genes is directly due to mutation(s) in *LYS* element. Grundy et al. (2003) have confirmed the hypothesis showing that in *B. subtilis* lysine directly promotes transcription termination causing structural shift in the *LYS* element of *lys*C. In contrast, *LYS* element in Gram-negative bacteria appeared to be regulated at the level of translation initiation rather then transcription termination. However, there are no reports on regulation of aspartokinase on a molecular level in lactobacilli.

2.2.2. Aminoadipic acid (AAA) pathway

AAA is found in prokaryotes and eukaryotes that do not contain diaminopimelic acid in the cell wall. It occurs in yeast and certain fungi. Similarly, the first six steps of the DAP pathway, from 2–ketoglutarate to α–aminoadipate, are common for prokaryotes and eukaryotes that use the AAA pathway for lysine biosynthesis (Figure 2.6). Lysine biosynthesis IV was typically found (and studied the most) in yeast and filamentous fungi. However, in filamentous fungi that produce penicillin, this pathway is branched at the step of α–aminoadipate synthesis leading to lysine and penicillin (Figure 2.7). While penicillin biosynthesis and regulation was studied for decades, lysine biosynthesis in filamentous fungi is somewhat neglected as enzymes and corresponding genes were not characterized.

Actinomycetes are interesting microorganisms with respect to lysine biosynthesis. These bacteria synthesize penicillin and therefore require both diaminopimelic acid for cell wall synthesis and α–aminoadipate as a precursor of β-lactam ring. Actinomycetes synthesize lysine (and diaminopimelic acid) via succinylase variant (Figure 2.3), but unlike fungi, actinomycetes convert L–lysine by lysine–6–aminotranferase to piperidine–6–carboxylate. Piperidine–6–carboxylate is oxidized to AAA, which is the direct precursor of cephalosporin C (Hernándo-Rico et al. 2001).

The branching point in lysine and penicillin biosynthesis in fungi and Actinomycetes has consequence which impact lysine and penicillin inter-regulation.

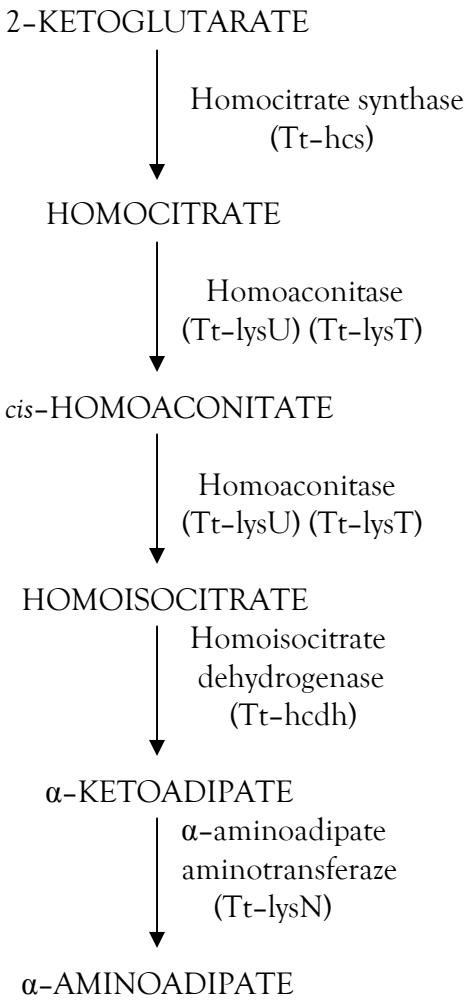

Figure 2.6. The first six steps in AAA pathway common for prokaryotes, fungi and yeasts. Enzymes and genes are listed as per *Thermus thermophilus* (Nishida et al. 1999).

While in fungi lysine and penicillin are synthesized in a branched pathway where lysine inhibits penicillin biosynthesis and vice versa (Brakhage and Turner 1992), in actinomycets lysine and penicillin are synthesized in a linear pathway where lysine seems to promote cephalosporin C biosynthesis (Leitão et al. 2001).

Lysine biosynthesis V (Figure 2.8) is found in *Deinococcus radiodurans* as well as in several archea, namely *Pyrococcus abyssi, Pyrococcus horikashii, Sulfolobus solfataricus, Sulfolobus tokadaii* and *Aeropyrum pernix* (Nishida et al 1999). This variation is somewhat similar to arginine biosynthesis in bacteria and it is characteristic for prokaryotes and some archea.

The fact that the prokaryote *Thermus thremophilus* synthesize lysine via AAA was significant in terms of understanding the lysine biosynthesis evolution and dichotomy. It was firstly believed that DAP pathway preceded AAA, due to the fact that DAP is characteristic for prokaryotes (Vogel and Shimura 1964). However, enzymes of *T. thermophilus* showed broad range of substrate specificity; this bacterium with optimal growth temperature of 100 °C populates habitats that most likely were the environment of early forms of life (Nishida et al. 1999). This gives enough of indication that in fact AAA preceded DAP and DAP evolved later, when was need for peptidoglycan cell wall.

Similar to the DAP; the AAA pathway is regulated in *S. cerevisiae* by feed-back inhibition of the Homocitrate synthase. However, there is no firm evidence that lysine biosynthesis in fungi and yeast has another riboswitch. The diversity that microorganisms and higher plants exert in lysine biosynthesis speaks for the biological importance of this pathway.

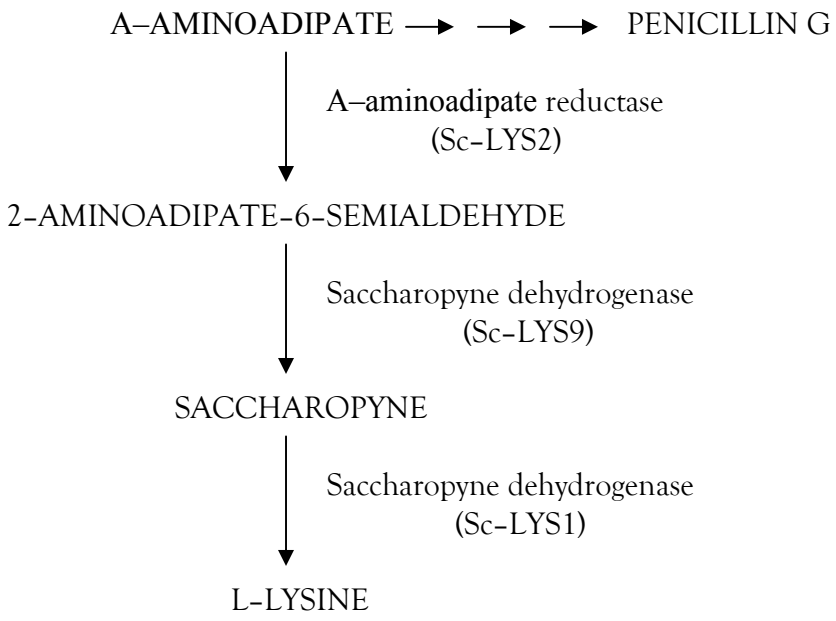

Figure 2.7. Lysine biosynthesis, variant IV. The enzymes and genes are shown as in *Saccharomyces cerevisiae*. The branch leading from α–aminoadipate to penicillin G is characteristic for filamentous fungi (Caspi et al. 2008).

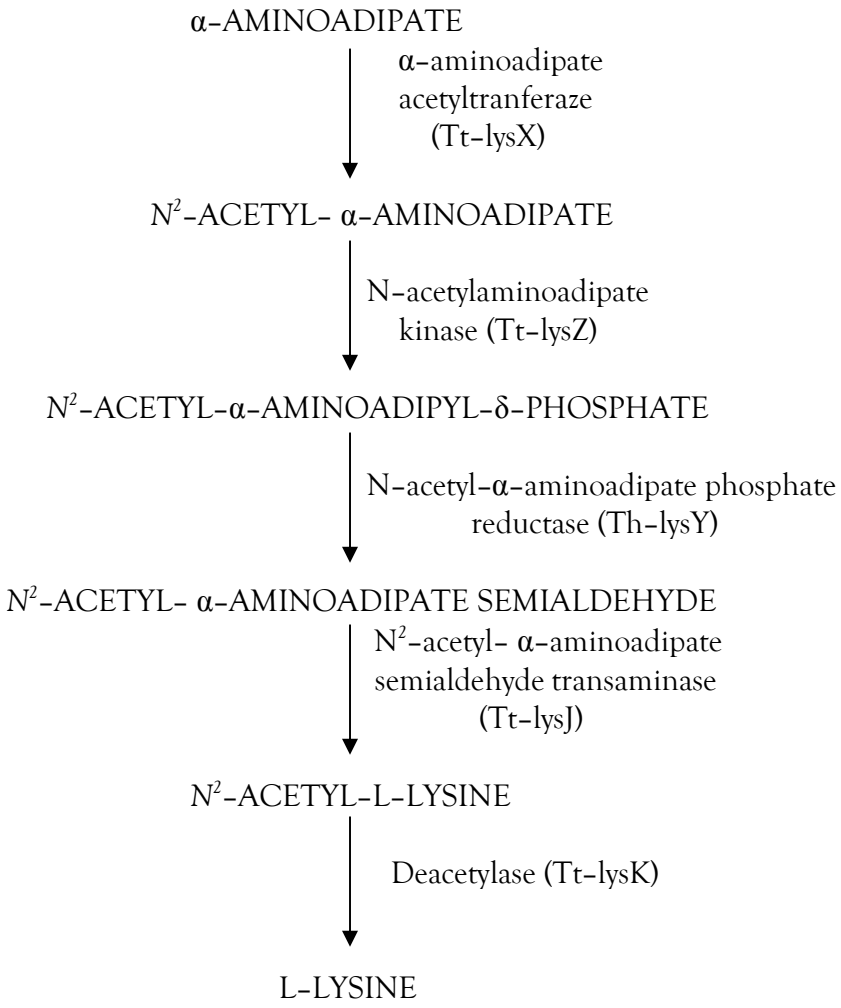

Figure 2.8. Lysine biosynthesis variant V. Enzymes and corresponding genes are listed as in *Thermus thermophilus* (Nishida et al. 1999).

Lysine is not only important for amino acid(s) synthesis, but it is strongly connected to industrially and medically important secondary metabolite such as antibiotics. These attributes undoubtedly place lysine biosynthesis and its regulation as a top priority of scientific research, from decades ago until today.

2.3. Availability of lysine in feed and food

Inability of higher animals and humans to synthesize lysine makes it one of the ten essential amino acids. The only way to obtain lysine (and other essential amino acids) is through a proper diet. However, there have been recognized two major problems associated with lysine supplementation of food and feed (i) lysine is the first limiting amino acids in most cereal crops and (ii) its bioavailability. Both of these problems were addressed many times using different approaches and ideas. The question of quantity of lysine in feed and food can be addressed either by increasing the content of proteins rich in lysine, increasing of free amino acid pool in plants that are used for feed and food or by fermentation with microorganisms that have increased lysine content. The latter is the approach taken by this thesis.

Bioavailability of amino acids is defined as the fraction of total dietary amino acids that can be utilized for protein synthesis by the various tissues by the animals (D'Mello 2003). Low bioavailability of lysine is connected to its thermal instability. Heat processing of feed leads to significant loss of lysine. Further, moist-heat processing of cereal crops and feeds favors Maillard reactions and lysine is commonly involved in it by reacting with the reducing sugars and forming complexes that cannot be digested or absorbed by animals (Larsen et al. 2002). It

is worth mentioning that only protein-bound lysine has low bioavailability, while crystalline lysine is considered 100 % available.

2.3.1. Lysine in animal feed

It is clear that the amounts of lysine needed in animal diets depend highly on animal growth stage and the expected performance. However, there is a remarkable consistency across species in the amino acids composition in mixed body protein from fetal, growing and mature animals (Bequette 2003). This suggests that the minimal qualitative requirement for essential amino acids for growth will be similar. Following this fact, three types of amino acids were recognized. One that could not be synthesized and is needed entirely for growth (lysine). One is needed for maintenance and for growth (tryptophan). The last one could be synthesized and is not limiting (glycine). Table 2.1 and Table 2.2 show that lysine and tryptophan are the first limiting amino acids while, threonine and methionine are listed as the second and third limiting amino acids in pigs. As mentioned above, lysine is much needed for the growth of monogastric animals as well as ruminants. Important protein supplements in feed such as corn, soybean, safflower, fish, meat and cotton seed meal, dried brewers yeast and skim milk contain different amounts of lysine, which makes them more or less suitable as protein supplements. Among these, fish meal is the richest in lysine containing 6.2 % and corn is the poorest containing 0.9 % based on the percentage in crude protein. All other protein sources fall in between 1-3 % based on crude protein percent (Kellems and Church 2002). However, well-balanced feed formulation is not based solely on high lysine content, but is certainly an important parameter under consideration.

Table 2.1. Limiting amino acids in cereal grains for pigs.

Cereal grain	Limiting amino acids		
	First	Second	Third
Barley	Lysine	Threonine	Histidine
Corn	Lysine and Tryptophan	Threonine	–
Oats	Lysine	–	–
Sorghum	Lysine	Threonine	Tryptophan
Triticale	Lysine	Threonine	–
Wheat	Lysine	Threonine	–

Adapted from Kopinski (2008).

Table 2.2. Limiting amino acid in protein supplements for pigs.

Protein	First Limiting Amino Acid	Protein	First Limiting Amino Acid
Corn gluten	Lysine	Sunflower	Lysine
Cottonseed	Lysine	Canola	Lysine
Linseed	Lysine	Soybean	Methionine
Peanut	Lysine	Meat Meal	Tryptophan
Safflower	Lysine	Blood Meal	Isoleucine
Sesame	Lysine	Legumes	Methionine, Cystine

Adapted from Kopinski (2008).

2.3.2. Lysine in food

Considering that lysine metabolism is the same in humans and higher animals, it is expected that its function and benefits in humans are identical to those in animals. Therefore, infants (3-6 months) require high amounts of lysine, 97 mg/kg of body weight compared to children (10-12 year) who require 44 mg/kg of body weight, due to its role in collagen formation and consequently, growth. Adults, on the other hand, require approximately 12 mg/kg of body weight.

Human diet seems more versatile than animal diet, consisting of fruits, vegetables, meats, dairy products and sweets. As shown in Table 2.3, lysine is readily found in Atlantic fish, soy and egg white, which are the richest sources of lysine. If one takes into account the food groups, lysine can be obtained through meats (especially white meat), soy and dairy products easier than through vegetables and fruits. Lysine toxicity may lead to diarrhea and abdominal cramps. However, this is not frequently reported and it is associated with uptake of extremely high doses of lysine, 15-40 g per day ((Foster 2008).

2.4. Supplementation or enhancing of lysine in food and feed

The world's lysine production was estimated at 600 000 metric tones/year (Koffas and Stephanopoulus 2005). Major producers are Ajinomoto (Japan), Archer Daniels Midland (ADM) Company (USA), Cheil-Jedang (South Korea), BASF and Degussa (Germany).

Table 2.3. Food sources rich in lysine.

Food group	Source	Lysine (mg/100g)
Fish	Cod, Atlantic	5769
Dairy and egg products	Egg (white)	5515
	Cheese (parmesan, shredded)	3844
	Egg (whole)	3402
Meats	Turkey (breast, cooked)	4432
	Pork (bacon)	3180
	Veal (shoulder, arm)	3138
	Beef (lean only, cooked)	3053
Sweets	Gelatin (unsweetened)	3460
Bakery	Yeast, active dry	3158
Vegetable	Parsley	3115
	Seaweed	3025

Data obtained from Foster (2008).

Market shows a potential of 7-8 % of growth per year; it is expected to reach the volume of $1 billion US by the year 2009 (Leuchtenberger et al. 2005). More attention has been paid to lysine enrichment in feed, due to higher lysine deficiency in feed than in food.

It is interesting that the world's lysine is produced solely by *Corynebacterium glutamicum* fermentation. Indeed, the mutants of *C. glutamicum* produce yields of 44 g/L, which means that almost 50 % of sugars are converted directly to lysine. There were attempts to introduce other sources of lysine to market, such as transgenic maize, but large-scale production is still not feasible, apart from the controversial issues associated with genetically modified crops. On a world scale, corn yield exceeds that of soybean yield, making corn the third largest produced crop, after rice and wheat. On the other hand, low lysine content imposes the question on its performance as a feed. Therefore, soybean or fish meals are frequently used as a protein supplement to corn meal. However, the low price of feed grade crystalline lysine, $1.5 U.S/kg, (Ajinomoto, Japan) still makes it the best lysine supplement (Koffas and Stephanopoulos 2005). One has to keep in mind that in meal protein, lysine undergoes Mailliard reaction, which decreases its bioavailability, while crystalline lysine has bioavailability of virtually 100 %. This strongly favors usage of crystalline lysine as opposed to soybean and fishmeal supplements or transgenic plants.

2.5. Genus Lactobacillus

The first comprehensive classification and description of lactic acid bacteria (LAB) was provided by Orla-Jensen in 1919. This early classification could have only taken into account phenotypic characters and evaluated them as physiological markers. Therefore, LAB were

classified according to morphology (rods, cocci or tetrad formation), mode of glucose fermentation (homo- or hetero-fermentative), range of sugars used for fermentation and growth at minimal and maximal temperature (10°C or 45°C). Historically, the genera *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* formed the core of the group, where the boundaries were subjected to some controversy. However, from the practical, food-technology point of view, genera *Aerococcus, Carnobacterium, Enterococcus, Weissella, Oenococcus, Vagococcus* and *Tetragenococcus* are also considered principal LAB (Axelsson 2004). Oral-Jensen's classification undoubtedly made a large impact on the systematics of LAB, which are accordingly described as Gram-positive, non-sporulating, catalase negative, non-respiring cocci or rods with lactic acids as a major product of fermentation. Yet, since the first classification, new data supporting the diversity of LAB have emerged. One of the key features in LAB taxonomy is certainly the LAB inability to synthesize the porphyrin group, such as hemes due to the absence of catalase. However, if heme is added to the medium, a true catalase or even cytochromes will be formed (Gaudu et al. 2002), which questions the description of LAB as catalase negative, non-respiring bacteria. The relatively recently described genus, *Weissella*, is the first genus in LAB that includes both rods and cocci (Collins et al. 1993) excluding morphology as a strong point in the taxonomy of LAB. *Streptococci,* unlike other lactic acid bacteria, have reasonably simple nutritional requirements where sugar fermentation in some species can result in the formation of small amounts of lactic acid under certain conditions. Lactic acid bacteria are typically designated as non-spore formers. However, *Sporolactobacilli* described by Kitahara and Suzuki (1963) share many similarities with LAB such as low G+C content, lactic acid production and tolerance of oxygen and are therefore designated as subgenus (Sanders et al. 2003). The

definition of lactic acid bacteria seems accurate only under 'normal' conditions often imposed upon them in a laboratory environment and obviously, many variations were found. Ironically, only the Gram-positive characteristic of LAB cannot be challenged.

Ever since their role in acidification of food and feed was discovered, LAB have been of interest. In recent years, as their pre- and pro-biotic role in human metabolism has been established, LAB have become not only the interest of science, but as a highly profitable industry as well. At this point, although contemporary science has met challenges regarding LAB, it seems that basic knowledge and profound understanding of the physiology of LAB is still missing. Unlike *E. coli, S. cerevisiae* and *B. subtilis,* whose metabolism was the subject of extensive studies for decades, in LAB there is a lack of the same level of information. Instead, recombinant DNA techniques and the genetic manipulation of LAB were developed. The trend for LAB in the 2000's is certainly genomic sequencing. The vast amount of information on LAB genomes provides enormous potential to understand this remarkable genus; yet it is meaningless if it is not put in a physiological context. Indeed, one can take many directions when discussing LAB. This thesis strongly focuses on their uniqueness, diversity and adaptability.

2.5.1. Habitats and evolution

It has been stated many times that LAB populate habitats rich in carbohydrates, proteins and their breakdown products, low oxygen tension and a mesophilic to slightly thermophilic temperature range. Therefore, LAB are associated with plants, milk and dairy products, humans and animals.

LAB occurring in humans and animals are most likely introduced through food and feed. LAB are only seldom present on intact plant tissue, where *Leuconostoc* accounts for 80 % of the mutants and *Lactobacilli* only 10 %. Among *Lactobacilli*, *L. plantarum* and *L. fermentum* are the predominant species. The data obtained recently for *L. plantarum* WCFS1 (Kleerebezem et al. 2003) showed that it has enormous potential for carbohydrate utilization, as genomic data revealed a possibility to utilize 25 carbohydrates, but proteolytic activity seems far less developed than that of *Lactococcus lactis* for example. This explains the occurrence of *L. plantarum* on plants to more extent than other species of LAB. On cut or bruised plant tissue, however, LAB becomes more prevalent. Occurrence of *Leuconostoc* and *L. plantarum* in plants can be extended to silage and fermented beverage and vegetables. Note that other bacterial species are also found, which depends on crops used for silage preparation or parameters applied for production of fermented beverages. Only the predominant species will be presented here. Considering the enzymatic system of LAB, where proteolytic extracellular enzymes dominate, this is not surprising. Lactobacilli are well equipped for uptake of peptides, free amino acids, vitamins and carbohydrates, but not for complete degradation of plant tissue. Therefore, plants are not considered as a prime and natural reservoir of LAB.

Milk and dairy products, due to rich amino acids and vitamins content, are undoubtedly an ideal environment for bacterial species. Therefore, lactobacilli are found in abundance in these environments that can be considered as their natural milieu. The most frequently occurring species are *L. plantarum*, *L. casei, L. brevis, Streptococci* and *Lactococcus* spp (Wood 1995). Due to acidophilic or aciduric nature of LAB, they are highly preferred bacteria

in fermentation of milk and cheese production. This subject is indeed broad. This thesis however, cannot get into details of the role of lactobacilli in fermented dairy products, cheese production and their flavor.

Some LAB are highly specialized in adaptation to a particular ecological niche and exhibit narrow range of adaptation. Typical examples are *L. sanfrancisco*, the dominant acid producer in Californian sour dough, and *L. delbrueckii* subs. *delbrueckii* found in potato and grain mashes fermented at 40-55 °C. Other examples are *L. delbrueckii* subsp. *bulgaricus* and *L. kefir* found exclusively in yoghurt and in Caucasian sour milk kefir respectively (Wood 1995). In humans and animals LAB are mostly found as a part of intestinal and oral microflora in healthy individuals. Many species are recognized and their balance is influenced by diet, physiology and immunological parameters. Lactobacilli colonize different parts of gastrointestinal tracts of all animals: stomach, small intestine, large intestine, ileum, cecum and colon. Each one of these habitats has specific characteristics, which impose challenges to LAB. Dominant species considered in humans and animals include *L. acidophilus*, *L. fermentum* and *L. salivarius* due to their ability to cope with high acidity of part of gastrointestinal tract (Wood 1995). Lactic acid bacteria, being mesophils to thermophiles, are not found in poikilothermic animals such as frogs, fish and tortoises.

As it was stated above, LAB populate unusually broad range of habitats. We find them as highly desirable bacteria in the fermentation processes, typically performed at elevated temperatures (above 40°C) as well as spoilage bacteria in refrigerated meats (≈4°C) and fermented beverages where they can successfully cope with high ethanol concentrations (4-6 % v/v). However, the common denominator of all these habitats is abundance of vitamins,

peptides and free amino aids. Adaptation of LAB exclusively to the nutrient-rich habitats imposes the questions about their adaptative abilities. This genus has been studied for more than 100 years and one of the first observations about LAB applicable to all the species is their multiple auxotrophy. There is no doubt that this very characteristic was responsible to some extent for LAB being neglected from scientific point compared to *E. coli, S. cerevisiae* and *B. subtilis*. The first insight into a genetic basis for multiple auxotrophy in LAB came from Morishita et al. (1981) who showed that the small genetic lesions that LAB harbor cause multiple auxotrophies. Furthermore, if amino acid prototrophs obtained by mutagenesis were returned to rich medium, they readily reverted to the auxotrophic state. It was inferred that LAB were evolved in this manner because of their adaptation to the rich environments, which undoubtedly has shaped the perception of LAB in terms of their uniqueness and high survivability. Morishita et al. (1981) made a strong impact on the science of LAB in the early '80s. Since that time, many studies were conducted with one single goal: designing the chemically defined medium for LAB in order to define their requirements and finally, make the connection with their habitats. Prior to this study, there was an inevitable comparison with *E. coli* and overemphasizing the mechanisms of nutritional requirements found in *E. coli*. The question of multiple auxotrophy in LAB, in spite of its significance, has not received the attention one would expect. However, in 2003, Bringel and Hubert examined the genetic lesions in arginine and pyrimidine biosynthetic pathways. They performed an extensive study using 207 strains of *L. plantarum* originating from diverse habitats. The study showed that *L. plantarum* strains that are *Arg*$^{-}$ revealed no preferable gene specific alteration with respect to the

particular habitat. The conclusion was that LAB metabolic diversity is the result of a stress-induced evolution. As much as one has to admire the thoroughness and breadth of the study, the conclusion does not seem to have much weight. The conclusion is true, but only because it is too general. One can easily say that everything that has ever occurred in evolution is due to stresses. At this point, it is interesting to compare LAB with *E. coli.*

Escherichia coli is a bacterium that populates a broad range of habitats and it is able to cope with many stresses accordingly, such as variations in pH, temperature and osmotic pressure. *Escherichia coli* is very important urinary pathogen. Yet, the features typically found in LAB (multiple auxotrophy as a stress response) are not found in *E. coli*. If stress promotes mutation(s) or loss of genes, why does *E. coli* respond to a stress in a different manner? When a certain characteristic is elucidated from the evolutionary prospective, one has to keep in mind that only desirable mutations or the ones that will ensure the continuation of the species will be retained. This obviously means that multiple auxotrophy provide advantages for LAB, since these mutations were evolved and kept, regardless of the seemingly restricted choice of habitats they impose. Synthesis of amino acids and vitamins is an anabolic process that requires energy. LAB certainly save energy in form of ATP by not synthesizing a high number of amino acids (or none of the 20). On the other hand, amino acids are primary metabolites and crucial for bacterial growth making the choice of multiple auxotrophy as a risky survival strategy. In general, microorganisms obtain energy in a form of ATP by fermentation and/or respiration. Lactobacilli are at the threshold of anaerobic to aerobic lifestyle, which allows them to use fermentation as well as respiration to obtain ATP. Aerobic respiration, employing glycolysis followed by tricarboxylic acid cycle (TCA, citric acid cycle) is far more effective

providing 36 ATP vs. fermentation that provides only 2 ATP. However, the fact that is well known about LAB, but not often discussed from this prospective, is that the TCA cycle is incomplete in LAB. It has been shown in several species of LAB that, among other, the activity of 2-oxoglutarate dehydrogenase was not detected (Morishita and Yajima 1995). This is closely related with their inability to synthesize glutamate; the trait so conserved in LAB that was proposed to be used as a phylogenetic characteristic (Ledesma et al. 1977).

The citric acid cycle accounts for a major portion of carbohydrate, fatty acid and amino acid oxidation and generates numerous biosynthetic precursors. Eleven amino acids are synthesized from the precursors provided by the TCA cycle. Obviously, the absence of the cycle important for providing energy and biosynthetic precursors makes microorganism strongly dependant on external pools of primary metabolites. In general, lactobacilli are fermentative bacteria; they do not possess electron transport chain or ATP synthetase and therefore cannot synthesize ATP in this manner. Instead, ATP is obtained at the level of substrate-phosphorylation. Konings et al. (1989) reported that LAB possess an enzyme similar to ATP synthetase, but its major role is reverse reaction. Lactobacilli ATP synthetase catalyzes rather hydrolysis of ATP providing energy for a transport of metabolites and ions. However, typical ATPase activity can be restored under certain conditions in *L. lactis* (Gaudu et al. 2002). It was also established that ATPases of *E. faecalis*, *L. lactis* subsp. *cremoris* and *L. casei* share basic structure with ATPase from mitochondria, chloroplast and photosynthesizing eubacteria (Muntyan et al. 1990). Metabolism of LAB is directed (and evolved) to saving and maintaining the energy; they have created alternative pathways for restoring energy. Indeed, beside fermentation, LAB, at certain conditions, also perform respiration using oxygen as an external

electron acceptor, but they are not restricted to it (Axelson 2004). Heterofermentative LAB, for example, use acetyl phosphate as an external electron acceptor whose presence or absence will determine whether ethanol (no ATP) or acetate (1 ATP) is formed (Lucey and Condon 1986). Genomic sequence of *Lactococcus lactis* II 1403 has revealed that cytochrome oxidase can be synthesized allowing lactococci to switch from less efficient fermentative to more efficient respiratory mode (Bolotin et al. 2001).

The unique way that LAB have evolved is not only shown in diverse pathways for energy maintenance, but where and when they use their pathways. It is clear that LAB, due to their inability to perform TCA cycle, are perfectly adapted to nutrient-rich environments. Recently available genome sequence from several strains show that most of the pathways for amino acid biosynthesis exist; however, nutritional requirements in chemically defined media are greater than that implied by sequence (Teusnik et al. 2005). This has confirmed that gene silencing in LAB indeed takes place, possible largely than firstly anticipated. Silencing of genes encoding for secondary metabolites is common in microorganisms, but silencing of genes encoding for primary metabolites such as amino acids could be considered as a particular lifestyle that again, contributes to energy saving. There is no doubt that this issue has evolutionary implications. More importantly, it suggests that there has been strong selection for cells committed to life in a rich environment.

2.5.2. Plasmids and mobile DNA elements

The singular characteristic coupled with lactobacilli and their manipulation is genetic instability. Roussel et al. (1994) had reported, in genetic mapping of spontaneously obtained clones by sub-culturing of *Streptococcus thermophilus* A054, two deletions occurred in each clone, one of which was due to the recombination between adjacent *rrn* operones. Genetic variability is also manifested in differences in genome size. In general, when closely related strains are analyzed, differences in 2-3Kb have been detected (Morelli et al. 2004). Functional and comparative genomics has established that LAB have small genome size, ≈2 Mbp (Roussel et al. 1994), which is in concordance with their multiple auxotrophy, where a number of biosynthetic pathways are absent. The exception is *L. plantarum* WCFS1 as the only LAB with genome size exceeding 3Mbp (Kleerebezem et al. 2003). To date, the sequences of other *L. plantarum* strains are not available in order to confirm that this unusually large genome linked with the number of habitats it populates. However, it is reported that near the origin of replication, *L. plantarum* WCFS1 harbor a large repetitive sequence designated as a 'lifestyle adaptation region' (Molenaar et al. 2005) and a gene cluster encoding nonribosomal peptide synthesis, the first example of such a system in LAB.

Plasmids, and especially DNA mobile elements profile, contribute to variability and instability of LAB chromosomes. The majority of LAB plasmids belong to the standard type, circular autonomously replicated plasmids. The rolling circle type of replication seems typical for LAB small plasmids. Large plasmids in LAB are usually theta-replicating and encode for metabolic reactions such as lactose fermentation and proteinase plasmids, citrate permease and

phage resistance plasmids (Morelli et al. 2004). Antibiotic resistance plasmids are relatively rare in lactococci, but quite common in lactobacilli and enterococci. Resistance to erythromycin and chloramphenicol is probably plasmid- associated in *L. plantarum, L. fermentum* and *L. acidophilus* (Morelli et al. 2004). Theta-replicating plasmids characterized in other species of LAB share high degree of homology especially in their *repB* gene. The host range of these plasmids is rather limited compared to the rolling circle plasmids. Interestingly, presence of linear plasmid has been reported in *Lactobacillus gasseri* (Roussel et al. 1993). To date, this is the only lactobacillus harboring a linear plasmid.

Another interesting feature is that *L. bulgaricus* spp. and *Streptococcus thermophilus,* used as starter co-culture in yogurt, contain a low number (1-5) or no plasmids (Aslim and Beyati 2004, Soomro and Masud 2007). They show no homology with plasmids of *Lactobacillus* and *Lactococcus* strains. One of these plasmids, such as pA33 obtained from *S. thermophilus,* is indeed unusual revealing 5 open reading frames and 22 bp directly repeated sequences with no *repA* plus and minus origins that are associated with Gram-positive bacterial small plasmids. In spite of the fact that the function of pA33 remained unclear, its presence seemed to have an effect on physiological traits of the host cell such as cell-chain length, antibiotic and phage resistance and milk clotting time (Mercenier et al. 1994). However, many authors have been reported difficulties associated with isolation, stability and manipulation of LAB plasmids (Posno et al. 1991, Soomro and Masurad 2007). This is not accidental; it has been reported that stress conditions markedly influence the stability of the bacterial plasmids (Brown et al. 1990). It seems that dynamic exchange and instability of plasmids

in LAB is the result of the overall lifestyle as multiple auxotrophs. Due to their nature, insertion sequences, transposons and introns certainly have much influence on genetic variations in LAB. Genome sequence of *L. lactis* II 1403 was one of the first available, revealing massive insertion sequences (IS): 43 IS for a total of 42 Kbp belonging to six different IS groups (Bolotin et al. 2001). The distribution of the IS, present as a multiple copies, suggested that this genome may be the result of a recent recombination between closely related genomes. Surprisingly, group II introns, characteristic for eukaryotes, plants and only several species in Eubacteria are found in *L. lactis* suggesting vertical gene transfer (Morelli et al. 2004).

Claesson et al. (2006) reported interesting findings of a 242 Kbp megaplasmid pMP118 in *L. salivarius* UCC118. Genes for completing the pentose phosphate pathway, carbohydrate utilization and bacteriocins are some of the genes located on pM118. Prior to this report, megaplasmids were not found in LAB. At the same time, the chromosome of *L. salivarius* UCC118 (2.13 Mb) is the smallest LAB chromosome sequenced so far. Considering that *L. salivarius* populates the GI tract, it obviously undergo extreme changes in the genome size, as the result of the competitiveness and adaptation to the harsh environment that GI tract imposes to microflora. Note that 16 different IS elements recognized were present in a 43 copies (11 of which were non-functional due to frameshift mutations, truncation and in-frame stop codons).

2.5.3. Bacteriocins

Environments in which LAB are typically found are rich in nutrients and can support the growth of numerous bacterial and fungal species. Therefore, one can speculate that the major stress LAB face in a nutrient-rich environment is competition. What strategies do LAB use in order to ensure their dominancy over other bacterial and fungal species? The most obvious one is production of organic acids. In homofermentative LAB, lactic acid is the major product of metabolism, while heterofermentative species produce acetic acid/ethanol in equimolar amounts as well. Of the two organic acids, acetic acid is a stronger inhibitor with a wide range of inhibitory effects on yeast, molds and bacteria. It has been reported that weak acids have a more powerful antimicrobial activity at low pH than at neutral pH due to lower pK_a (Ouwehand and Vesterlund 2004). However, when a mixture of organic acids is present, the role of lactic acid is probably acidifying the medium making favorable conditions for antimicrobial activity of acetic acid. This suggests synergistic activity of organic acids in heterofermentative LAB in order to provide dominancy of its species. Lactobacilli are also known producers of CO_2 and H_2O_2 that exert antimicrobial activities as well. Axelsson et al. (1989) have reported that a low molecular weight compound produced by *L. reuteri* had a broad range of antimicrobial activity effective against bacteria, fungi, protozoa and viruses. The compound, hydroxypropionylaldehyde, was accordingly named reuterin. The broad spectrum of antimicrobial activity is the result of its mechanism of action; reuterin is shown to be an inhibitor of substrate binding subunit of ribonucleotide reductase therefore interfering with the DNA synthesis (Ouwehand and Vesterlund 2004). Similar compound synthesized by the same strain, (5R)1-(2-decanoyl)-2-hydroxy-3-acetyl-5-isobutyl-2δ-pyroline-4-on or reutericyclin,

also showed antimicrobial activity, but not as broad as that of reuterin (Ouwehand and Vesterlund 2004).

However, antimicrobial compounds synthesized by LAB that have attracted most of the interest are certainly proteinaceous bacteriocins. Bacteriocins are antibiotics typically produced by bacteria with the purpose to inhibit or kill closely related species or even different strains of the same specie. Many bacterial species produce bacteriocins, but LAB bacteriocins are especially interesting due to desirable properties of LAB, that at the same time inhibits the growth of pathogens such as *Listeria* or *Salmonella*. Lactobacilli produce wide range of bacteriocins that can be classified into (i) class I or lantibiotics, (ii) class II or small heat-stable peptides and (iii) class III or large heat-labile proteins. A fourth class of bacteriocins with a complex structure has also been suggested, but not generally accepted. Although knowledge about bacteriocins has increased greatly, there are still many questions regarding self-protection and the molecular basis of target-cell specificity.

Class I bacteriocins contain unusual amino acids synthesized ribosomaly by posttranslational modifications. They are small peptides that require many genes for their production. The best studied lantibiotic is nisin, produced by several strains of *L. lactis.* Genes encoding for nisin are located on chromosome and carried by a large conjugative transposon (Dodd and Gasson 1994). Nisin is a bacteriocin of broad spectrum that inhibits peptidoglycan biosynthesis and therfore responsible for pore formations in the cell wall (Ouwehand and Vesterlund 2004). Other class I bacteriocins include mutacin 1140 and B-Ny266 produced by *Streptococcus mutants*, carnocin U149 produced by *Carnobacterium piscicola* U149 and salivaricin A produced by *S. salivarius* 20P3 (Ouwehand and Vesterlund 2004).

Class II bacteriocins are also small, heat-stable and membrane-active peptides. They typically have a narrow host range, which has raised the question whether class II bacteriocins require a receptor molecule in the target cell membrane. However, this is still an unresolved issue. Mode of action of class II bacteriocins is disruption of the protein motif force of the target cell. Class II is divided into three subclasses, class IIa, class IIb and class IIc. Class IIa is the largest group that is typically effective against *Listeria*, which makes them promising for industrial application. They are also referred to as pediocin-like as pediocin PA-1 is the best studied. Class IIb includes bacteriocins with two peptides such as acidocin J1132 produced by *L. acidophilus* JCM 1132 and active against *L. acidophilus* and lactacin F produced by *L. johnsoni* 11088 active against *L. fermentum, L. delbrueckii* and *L. helveticus* (Ouwehand and Vesterlund 2004). Class IIc contains non-lantibiotic bacteriocins and miscellaneous peptides that do not belong in class IIa and b. Class II bacteriocins are mostly plasmid encoded (Ouwehand and Vesterlund 2004).

Class III bacteriocins are defined as large (>30 KDa) heat-labile proteins. So far, they have been obtained only from members of the genus *Lactobacillus*. It has been suggested that extracellular enzymes, such as muramidases and hemolysins, may be classified as class III bacteriocins (Jack et al. 1994). Compared to other classes, not many bacteriocins are classified in this group. To date, helvetican J, produced by *L. helveticus* 481 has been studied to greater extent. It has been found that helvetican J exerts narrow host range; it is active against closely related *Lactobacillus* species. It was firstly believed that helveticin J was chromosome encoded, but failure to produce active bacteriocin by certain hosts suggested that not all the genes were present on the cloned chromosomal fragment (Dodd and Gasson 1994). Along with *L.*

helveticus, *L. delbrueckii* species synthesize lactacin A and B also classified as class III bacteriocins active only against other strains of *L. delbrueckii*. Interestingly, they are chromosome encoded as well.

In summary, LAB exert several indeed unusual features when speaking of adaptation abilities. Their respiratory system is merely a reflection of their unusual mode of metabolism. Lactobacilli certainly cannot be classified as obligate anaerobs, although the conditional absence of TCA cycle, electron transport chain system and ATP synthetase may imply that. On the other hand, their fermentative nature and complex nutritional requirements seem to be expected consequences of an incomplete TCA cycle, which is typical for obligate anaerobes such as *Clostridium* species (Whitmer and Johnson 1988). However, existence of biosynthetic pathways for primary metabolites has never been reported in *Clostridium* or other anaerobes. Lactobacilli are frequently described as facultative anaerobes, but compared to typical facultative anaerobes such as *E. coli* and *S. cerevisiae*; they differ in their inability to perform complete aerobic respiration. Instead, LAB perform fermentation, but in favorable conditions aerobic respiration occurs. Oxygen may or may not be used as an electron acceptor and ATPase performs a reverse reaction compared to other bacteria. It almost seems that new a term is needed to describe the respiration and energy producing cycle in lactobacilli. Information on more strains, closely and distantly related, is needed to generalize the conclusion.

Even though Orla-Jensen's classification of LAB persisted a whole century and its basis remains remarkably unchanged, the modern tools of molecular biology revealed diversity in the genus that could not possibly be anticipated by relying on classical systematic. Multiple auxotrophy and gene silencing seem adopted as a convenient means of saving energy needed

for biosynthesis of macromolecules such as bacteriocins, making the species safe from the competitors found in rich environment. However, it is not known to what extent LAB would silence the genes or if any priority would be given to some genes over others. At this point, classical studies on physiology, performed on *E. coli* and *S. cerevisiae* 50 years ago, would be highly fitting. Drastic changes in the chromosome size may also be part of the 'energy saving program' typical for LAB and not found in the other bacteria. Significant numbers of IS and transposons contribute to the genomic plasticity, allowing dynamic changes, accumulation of recessive mutations and adaptation to the environment in a very strict sense. According to the drastic changes that LAB genomes are going through, this is probably a young genus in an evolutionary sense.

2.6. Genus Aspergillus

Mitcheli, a Florentine-priest mycologist, first described the genus *Aspergillus* in 1729. The conidiophore that characterizes the genus reminded him of an aspergillum, a liturgical device used to sprinkle holy water and decided the name. Aspergilli are the first fungal organisms that were cultivated on artificial media and studied for their biochemical properties. They are one of the most common fungi in the human environment. The knowledge of the genus *Aspergillus* is owed to the four thoroughly studied species: *A. nidulans, A. niger, A. oryzae* and *A. fumigatus*. Similar to lactobacilli, which are seen as a 'working horse' of the fermentation industry, *A. ficuum* is appreciated only for its high phytase activity, while in both cases basic knowledge is lacking.

Aspergillus nidulans was found especially useful for studies of cell biology and gene regulation due to the availability of diploids and the effectiveness of the transformation system. The versatile metabolism of Aspergilli made them highly important from the medical and industrial aspects; *A. niger* is widely used for production of citric acid, *A. oryzae* is important for production of Oriental fermented foods and beverages and *A. flavus* is used for aflatoxin production. Here, a brief overview of the physiology and practical application of *Aspergillus* spp. will be provided.

2.6.1. General physiology

Vegetative growth of filamentous fungi is initiated by germination of the spore, followed by formation of hyphae, growing in the polar fashion by apical extension and branching. Hyphae form a network of mycelium whose purpose is to acquire nutrients from the environment.

Aspergillus is a genus of asexual fungi, some of which have sexual stages classified among the Ascomycetes. It was firstly assumed that fungi reproduce asexually or sexually. However, it has been shown that both reproductive cycles can coexist in *A. nidualns* giving offspring of the same genotype (Bruggeman et al. 2003).

In asexual cycle, vegetative spores or mycelia fragments produce offspring by mitosis and therefore, they are genetically identical. Asexual reproduction certainly allows rapid dispersion by taking up to 25 h at 37°C, but evolutional implications of sexual reproduction are more significant. *Aspregillus fumigatus*, a mycotoxin producer, is characterized as asexual, but its genome contains many genes related to mating and sexual development. *Aspergillus oryzae* is

also found to have potential for sexual reproduction. *Aspergillus ficuum*, the species used in this Thesis, is considered as a variety of *A. niger* and therefore, asexual.

In sexual reproduction, Ascomycetes undergo both haploid and diploid stages. The nuclei fusion (karyogamy) occurs in ascus, which is immediately followed by meiosis. Unlike mitosis, meiosis allows genetic recombination between two different (parental) nuclei and therefore, produces genetically distinct offspring. Dispersed ascospores form new haploid mycelia after germination. Sexual sporulation is late event that, depending on the genetic background and cultural conditions, will occur several days to more than a week after conidiation is complete. Sexual spores (ascospores) will develop within asci located throughout the closed spherical shells (cleistothescia) composed of fused hyphae (Champe et al. 1981). At 37 °C sexual spore formation takes 100-150 h. Sexual cycle has been established in Basidiomycetes such as yeast and some Ascomycetes such as *A. nidulans*.

Pontecorvo (1953) observed that in *A. nidulans*, reproduction, along with the sexual, occurs in a similar cycle, but with a few distinct differences. In order to accentuate the similarities and the differences, the cycle was named 'parasexual'. In this cycle, two vegetative haploid nuclei fuse into vegetative diploid nuclei that can give a rise to vegetative diploid mycelium. Diploid mycelium is of the same architecture as the haploid mycelium except that it has a half of number of nuclei. During mitosis, diploids may repeatedly fail to split (nondisjunction), but they can revert to haploids by random loss of whole chromosome leading to the recombination at the chromosome level. Apart from the mitotic recombination, the difference between sexual and parasexual cycle is that latter cycle occur in mycelium and spores. Parasexual, along with the sexual cycle in *A. nidulans* was quickly adopted as a tool for

genetic studies making *A. nidulans* the model microorganism. However, its contribution to genetic diversity of Ascomycetes proposed by Pontecorvo, was denied for a long time due to the argument that somatic incompatibility and thus heterokaryons are seldom seen in nature. Finally, Schoustra et al. (2007) showed that parasexual cycle indeed contributes to fitness and environmental adaptability in *A. nidulans.* It is interesting how this work nicely demonstrated the principle in which asexual (typically mitotical and prokaryotic) and sexual (meiosis or meiosis-like and typically eukaryotic) cycle contribute to the adaptation fitness of the species. The authors stated that haploid strains show fast response and changes occur gradually and in a relatively small steps. Diploid stains however, showed delayed response, but changes in fitness occur in large steps. Parasexual cycle is found in *A. flavus, A. fumigatus, A. niger, A. parasiticus* and *A. awamoni* var. *kawachi*. There are no reports on parasexual cycle in *A. ficuum.*

The ability of *Aspergilli* to use all three reproduction cycles certainly explains the ability to cope with the extreme environments. Some *Aspergillus* species can grow over a wide pH range 1.5-9.8 and at temperature between 15-30 °C, but a few species are known to grow at temperatures outside this range. However, their growth is determined by water availability. *Aspergillus* species can grow and tolerate adverse levels of one variable, if the other variables are optimal. Apart from diverse reproductive cycles, survival abilities of *Aspergilli* are due to spore formation. It is known that the thick wall of spores protects them from adverse conditions; spores can, depending on favorable physical parameters and nutrient availability, stay dormant for long periods or decide to germinate when favorable conditions are met. It has been observed quite early that sporulation occurs with precise timing. If spore from liquid medium has been transferred to solid medium at any time before 20 h, sporulation begins at 24 h. If,

however, spore is transferred to the solid medium after 20 h, sporulation commences 4 h later. This is known as the time for competence and is affected by temperature and cell density, but appears to be independent of nutrient status.

As described above, versatile reproductive systems that Aspergilli use, contributes to their genomic plasticity and is important from an evolutionary perspective. Aspergilli are ubiquitous, saprophytic fungi populating numbered habitats and are able to overcome many stresses. However, the mechanisms Aspergilli employ for short-term survival and growth on a specific habitat rely heavily on signal transduction. It certainly drives complex functions in fungi ranging form pathological and morphological development to reproduction. Signal transduction processes are usually rapid, measured by milliseconds in the case of ion flux, minutes for the activation of protein-and-lipid- mediated kinase and hours and days for development specific gene expression. In short, every major event in the cell is triggered by the environmental stimuli of the enzymes. Stimulation of the enzymes is followed by activation of the second messenger(s) resulting in the signal transduction pathway. For a very thorough review on signal transduction in Aspergilli, the reader is referred to the review of Ward et al. (2006).

Aspergilli are able to utilize numerous carbon and nitrogen sources to support sufficient growth. However, not all carbon and nitrogen sources are utilized in the same manner and pace, suggesting that Aspergilli have system to distinguish more from less favorable C/N sources. It has been established quite early that Aspergilli, as well as many other microorganisms, use carbon and nitrogen catabolite repression in order to distinguish available C/N sources. This is indeed a wide spread phenomenon where repression of the synthesis of a

range of enzymes required for utilization of less favorable C/N source when more readily utilized source is in the environment occur. With respect to nitrogen metabolism, Aspergilli are capable to utilize ammonia, nitrate, nitrite, purins, amides, and most of amino acids as a sole nitrogen source. Preferred nitrogen sources, however, are ammonium, glutamine and glutamate. The utilization of alternative secondary nitrogen sources is highly regulated. Expression of the genes involved in the utilization of the less favorable carbon source is repressed by glucose irrespective of the nitrogen source in the medium. For example, genes in ethanol regulation are repressed by glucose. Conversely, genes involved in the utilization of the compound that serves as a nitrogen source only are repressed by ammonium or glutamine irrespective of the carbon source. Therefore, genes involved in the regulation of nitrate or purine are repressed by ammonium. Interestingly, when compound serve as carbon and nitrogen source, than is repressible by both favorable sources. Indeed, the expression of *prn* cluster (responsible for proline metabolism) is repressible by the simultaneous presence of glucose and ammonium (Gonzalez et al. 1997). This allows the use of proline as a nitrogen source even if rich carbon source is present and its use as a carbon source even if rich nitrogen source is present. The utilization of other metabolites that can serve as C/N source is regulated in the same manner as proline utilization.

2.6.2. Industrial application

Due to their versatile enzymatic systems, there are certainly many applications for Aspergilli. One of the industrially most significant applications is citric acid production. Citric acid is, due to low toxicity and pleasant flavor, widely used as a flavoring compound in production of beverages, juices, jams, jellies, and other products based on fruits and vegetables. In 1917, food chemist James Currie found that some species of *A. niger* excrete citric acid into glucose containing medium, which become the basis for the extensive research ever since. As a result, modern fermentation technology can yield up to 95% of citric acid form sugar with high producer strains (Ward et al. 2006). Much of the published work showing high yields are performed in laboratory environment using glucose as a carbon source. However, it has been shown recently over-producing *A. niger* strains that utilize corn and potato starch, which is undoubtedly an improvement in economic rationale for citric acid production (Haq et al. 2003). The potential Aspergilli offer is enormous; database mining *A. niger* genome resulted in the identification of 12 new starch-modifying enzymes (Yuan et al. 2005).

Along with the citric acid, the extra-cellular enzymes, mainly amylases and proteases of Aspergilli received a lot of attention. Aspergillus species, especially GRAS-designated species, are used in large scale fermentation producing number of α-amylases, glucoamylases, cellulases, pectinases, xylanases, hemucellulases and proteases. Fungal α-amylases and proteases are substantially used in the baking industry to increase the concentration of simple sugars and the elasticity of gluten. Pectinases have wide applications in fruit processing, puree preparation, fruit juice recovery, clarification and plant oil recovery. The groups of enzymes that attract more attention lately are cellulases allowing utilization of cheap substrates for the bio-ethanol

industry. Singh et al. (1990) reported that Aspergilli possess all four classes of enzymes involved in degradation of cellulose: endoglucanases, exoglucanases, cellobiohydrolases and β-glucosidase.

Since the early 1970's there has been a dramatic increase in the use of plant protein sources in animal feed. The salts of phytic acid are common ingredients in plant tissue as a major storage of phosphorous. However, they are not digestible by animals making the essential mineral phosphorous unavailable. Due to this, phytases secreted by *A. ficuum* were studied extensively. This has created a market potential of $500 million US for animal feed industry (Ward et al. 2006).

One aspect of practical application of Aspergilli that certainly must not be neglected is the variety of biotransformation they are capable of performing. By definition, biotransformation exploits the versatility and high reaction rates achievable under mild conditions to catalyze reactions that are highly region- and stereo-specific (Ward et al. 2006). Aspergillus species have been employed in biotransformation of alicyclic insecticides, aromatic and phenoxy herbicides, organophosphorous and other pesticides, terpens and alkaloids.

While Aspergillus species are not an industrial host used for antibiotic production, their biosynthesis in *A. nidulans* presents the most advanced model system for studying the biosynthesis and regulation of antibiotics. *A. terreus* has been identified as a new producer of cephalosporin A (Sallam et al. 2003) and lovastatin (Lai et al. 2003). Secondary metabolites of high medical importance produced by Aspergilli are mycotoxins. Mycotoxins are a diverse group of bioactive fungal secondary metabolites of a small size (<700 Da) that cause pathological conditions in humans and animals. Mycotoxin production in toxigenic strains is

determined by the availability of the nutrients, environmental factors such as water, temperature, host resistance and interactions with other fungi. Toxigenic fungi in rare situation appear in isolation; their ability to compete with other organisms will affect toxin production. Aflatoxins are the most important mycotoxins, especially aflatoxins B and G, which are strong liver carcinogens. There are only three *Aspergillus* species known to be aflatoxin producers: *A flavus* (aflatoxins B1 and B2), *A. parasiticus* and *A. nomius* (aflatoxins B1, B2, G1 and G2). From the medical point, pathogenic species, mainly *A. fumigatus,* received a lot of attention. It is designated as causing agent of invasive aspergillosis, which accounts for major cause of morbidity and mortality in immuno-compromised patients (Antachopoulos et al. 2006).

3. NUTRITIONAL REQUIREMENTS OF LACTOBACILLI

3.1. ABSTRACT

This study was undertaken to design chemically defined medium optimized for the growth of lactobacilli of various origins. Therefore, were studied lactobacilli associated with plants, lactobacilli used in fermentation, dairy and non-dairy lactobacilli. The single omission technique revealed the requirements of nine strains, allowing further optimization and simplification of the medium. It was found that Asp, Val, Leu, Cys, Phe, Gln and Thr are essential amino acids for all of the strains tested, while pyridoxine was the only vitamin for which lactobacilli showed an absolute requirement. Lysine was the least required amino acid; it exerted inhibition of the growth in *Lactobacillus zeae*, *Lactobacillus acidophilus* and *Lactobacillus casei*. The chemically defined medium, M3, supported the growth of nine strains with growth rates of 0.26 h^{-1} to 0.08 h^{-1} and with the maximal total cell count of 10^9 cells/mL. The growth rates and optical densities at 620 nm (OD_{620}) remained consistent throughout at least 5 successive transfers. Optimal growth conditions for nine lactobacilli could be accomplished in M3 containing as few as 29 constituents. To our knowledge, this is the first time that nutritional requirements of *Lactobacillus agilis*, *Lactobacillus frigidus* and *Weissella confusa* were discussed.

3.2. INTRODUCTION

Lactic acid bacteria (LAB) are found in a variety of habitats including intestinal mucosal membranes of human and animal, plants, dairy products, wine and feed (Wood 1995). They

are natural auxotrophs for several amino acids, purines, pyrimidines and vitamins (Morishita et al. 1974, Morishita et al. 1981, Wood 1995). Considering the great importance LAB have in industry and medicine, the genetics and physiology of this remarkable genus is still not well understood. A critically important requirement for undertaking physiological studies is the availability of a common defined minimal medium, which can provide insight into the influence of the nutrients on the growth, the metabolic pathways and the products of metabolism.

Chemically defined media have been developed for several LAB including three subspecies *of L. delbrueckii* (Peters and Snell 1953, Grobben et al. 1998, Chervaux et al. 2000, Hébert et al. 2004), *L. plantarum* (McFeeters and Chen 1986, Ruiz-Barba and Jimenez-Diaz 1994, Teusnik et al. 2005), *L. johnsonii* (Elli et al. 2000), meat lactobacilli (Møretrø et al. 1998), *L. helveticus* (Hébert et al. 2000), *Lactococcus lactis* (McIntyre and Harlander 1989), *Streptococcus thermophilus* (Garault et al. 2000, Letort and Juillard 2001) and *Leuconostoc mesenteroides* (Foucaud et al. 1997). Due to their industrial importance, nutritional requirements of dairy LAB were more frequently studied, e.g. in terms of a cost or complexity of the production. A novel approach taken on this subject is the *in silico* reconstruction of the pathways of the *L. plantarum* WCFS1 via an available database (Teusnik et al. 2005). According to the authors, a lack of a high quality organism-specific database constructed for Gram positive bacteria prevents extending this approach to other species of LAB at the moment. Oddly enough, even if genome sequencing was available to be used for practical purposes such as definition of growth requirements and media design, the connection between genetic imprint and nutritional requirements remains to be determined.

However, many other industrially or medically important LAB strains have been neglected. Non-dairy LAB, thanks to their amyloytic activity, offers many possibilities in terms of new substrates for lactic acid production (Guyot et al. 2000). Yet, very little is known about their nutritional requirements and consequently, metabolism. The aim of this study was to determine the nutritional requirements of twelve LAB species isolated from different habitats in a chemically defined medium that contains as little as 29 nutrients. To our knowledge, this is the first time that nutritional requirements of *L. agilis, L. frigidus* and *W. confusa* have been discussed.

3.3. MATERIALS AND METHODS

3.3.1. Bacterial strains and growth conditions. The following strains were used: *Lactobacillus acidophilus* NRRL B 4495, *L. agilis* NRRL B 14856, L. *amylophilus* NRRL B 4437, L. *amylovorus* NRRL B 4540, *L. casei* NRRL B 1922, *L. casei* subsp. *rhamnosus* ATCC 7469, *L. delbrueckii* subsp. *delbrueckii* ATCC 9649, *L. frigidus* NCIB 8518, *L. plantarum* NRRL B 4496, L. *zeae* ATCC 15820 (previously designated as *L. casei* subsp. *rhamnosus* ATCC 15820) *Pediococcus pentosaceus* NRRL B 14009 and *Weissella confusa* NRRL B 1064. Strains were obtained from Northern Regional Research Laboratory (NRRL, now the National Center for Agricultural Utilization Research) and American Type Culture Collection (ATCC). The strains were stored in de Man, Rogosa and Sharpe (MRS) broth (Sigma Aldrich) in 25 % (v/v) glycerol at -30°C and maintained on MRS plates supplemented with 1.5 % (w/v) agar. For the experiments performed here, a colony from agar plate was grown in 10 mL of MRS broth in statically tube

until early stationary phase was reached. *Lactobacillus delbureckii, L. plantarum, L. amlylophilus, L. zeae, P. pentosaceus* and *W. confusa* were cultivated at 30˚C. *Lactobacillus rhamnosus, L. amylovorus, L. acidophilus, L. agilis* and *L. casei* were cultivated at 37˚C and *L. frigidus* at 26˚C. The growth rate was calculated as $\mu=\ln 2/g$, where g, mean generation time, was calculated by the equation: $g= (\log N_t - \log N_0)/\log 2$. N_t was the number of the cells at the early exponential phase of growth, while N_0 was the number of the cells at the end of exponential phase of growth.

3.3.2. Chemicals. All chemicals were of Sigma and reagent grade (Sigma-Aldrich, St. Louis MO). Amino acids, vitamin and solutions of precursors of nucleic acids were made as a hundred fold stock solutions of the final concentration used in the medium and kept at -30˚C until use. Only L-cysteine was added fresh.

3.3.3. Media. Several variations of media adapted from Morishita et al. (1981) were used to develop the Medium 3 (M3) that supported the growth of nine strains of LAB. Medium 3 was filter-sterilized (Millipore, 0.22 µm pore size) and stored at +4˚C, protected from light. Solid medium was prepared with addition of 1.5 % (w/v) agar.

3.3.4. Assessment of the requirements. In order to avoid carryover of the nutrients, 1 mL aliquot of early stationary culture from MRS was harvested by centrifugation at 8000×g (IEC Micromax) for 5 min, washed twice in 0.9 % (w/v) NaCl and re-suspended to 1 mL volume. Screening for nutritional requirements was performed in a sterile Titertek U-bottom 96 wells plate in a set of four replicates with the final volume of 150 µL with 1 % (v/v) inoculum. Positive control was complete M3 and negative control had the inoculum added to M3, which OD_{620} was read at zero time. Using the single omission technique (Hébert et al. 2004), specific

nutritional requirements of LAB were determined. A constituent was considered essential (E) if its omission caused less then half of the maximal OD_{620} of the positive control (complete M3). Stimulatory (S), if its omission caused between 50-80 % of the maximal OD_{620} compared to the positive control and nonessential (NE) when the growth remained 80 % or more upon its omission (Hébert et al. 2004). In the case when omission of the constituent increased the growth of LAB, the constituent is considered inhibitory (INH).

3.4. RESULTS

Several different formulations of medium developed by Morishita et al. (1981) were the baseline for further development. The one that supported the growth of nine strains, was named M3 and was a basis for the study of the essential nutrients required for LAB growth. The composition of M3 listed in the Table 3.1 appeared to be adequate since the addition of double amount of amino acids did not enhance the growth to a higher extent for any LAB strain. Medium 3 supported the growth of *L. rhamnosus, L. delbrueckii, L. plantarum, L. zeae, L. frigidus* and *W. confusa* with the growth rates 0.1 h^{-1} - 0.26 h^{-1} (Table 3.2). On the other hand, *L. agilis*, *L. casei* and *L. acidiphilus* were shown to be more demanding, with the growth rates ranging from 0.06 h^{-1} – 0.08 h^{-1}. The pH of M3 was 6.25, which is a favorable pH for most LAB. In stationary phase cultures, pH dropped to 4.51 to 4.62, with the exception of *L. zeae*, where the final pH was 5.29. The pH values obtained in M3 were highly comparable with the pH values obtained in MRS. This suggested that pH was not a limiting factor for growth, providing sufficient buffer capacity necessary for neutralizing organic acids produced during

the growth. Therefore, the use of phosphate buffered medium of pH 6.25 for these LAB was justified. In contrast, it has been suggested that phosphate buffered medium does not provide satisfactory buffering capacity for meat LAB (Møretrø et al. 1998).

M3 provided maximal cell counts of 10^9 cells/mL for *L. delbrueckii*, 7×10^8cells/mL for *L. plantarum*, 6.5×10^8 cells/mL for *L. rhamnosus* and 10^8 cells/mL for the rest of LAB, while MRS, a commonly used rich medium, provided maximal cell count of $1\text{-}1.6\times10^9$ cells/mL for these strains. *Lactobacillus rhamnosus* and *L. delbrueckii* showed no lag phase and reached early stationary after 32 h and 36 h respectively while, *L. plantarum* and *L. zeae* had 4 h of lag phase and the growth ceased after 36 h and 48 h respectively. The rest of LAB showed lag phase of 8-10 h and reached early stationary after 48 h of growth. The morphological appearance of the colonies on M3 plates was comparable to the appearance on the MRS plates, except that the size was smaller by roughly 20 %.

3.4.1. Amino acid requirements. In this formulation, 14 amino acids were found to support growth to an OD_{620} range between 0.05 to 0.41 for all strains except for *P. pentosaceus*, *L. amylovorus* and *L. amylophilus*. Amino acids that were not used here, namely L-alanine, L-glycine, L-serine and L-asparagine, were seldom reported as essential in different lactobacilli (Wood 1995). In order to simplify the medium, above-mentioned amino acids were omitted. For example, Asp was included into M3, but its uncharged derivative L-asparagine was excluded. It is well established that in a chemically defined medium lactobacilli require L-glutamine or L-glutamic acid or glutamate (Morishita et al. 1981), thus, all three are not necessary. In M3 glutamine alone was used. In this system, seven amino acids were shown to be essential to all the strains: Asp, Val, Leu, Cys, Phe, Gln and Thr (Table 3.3).

Table 3.1. The composition of M3.

Constituent	Chemicals	Conc (g/L)
Carbon source	Glucose	10
Buffer	Sodium acetate	15
	Ammonium citrate	4.6
Minerals	Magnesium sulfate heptahydrate ($MgSO_4 \times 7H_2O$)	0.1
	Manganese sulfate monohydrate ($MnSO_4 \times H_2O$)	0.05
	Ferric sulfate heptahydrate ($FeSO_4 \times 7\ H_2O$)	0.02
	Di-potassium phosphate dibasic (K_2HPO_4)	3
Fatty acid	Tween 80	5
Amino Acids	L-glutamine (Gln)	0.5
	L-aspartic acid (Asp)	0.2
	L-cysteine HCl (Cys)	0.2
	L-valine (Val)	0.1
	L-tyrosine (Tyr)	0.1
	L-isoleucine (Ile)	0.1
	L-leucine (Leu)	0.1
	L-tryptophan (Trp)	0.1
	L-arginine (Arg)	0.1
	L-phenylalanine (Phe)	0.1
	L-methionine (Met)	0.1
	L-threonine (Thr)	0.1
	L-proline (Pro)	0.1
	L-histidine (His)	0.1
Vitamins	Pyridoxine HCl (B6)	0.02
	Ca-pantothenate	0.021
	Niacin (B3)	0.001
	Riboflavin (B2)	0.001
	Cyanocobalamine (B12)	0.001
	Folic acid	0.0001
Nucleoside	Thymidine	0.005

Table 3.2. Maximal OD_{620} and the growth rate of selected lactobacilli.

Lactic acid Bacteria	OD_{620}	μ_{max}
L. delbrueckii	0.41 ± 0.03	0.20 ± 0.00
L. plantarum	0.38 ± 0.04	0.18 ± 0.00
L. rhamnosus	0.32 ± 0.02	0.26 ± 0.01
W. confuse	0.17 ± 0.02	0.11 ± 0.00
L. zeae	0.13 ± 0.02	0.17 ± 0.01
L. frigidus	0.11 ± 0.01	0.10 ± 0.00
L. agilis	0.07 ± 0.00	0.06 ± 0.01
L. casei	0.07 ± 0.00	0.07 ± 0.00
L. amylophilus	0.06 ± 0.01	–
L. acidophilus	0.05 ± 0.00	0.08 ± 0.00
P. pentosaceus	0.05 ± 0.01	–
L. amylovorus	0.03 ± 0.01	–

The growth rate was calculated as described in Materials and Methods. Data are expressed as an average from two independent experiments ± standard deviation.

Note that this is versatile group containing acidic (Asp), polar (Gln and Thr) and non-polar (Val, Leu, Cys and Phe) amino acids. The essentiality of aliphatic amino acids, Leu and Val, is in concordance with the findings of Bringel and Hubert (2003). L-isoleucine is an exception to this observation, as this study showed that it is stimulatory in *L. agilis* and nonessential in *L. acidophilus*. Amongst aromatic amino acids, Phe is the only essential amino acid for the lactobacilli examined. Tryptophan is stimulatory for *L. rhamnosus,* Tyr is stimulatory for *L. agilis* and nonessential for *L. acidophilus* and His is nonessential for *L. rhamnosus* and stimulatory for *L. delbrueckii* and *L. plantarum*. In the case of Met and Cys both sulfur containing and hydrophobic amino acids, Cys only was required by all of the strains tested, while Met was stimulatory in *L. plantarum* and *L. rhamnosus*. If one takes into account the biosynthetic pathway that these amino acids share, it can be appreciated from Table 3.3 that none of the biosynthetic pathways are completely and functionally tested in lactobacilli. Peters and Snell (1953) discussed the nutritional requirements of *L. delbrueckii* ATCC 9649. It was stated that both L-alanine and D-alanine supported the growth, implying that this microorganism possessed a racemase. In this study, DL-alanine had no influence on the growth of *L. delbrueckii*. Belgian Co-ordinated Culture Collection provided information that *L. delbrueckii* ATCC 9649 (deposited as LMG 6412) has two stable types of colonies, which may suggest two stable phenotypes with different requirements. Some studies have shown that reversion rate is 10^{-11} that is usual for the occurrence of spontaneous mutations suggesting that while auxotrophy is stable reversion to prototrophy can occur (Morishita et al. 1981, Chervaux et al. 2000).

Footnote to Table 3.3. Amino acids are clustered according to biosynthetic pathway they share. Importance of amino acids is designated as described in Materials and Methods; nonessential (NE), stimulatory (S) and essential (E). Numerical values of importance to the constituents are provided in Appendix 10.1

Table 3.3. Importance of amino acids for the growth of lactobacilli.

Omitted L-amino acid	Lactobacillus species and importance of amino acids								
	L. delbrueckii	*L. plantarum*	*L. rhamnosus*	*W. confusa*	*L. zeae*	*L. frigidus*	*L. agilis*	*L. casei*	*L. acidophilus*
Glu	E	E	E	E	E	E	E	E	E
Pro	E	E	NE	E	E	E	E	E	E
Arg	E	E	S	E	E	E	E	E	E
Asp	E	E	E	E	E	E	E	E	E
Ile	E	E	E	E	E	E	S	E	NE
Thr	E	E	E	E	E	E	E	E	E
Met	E	S	S	E	E	E	E	E	E
Val	E	E	E	E	E	E	E	E	E
Leu	E	E	E	E	E	E	E	E	E
His	S	S	NE	E	E	E	E	E	E
Phe	E	E	E	E	E	E	E	E	E
Tyr	E	E	E	E	E	E	S	E	NE
Trp	E	E	S	E	E	E	E	E	E

3.4.2. Vitamin and mineral requirements. In this thesis was found that vitamins and minerals stimulate the growth of LAB rather then being essential (Table 3.4). The inorganic component essential for all tested strains was K_2HPO_4 as the only source of phosphorus in this formulation and its omission almost completely aborted the growth. In addition, K_2HPO_4 along with ammonium citrate and sodium acetate ensured buffer capacity of the medium, which was highly important knowing that pH below 4.5 is detrimental for the growth (Guyot et al. 2000). Omission of ammonium citrate resulted in pH change from 6.25 to 7 that supported the growth of only *L. acidophilus* indicating that this strain favored slightly higher pH (Morishita et al. 1981). This is surprising, as the name of this lactobacillus species suggests 'acid-loving' bacterium. Furthermore, in the genomic sequence were found genes for acid-resistance mechanisms typical of Gram positive bacteria (Altermann et al. 2005). For *L. amylophilus, L. agilis, L. zeae* and *W. confusa*, ammonium citrate was not necessary for the growth, but it stimulated the growth of *L. delbrueckii, L. plantarum* and *L. casei* and was essential for *L. frigidus*. Sodium acetate was nonessential for *W. confusa, L. casei, L. agilis* and *L. zeae*, but stimulated the growth of *L. plantarum, L. delbrueckii* and *L. amylophilus*, while it was slightly inhibitory for *L. rhamnosus* and *L. acidophilus*. It is generally accepted that lactobacilli are among the few bacteria, which do not require iron. One of the studies showed that iron requirement depends on the purines and pyrimidines present in the medium (Elli et al. 2000). In the formulation used in this thesis, iron was nonessential only for *L. rhamnosus* and *L. amylophilus.*

It stimulated the growth of *L. zeae, L plantarum, W. confusa, L. agilis* and *L. acidophilus*, but was essential for *L. frigidus* and *L. casei*.

Table 3.4. Importance of vitamins and minerals for the growth of lactobacilli.

Omitted constituent	Lactobacillus species and importance of the constituent								
	L. delbrueckii	*L. plantarum*	*L. rhamnosus*	*W. confusa*	*L. zeae*	*L. frigidus*	*L. agilis*	*L. casei*	*L. acidophilus*
Tween 80	NE	S	S	S	S	S	S	E	NE
Ammonium citrate	S	S	NE	NE	NE	E	NE	S	INH
Sodium acetate	S	S	INH	NE	NE	E	NE	NE	INH
K_2HPO_4	E	E	E	E	E	E	E	E	E
$MgSO_4 \times 7H_2O$	S	S	S	E	E	E	E	E	E
$MnSO_4 \times H_2O$	NE	NE	NE	S	NE	E	E	E	E
$FeSO_4 \times 7\ H_2O$	E	S	NE	S	S	E	S	E	S
Ca pantothenate	S	S	NE	S	S	E	S	E	S
B6	E	E	E	E	E	E	E	E	E
B12	S	S	NE	S	NE	E	E	E	S
B3	S	S	NE	S	S	E	E	E	S
B2	S	NE	NE	S	S	E	E	E	E
Folic acid	S	S	NE	E	S	E	E	E	S
Thymidine	S	S	NE	S	S	E	E	E	S

Importance of amino acids is designated as described in Materials and Methods; nonessential (NE), stimulatory (S) and essential (E).

Numerical values of importance to the constituents are provided In Appendix 10.2.

Manganese was shown to be essential for four strains, *L. casei, L. frigidus L. acidophilys* and *L. agilis*. It stimulated the growth of *L. amylophilus* and *W. confusa*, while it was nonessential for *L. rhamnosus, L. delbrueckii* and *L. plantarum*. Magnesium stimulated the growth of *L. rhamnosus, L. delbrueckii* and *L. plantarum*, while the growth of other lactobacilli was completely aborted upon magnesium omission. It was striking that from six vitamins included in M3, only pyridoxine HCl was found to be essential for all nine strains. Indeed, the role of pyridoxine in amino acid metabolism is a unique one among the vitamins. Pyridoxine is involved in transamination, decarboxylation and racemization of amino acids. Furthermore, it plays role in metabolism of Thr, Cys, Met, Ala, Gly, Ser and Trp (Meister 1957).

It was interesting that, although the medium was supplemented with all essential amino acids, pyridoxine was still needed for the growth of lactobacilli, indicating that pathways for amino acid catabolism might have been impaired. The least required vitamin was calcium pantothenate, as it was found to be essential only in *L. frigidus* and *L. casei*. Thymidine was the only nucleoside in this formulation and was shown to be essential for *L. frigidus, L. agilis* and *L. casei*, nonessential for *L. rhamnosus* and stimulatory to the remaining lactobacilli tested. Addition of uracil, adenine and guanine did not yield increase the growth of any LAB strain. Despite the fact that essential role of uracil was frequently reported for *L. delbrueckii* strains (Grobben et al. 1998, Hébert et al. 2004, Peters and Snell 1953), this thesis did not confirm that.

The growth requirements of *L. rhamnosus* were found interesting; it required only K_2HPO_4 and pyridoxine, although magnesium and Tween 80 were stimulatory and the remaining minerals, vitamins and nucleoside used in this medium were nonessential.

However, if all nonessential constituents were omitted simultaneously, the growth would be aborted due to overlapping and possible interaction between the biosynthetic pathways. On the other hand, this study showed that medium for *L. casei* subsp. *rhamnosus* ATCC 7469 can be further simplified and customized. Finally, in Association of Official Analytical Chemists (AOAC) was indicated that this lactobacilli was used for microbiological assay of riboflavin inferring a requirement. In this study, omission of riboflavin did not abort the growth of *L. rhamnosus* ATCC 7469. It is worth mentioning that OD_{620} and the growth rates of LAB in the M3 remained the same for at least 5 successive transfers.

3.4.3. Lysine requirements in lactobacilli. Considering the aim of this thesis, it was necessary to design the culture medium for LAB without exogenous lysine in order to perform screening of lysine secretion. For this reason, the chemically defined medium used for the lysine over-producers and DAP pathway regulation studies (Chapters 4 and 5) did not contained lysine. Regulation of the DAP pathway (Chapter 5) suggested that lysine plays an important role in its regulation, which was confirmed in the study of growth. Due to this, the influence of lysine on the growth of lactobacilli studied here was determined and presented separately. In the chemically defined M3, lysine was added to a final concentration of 0.1 g/L. Lactobacilli were grown, washed and the growth response was measured as previously described.

Table 3.5. Impact of the addition of lysine in M3 on the growth of lactobacilli.

Lactobacilli	Impact of lysine on the growth	Importance of lysine	Maximum OD_{620}	
			M3	M3 + lysine
L. delbrueckii	2 % ↓	NE	0.37 ± 0.02	0.37 ± 0.01
L. rhamnosus	13 % ↓	NE	0.34 ± 0.01	0.30 ± 0.02
L. plantarum	25 % ↑	S	0.30 ± 0.04	0.38 ± 0.03
L. agilis	43 % ↑	S	0.07 ± 0.01	0.10 ± 0.01
W. confusa	22 % ↓	INH	0.17 ± 0.02	0.13 ± 0.00
L. acidophilus	37 % ↓	INH	0.05 ± 0.00	0.03 ± 0.00
L. zeae	54 % ↓	INH	0.20 ± 0.05	0.10 ± 0.01
L. casei	59 % ↓	INH	0.07 ± 0.00	0.04 ± 0.00
L. frigidus	NA	NA	0.11 ± 0.01	NA

Lysine was considered inhibitory (INH) if its addition caused more than 20 % of the growth decrease. Nonessential (NE) if its addition caused up to 20 % of increase or decrease of OD_{620} compared to OD_{620} in M3, stimulatory (S) if the growth increased for 21-50 % and essential (E) if the growth increased for 51 % or more. 'NA' stands for 'not assayed'.

It can be appreciated from Table 3.5 that tested lactobacilli showed versatility in terms of lysine requirements. In three strains, *L. acidophilus, L. zeae* and *L. casei*, lysine caused strong inhibition, 37 %, 54 % and 59 % respectively. It stimulated the growth of *L. plantarum* and *L. agilis* and was shown to be nonessential for *L. delbrueckii* and *L. rhamnosus.* Common to all the strains tested was that lysine was not essential.

3.5. DISCUSSION

This study showed that a formulation with as little as twenty-nine ingredients supported the growth of nine LAB strains. Strains used here originated from different environments (Table 3.6). Morishita et al. showed in 1974 and confirmed in 1981 that complex nutritional requirements and polyauxotrophy of lactobacilli is due to adaptation to rich environments. This study showed that non-dairy lactobacilli originating from fermentation (*L. amylophilus, L. amylovorus, L. zeae* and *L. agilis*) are more nutritionally demanding that lactobacilli originating from dairy products and/or humans. This thesis was unsuccessful in spite of the effort, to support the growth of *L. amylovorus, L. amylophilus* and *P. pentosaceus* in the chemically defined medium. The medium containing all 20 amino acids and four bases (adenine, thymine, uracil and guanine) could not support the growth of the above-mentioned strains. One can speculate that either these lactobacilli have developed auxotrophy for highly specialized ingredient(s) found in the natural environment or that the transport of free amino acid(s) was absent or ineffective.

Table 3.6. The origin of the strains used in this study.

Lactobacilli	Mutant source	Origin
L. acidophilus	NRRL B 4496	Human
L. agilis	NRRL B 14856	Sewage, municipal
L. amylophilus	NRRL B 4437	Swine waste corn fermentation
L. amylovorus	NRRL B 4540	Cattle waste corn fermentation
L. casei	NRRL B 1922	Cheese
L. delbrueckii	ATCC 9649	Distillery sour grain
L. frigidus	NCIB 8518	Beer spoilage
L. plantarum	NRRL B 4496	Pickled cabbage
L. rhamnosus	ATCC 7469	Intestine
L. zeae	ATCC 15820	Corn steep fermentation
P. pentosaceus	NRRL B 14009	Ragi
W. confuse	NRRL B 1064	Sugar cane

It was reported that lactobacilli originating from wine require D-mevalonic acid and that *L. delbrueckii* species originating from cheese require orotic acid (Bringel and Hubert 2003). Peters and Snell (1953) had reported that *L. delbrueckii* ATCC 9649 had much higher growth rate when grown in the medium supplemented with partially hydrolyzed casein as opposed to the medium containing the same concentration of free amino acids. Similarly, Fernández et al. (2003) reported that the growth of *P. pentosaceus* c1 obtained from an Argentinean red wine was 50 % higher when the free amino acid Gly (that was essential for the growth) was replaced with di-peptide Gly-Gly.

Interestingly, lysine was the least required amino acid in LAB tested. Indeed, addition of lysine into M3 stimulated only the growth of *L. agilis* (43 % growth increase) and *L. plantarum* (25 % growth increase). For *L. delbrueckii* and *L. rhamnosus* addition of lysine made no difference while it inhibited the growth of other strains. Very few lysine auxotrophs were reported among the genus (Grobben et al. 1998, Hebert et al. 2000, Møretrø et al. 1998) compared to the auxotrophs for other amino acids. The reasons could be that (i) L-lysine, along with DAP is a very important constituent of the Gram positive bacterial cell wall and (ii) lysine is essential and the first limiting amino acid for most of the plants that produce crops or feed.

Another striking feature that is highly conserved trait in the genus is the absolute requirement for Gln, Val, and Leu. This study has also confirmed essentiality of the above-mentioned amino acids in all LAB studied. In fact, this phenomenon is so widely spread, that Ledesma et al. (1977) suggested that these nutrient requirements might be taken as a taxonomic characteristic of the genus *Lactobacillus*.

As mentioned before, while amino acids are mostly essential, vitamins were shown to be stimulatory for lactobacilli tested. The only essential vitamin for all the lactobacilli used in this thesis was pyridoxine, probably due to its significant involvement in amino acids metabolism. It has been suggested that polyauxotrophy of LAB is due to environmental stresses and hence, LAB associated with human origin are more fastidious than other species (Bringel and Hubert 2003). In this study, *L. rhamnosus* originating from intestine is the least amino acid demanding strain tested. On the other hand, *L. acidophilus*, originating from human (Table 3.6) was a moderately amino acid demanding strain. In fact, once again is confirmed that LAB display strong variability with respect to their requirements and consequently, metabolism.

Depending on the nature and extent of auxotrophic mutations, LAB can be reverted to prototrophy. It was reported that *L. delbrueckii* is auxotroph for alanine and uracil (Peters et al. 1954). In this study, however, *L. delbrueckii* did not exert requirements for alanine or uracil. According to AOAC *L. casei* subsp. *rhamnosus* ATCC 7469 is used for microbiological assay for riboflavin, while this study showed that riboflavin was nonessential for growth. This implies that strain(s) of interest should be individually characterized regardless of the previous findings or reports. Recent studies (Bringel and Hubert 2003, Molenaar et al. 2005, Callanan et al. 2008, Christiansen et al. 2008) that used contemporary microbiological tools have confirmed that not only do LAB harbor point mutations, as a result of high adaptation to the environment, but they also have numerous mutations caused by insertion sequence(s) and horizontal gene transfer. It was stated by Van de Guchte et al. (2006) that the genome of *L. delbrueckii* subsp. *bulgaricus* is in a relatively recent state of evolution and adaptation to the dairy environment showing the large number of pseudogenes and other features.

In conclusion, due to complexity and plasticity of the genus *Lactobacillus*, it is not possible to create a chemically defined and reasonably simple medium that supports the growth of all lactobacilli. On the other hand, lack of a high quality database still prevents accurate *in silico* reconstruction of the effective pathways in this genus. Thus, rational studies on growth, optimized for a larger number of lactobacilli, are still the most reliable tool in metabolic studies of lactobacilli.

3.6. RELEVANCE OF CHAPTER 3 TO THE THESIS

Selections of regulatory mutants, which are lysine over-producers, impose on the choice of the bacterium, i.e., those that do not have absolute requirement for provision of lysine to sustain the growth. Therefore, natural lysine auxotrophs cannot and should not be taken into consideration for obtaining lysine over-producer. On the other hand, multiple auxotrophy in lactobacilli is a well-established fact. In this context, the diversity of nutritional requirements amongst lactobacilli mutants leaves no choice, but to determine requirements for each strain used. This investigation was done in Chapter 3 for nine lactobacilli. Detailed information on their nutritional requirements was provided allowing customizing the medium for lysine over-producer selection and maintenance.

4. REGULATION OF LYSINE BIOSYNTHESIS IN RELATION TO *L. DELBRUECKII* AND *L. PLANTARUM*

4.1. ABSTRACT

Biosynthesis of L-lysine, L-threonine, L-methionine and L-isoleucine in most bacteria and in plants occurs through the diaminopimelic pathway, with aspartokinase (AK) as the key regulatory enzyme. Presence of AK isozymes was not known until now in *Lactobacillus plantarum* NRRL B 4496. Using zymographic analysis, the presence of four isozymes and their subunit composition have been shown. The AK activities of AK isozymes changed with respect to the growth stage. Two isozymes, AK I and AK III, were lysine sensitive and active only during stationary phase; AK I was not repressed by any amino acids of the aspartate family amino acids, while AK III was subjected to multiple repression by lysine or methionine or isoleucine. Aspartokinase II and AK IV were active in both exponential and stationary phases, repressed by lysine or threonine or isoleucine and sensitive to methionine and threonine respectively. In contrast to the other bacterial AKs, whose activities declined during stationary phase, the AKs in *L. plantarum* remained active throughout stationary phase, but remained unresponsive to their inhibitors, as was reported in plants. All isozymes exist as trimers with subunits of identical molecular weight generating a total molecular mass of 224 860 Da.

4.2. INTRODUCTION

According to IUPAC-IUB Commission on Biochemical Nomenclature, isozymes are defined as multiple enzyme forms in the single species catalyzing the same reaction. To date, seven varieties of isozymes have been identified: (1) genetically independent proteins encoded by separate genes and often of different locations, e.g. malate dehydrogenase (2), polymeric isozymes in which subunits are encoded by more than one locus, e.g. lactate dehydrogenase (3), isozymes encoded by allelic genes (4), isozymes consisted of a series of polypeptides of a single subunit e.g. glutamate dehydrogenase (5), isozymes formed as a result of post-translational modifications e.g. phosphatase and fucosidase (6), isozymes which arose by partial proteolysis of the original polypepetide, e.g. trypsin and chymotrypsin and (7) conformational isozymes (Markert 1977). High diversity in origin implies high diversity in structure and application. Indeed, most of the biological systems and biosynthesis, from prokaryotic to eukaryotic including mammals, are under regulation of isozymes. There is evidence that ancient enzymes did not show high specificity to substrates or single catalysis but rather that ancestral enzymes produced families of products from families of substrates (Jensen and Byng 1977). During the evolution, the differentiation of the habitats imposed microenvironments that are more specific; accordingly, bacteria have developed highly specialized biosynthetic machinery. The specialization of the enzyme species by narrowing and modifications of the substrate specificity, followed by the gene duplication or some other gene amplification, lead to the formation of isozymes.

The biosynthesis of L-lysine, L-threonine, L-methionine and L-isoleucine in most bacteria and plants occurs through the diaminopimelic (DAP) pathway, with AK as the key regulatory enzyme. Aspartokinase (EC 2.7.2.4) catalyzes the phosphorylation of L-aspartate to aspartyl-phosphate, the first step in the biosynthesis of aspartate family amino acids. Due to the complex nature of this pathway, bacteria AK activity is subject to a complex regulation by end-products and a multivalent repression. According to the current understanding, it seems that most bacteria share the DAP pathway for the biosynthesis of the aspartate family amino acids, with species-specific regulation. In *Escherichia coli*, there are three isozymes of AK and two homoserine dehydrogenases, that are regulated by different end-products (Theze et al. 1974). Lysine, threonine and methionine regulate AKs of *E. coli*. Aspartokinase I and homoserine dehydrogenase I are subject to divalent repression by L-threonine and L-isoleucine. This is because L-threonine is the precursor for L-isoleucine and product repression only by L-threonine could create difficulties for the pathway. The level of synthesis for aspartokinase II and homoserine dehydrogenase II is controlled by L-methionine and that of aspartokinase III of L-lysine. The branch of the pathway dealing with L-isoleucine, L-valine and L-leucine biosynthesis has four steps catalyzed by the same enzyme. Furthermore, α-oxo-β-methylbutarate is not only the precursor of L-valine, but also the starting material for L-leucine synthesis. In this case, a trivalent repression is installed in order to prevent a shortage of one of the three amino acids. L-leucine alone represses the synthesis of the enzymes leading from α-oxo-β-methylbutarate to L-leucine.

Serratia marcescens has two AKs inhibited and repressed by lysine and threonine (Komatsubara et al. 1979). In *Corynebacterium glutamicum*, single aspatrokinase is inhibited by

lysine alone (Cremer et al. 1988). In *Bacillus subtilis* there are three AKs inhibited synergistically by lysine and threonine, meso-DAP and lysine alone (Zhang et al. 1990). Hernando-Rico et al. (2001) have reported a single AK in actinomycetum, *Amycolatopsis lactamdurans* that is inhibited synergistically by lysine and threonine. Similar to bacteria, plants also synthesize the aspartate family amino acids via the DAP pathway and the number and sensitivity of isozymes differs among the plant species accordingly. In plants, specifically carrots and maize, it has been reported that expression of lysine and threonine sensitive aspartokinases' is tissue and age dependent (Relton et al. 1987, Dotson et al. 1989). In barley there are three AK isozymes, two are lysine sensitive and one threonine sensitive (Rognes et al. 1983).

There are only a handful of studies on the regulation of the DAP pathway in lactobacilli and their findings differ. Adebawo et al. (1997) found that none of the amino acids of the aspartate family amino acids inhibits or represses AK, while Cahyanto et al. (2006, 2007) found that AK is inhibited by lysine and repressed by threonine. Later work was based on analysis of genome sequence and gene expression; however, it is unclear if these are activities of isozymes. Presence of AK isozymes was not known previously in *L. plantarum* NRRL B 4496. While proteomic analysis and the use of zymograms could provide additional clarity, there are only two reports on the use of zymograms of AK, both relating to AK in plants (Relton et al. 1987, Dotson et al. 1989).

During the study of the regulation of the DAP pathway in *L. plantarum*, a changing relationship between the regulation of the pathway and the stages of culture growth was discovered. These results undoubtedly contribute to an understanding of the overall metabolic pathway and its dynamics. The objective of this study is to define the regulation of AK in *L.*

plantarum NRRL B 4496 at different growth stages and to characterize isozymes and subunit composition. The zymography coupled with the growth studies gave enough of an indication that *L. plantarum* adapts the regulation of a vital biosynthetic pathway, the DAP pathway, to its highly specialized nutritional requirements. Having in mind the versatility of the genus, the growth studies with respect to the DAP pathway regulation were performed on *L. delbrueckii* as well.

4.3. MATERIALS AND METHODS

4.3.1. Bacterial strains and growth conditions. *Lactobacillus plantarum* NRRL B 4496 and *L. delbrueckii* ATCC 9649 were stored and maintained as described in Chapter 1. The enzyme studies were performed with *L. plantarum* in M3. To study repression of AK, the same medium supplemented with 10 mM of the amino acid of interest was used. Growth experiments in MRS broth/agar as well as on the chemically defined medium were carried out at 30 °C.

4.3.2. Growth studies. LAB strains, *L. plantarum* and *L. delbrueckii*, were used in the comparative growth studies. The basal medium used was M3 with omitted aspartate family amino acids except L-isoleucine, where L-lysine, L-threonine, L-methionine, L-isoleucine and DL- diaminopimelic acid, which were added singularly or in combination.

The studies were performed in a total volume of 150 µL with 10^6 cells/mL inoculum concentration in a 96 well plate with U-bottom and in quadruplicates. The LAB cultures were incubated for 72 h at 30°C. The growth was estimated by the OD_{620} on Microtiter plate reader (Titertek Multiscan) against M3 as a control.

4.3.3. Crude cell extract. Cells grown in 200 mL of M3 were harvested by centrifugation 1998 (Sorvall, RC 5C) at 10 000 x *g* for 10 min and washed twice in 50 mM Tris HCl, pH 8.0. Washed cells were re-suspended in 100 mL of 50 mM Tris pH 8.0 containing 20 % sucrose and 0.4 mg/mL of lysozyme (Groot and De Bont 1998) and incubated 30 min at 30°C in water bath shaker (New Brunswick Scientific Co Inc, New York) at 150 rpm. Incubated cells were washed, re-suspended in 50 mM Tris as previously described and sonicated for 10 min with 30 sec pulses at 40 % power (Branson Sonifier 450) in an ice bath. The cell debris was removed by centrifugation for 30 min at 18 000 x *g* at 4°C. The clear crude cell extract was dispensed in 300 µL aliquots and stored at -30°C.

4.3.4. Partial purification of aspartokinase. The crude cell extract was brought to 30, 40, 50 and 60 % saturation with ammonium sulfate (MP Biomedical, ultra pure). After stirring the contents for 1 h in an ice bath, the precipitates were removed by centrifugation at 12 000 x *g*, 20 min (Sorval, RC 5C) at 4 °C and dissolved in 50 mM Tris (pH 8.0) containing 50 mM KCl, 1 mM EDTA, 10 % glycerol, 1 mM DTT and 0.1 mM lysine (Relton et al. 1989). Dissolved precipitates were dialyzed for 18 h at 4 °C against the same buffer and concentrated again with polyethylene glycol, 20 000. Fractions were assayed for the aspartokinase activity.

4.3.5. Aspartokinase assay. Essentially, the aspartokinase assay was performed according to the procedure outlined by Black and Wright (1955) with some modifications. The reaction mixture was the one described by Cahyanto et al. (2006) except that 50 mM Tris pH 8.0 was used. All chemicals were purchased from Sigma Aldrich. The pH of the stock solutions was adjusted to 8.0 using Tris base. The chemicals were aliquoted and stored at -30 °C with the exception of hydroxylamine, which was made fresh and pH was brought to 7.7. The assays

were performed in 1 mL of total volume containing 150 µL of the crude cell extract and incubated for 3 h at 37 °C. The reaction was terminated using 500 µL 50 % (w/v) trichloracetic acid adjusted to pH 0.9 with NaOH pellets followed by immediate addition of 200 µL of 1.67 M ferric(III)-chloride hexahydrate (Paulus and Gray 1967). The precipitate was removed by centrifugation, 3 min at 10 000 X *g* (IEC Micromax) and OD_{520} was read on titertek plate reader (Titertek Multiscan) within 20 min. Optical density readings were standardized against the control in which L-aspartate had been omitted. Enzymatic activity was expressed as µmol of hydroxamate $min^{-1}mg^{-1}$ using L-aspartic acid β-hydroxamate as a standard (Cahyanto et al. 2006). The protein content was determined by Bradford microassay with bovine serum albumin as a standard.

4.3.6. SDS-PAGE and zymograms. SDS-PAGE was performed according to Leammli under the reducing conditions with 12 % (w/v) acrylamide gels at the constant voltage (150 V). The gel was stained with Blue silver and scanned images were converted to the gray scale and adjusted using Adobe Photoshop Professional software, 6.0.

The crude cell extracts were concentrated by freeze drying (Labconco) to approximately 1/10 of the starting volume. Concentrated samples were run on 12 % acrylamide gel at native conditions at 4 °C and 10 mA of constant current for approximately 4 h. Prior to loading the samples, the gel was pre-run for 15 min to remove TEMED and ammonium persulfate, as they could interfere with the enzyme activity. The sample buffer used was made according to Qazi and Khachatourians (2007). To preserve enzyme activity, Triton X (1 g/L) was added to the running buffer. Zymograms were incubated for 6 h at 37 °C in the buffer described by Dotson et al. (1989) except 25 mM ATP was used instead of originally proposed 10 mM. In order to

see the inhibitory effect of lysine on AK isozymes, the buffer was supplemented with 10 mM lysine. The negative control was the same reaction mixture from which L-aspartate was omitted. Aspartokinase activity was visualized using calcium phosphate precipitation method (Relton et al. 1988). The excess calcium phosphate was removed by vigorous shaking of the gel in 50 mM Tris pH 8.0 for up to 2 h. The images were taken on Bio Doc IT Imaging System, and turned into gray scale using Adobe Photoshop Professional software, 6.0.

4.3.7. Recovery of AK from the gels and assay. The bands were cut from the zymogram and proteins of each band was extracted with 200 µL of a buffer containing 50 mM Tris, (pH 8.0), 1 mM DTT and 20 % (v/v) glycerol for 17 h at 4 °C on a reciprocal shaker at 300 rpm (New Brunswick Scientific, Inc). Aspartokinase activity was assayed using 20 µL of eluent in a total volume of 100 µL.

4.3.8. Statistical analysis. Statistics is performed in CoStat software, version 6.0. Analysis of variance (ANOVA) was performed using one-way completely randomized experimental design. The level of significance (a, b, c, d) was determined by Duncan's Multiple Range test where $p \leq 0.05$. The level of significance was used to assess inhibition and/or repression of AK and AK isozymes.

4.4. RESULTS

In order to provide more complete information on the regulation of the DAP pathway in *L. plantarum*, the details of regulation were observed with respect to the exponential and the stationary phases of growth. Cells after 18 h and 28 h of growth were used for studies performed in exponential and stationary phases respectively. The levels of AK in the crude cell extract were measured by spectrophotometric assays. The study of inhibition was performed by

adding the amino acids to the enzyme preparation from the exponential or stationary cell culture. The repression study was performed by addition of amino acids in a concentration of 10 mM to M3.

The results revealed that the level of AK activity depends on the bacterial growth stage (Table 4.1). In the crude cell extract obtained from exponential phase culture, the addition of isoleucine, methionine and lysine to the enzyme preparation showed some stimulation, while the addition of threonine caused highly significant inhibition (62.2 %) of AK activity. The presence of lysine or threonine in the growth medium caused the repression of AK 52.4 % and 32.9 % respectively, during the exponential phase (Table 4.1). Therefore, contrary to the finding of Cahyanto et al. (2006) who reported repression by threonine alone, lysine and threonine both exerted repression in the system used in this thesis. On the other hand, methionine and isoleucine brought the most significant de-repression.

A significant decline in the levels of AK specific activity was observed in the stationary phase cultures. In stationary phase culture, addition of lysine to the reaction mixture caused 65.8 % inhibition of AK (Table 4.1). This is in agreement with Cahyanto et al. (2006); although they did not specify the growth phase. However, repression was not expected nor observed in stationary phase.

Table 4.1. Regulation of aspartokinase in crude cell extracts of *L. plantarum* NRRL B 4496 in exponential and stationary stages of growth.

Amino acid added	EXPONENTIAL PHASE	
	Aspartokinase activity (μmol $min^{-1}mg^{-1}$)	
	Inhibition	Repression
None	7.48 ± 0.16 [a]	5.19 ± 0.03 [b]
Lysine	9.32 ± 0.54 [a]	2.47 ± 0.08 [d]
Threonine	2.83 ± 0.09 [b]	3.48 ± 0.19 [c]
Methionine	8.41 ± 0.27 [a]	12.01 ± 0.35 [a]
Isoleucine	7.95 ± 0.81 [a]	11.59 ± 0.28 [a]
	STATIONARY PHASE	
	Inhibition	Repression
None	2.84 ± 0.16 [c]	2.10 ± 0.03 [d]
Lysine	0.97 ± 0.02 [d]	4.55 ± 0.06 [c]
Threonine	3.28 ± 0.05 [b]	6.55 ± 0.33 [ab]
Methionine	3.74 ± 0.05 [a]	8.00 ± 0.53 [a]
Isoleucine	2.87 ± 0.02 [c]	5.96 ± 0.64 [bc]

AK activity is expressed as a mean ± standard deviation of two independent experiments, each performed in triplicate. Means followed by the same letter in a column do not differ significantly ($p \leq 0.05$).

The specific zymographic method was used to investigate the status of particular isozymes during different growth stages. Zymograms revealed four bands (Figure 4.1B) that were L-aspartic acid sensitive (Figure 4.1C) and showed AK activity in spectrophotometric assay. These bands were designated as AK I-IV based on the sensitivity to different amino acids of the aspartate family amino acids. It can be appreciated from Figure 4.1 that only isozymes AK II and AK IV were active in the exponential stage of growth, while all four isozymes were active in the stationary stage of growth.

Relton et al. (1987) and Dotson et al. (1989) reported that only light colored bands appearing as a white calcium phosphate precipitate showed the aspartokinase activity. Surprisingly, as depicted in Figures 4.1 and 4.2, dark bands also showed aspartokinase activity, but were of decreased amounts when compared to the bands appearing as the white precipitate (Table 4.2). As shown in Figure 4.1A, AK IV that appeared as a white precipitate, had an activity of 25.44 μmol $min^{-1}mg^{-1}$ while AK II appeared as dark band with an activity of 12.92 μmol $min^{-1}mg^{-1}$ (Table 4.2).

Next, amino acid inhibition, induction and repression of the purified AK isozymes were tested. The amounts of activity of specific isozymes differed with respect to the growth stage in concordance to the observation with the crude cell extract.

As shown in Figure 4.1A and Table 4.2 AK I was lysine sensitive and not active throughout the exponential phase unless the cells were subjected to an excess amount of the aspartate family amino acids in the growth medium (Figure 4.2B). The activity of AK I was strongly activated (41.53 μmol $min^{-1}mg^{-1}$) when cells were incubated with threonine. However, at this point it was not clear if this was due to induction or de-repression. Keeping in mind

that AK I was inhibited by lysine and activated by threonine, apparently its role was mainly to balance the pools of these two amino acids. From all of the aspartate family amino acids, isoleucine seemed to have the least inducing effect on AK I where the activity was 9.92 μmol $min^{-1}mg^{-1}$, two or three fold lower compared to the effects of methionine and lysine (Table 4.2).

The AK II was methionine sensitive, showing 84.5 % inhibition by methionine. It was repressed by lysine or threonine or methionine (Table 4.2). Threonine caused only slight increase of activity (approximately 20 %) in methionine sensitive AK, while isoleucine was shown to be its strong activator (41.4 %). AK II was active throughout both the exponential and stationary phases (Figure 4.1) suggesting that its main role is to ensure the pool of methionine for protein synthesis.

The AK III, similarly to AK I, was not active in the exponential phase (Figure 4.1), but differed in that unlike AK I, it was repressed by lysine or methionine or isoleucine. This isozymal activity at 7.96 μmol $min^{-1}mg^{-1}$ was observed only if cells were grown in the presence of threonine (Table 4.2). In addition, AK III was assayed by incubating the zymogram in the buffer containing 10mM lysine. It can be appreciated from Figure 4.3A and 4.3B, that the AK III band was absent on the zymogram incubated in a buffer supplemented with lysine. The spot densitometry confirmed inhibition of AK III by lysine to be greater than that of AK I. Both sets of data obtained by either spot densitometry or by spectrophotometric assay, confirmed the conclusion of lysine sensitivity on AK I and AK III (Table 4.2).

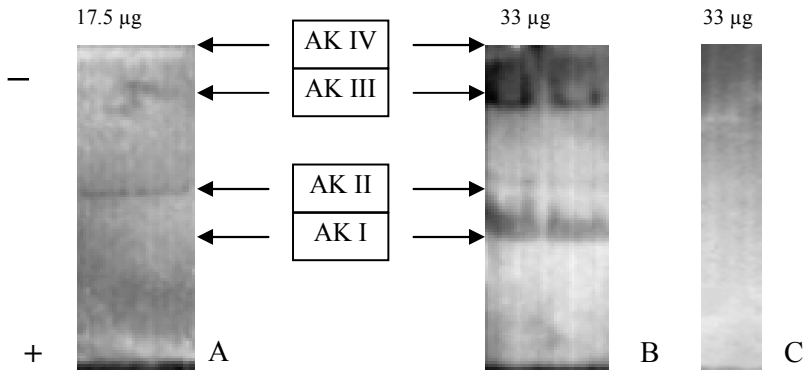

Figure 4.1. Aspartokinase isozymes in *L. plantarum* with respect to the growth stage. Figures are represented as follows: (A) exponential and (B) stationary growth stage, while (C) is negative control from which L-aspartate is omitted. Two lanes are represented for each run. The isozymes are numbered according to IUPAC-IUB Commission on Biochemical Nomenclature (CBN). The amounts of the total protein loaded in a well are indicated above the lanes.

Table 4.2. The inhibition and the repression of aspartokinase isozymes in *L. plantarum* NRRL B 4496 in the exponential growth stage[1].

Isozymes	INHIBITION				
	Aspartokinase activity (μmol $min^{-1}mg^{-1}$)/Amino acid added				
	—	Lysine	Threonine	Methionine	Isoleucine
AK I	9.92±0.82[a]	4.23±0.83[b]	8.77±0.36[a]	10.39±1.20[a]	8.69±0.18[a]
AK II	12.92±0.12[bc]	12.36±0.57[c]	15.45±0.53[b]	2.00±0.80[d]	18.27±0.71[a]
AK III	Nd	Nd	Nd	Nd	Nd
AK IV	25.44±0.94[a]	20.71±0.47[ab]	6.62±1.17[c]	20.60±1.24[ab]	18.47±1.89[b]
	REPRESSION				
AK I	Nd	27.07±1.34	41.53±3.10	21.05±3.82	9.92±0.82
AK II	12.92±0.12	Nd	Nd	Nd	Na
AK III	Nd	Nd	7.96± 0.70	Nd	Nd
AK IV	25.44±0.94[a]	7.01±0.99[c]	Nd	22.18±0.88[a]	17.03±0.15[b]

[1]The study of inhibition was performed on the eluents where amino acids were added into the reaction mixture in concentration of 10 mM. Isozymes AK II and AK IV were eluted from the cells grown in M3. Isozymes AK I and AK III were eluted from the cells grown with the addition of 10 mM isoleucine because these isozymes were not observed on the control zymogram (Figure 4.1). The study of repression was performed on the crude cell extract when 10 mM amino acids were added into the growth medium. The negative control was the crude cell extract obtained from the cells grown in M3. 'Nd' and 'Na' indicated 'not detected' and 'not assayed' respectively. The aspartokinase activity was expressed as a mean ± standard deviation of two independent experiments, each performed in duplicate. Means followed by the same letter in the row do not differ significantly ($p \leq 0.05$).

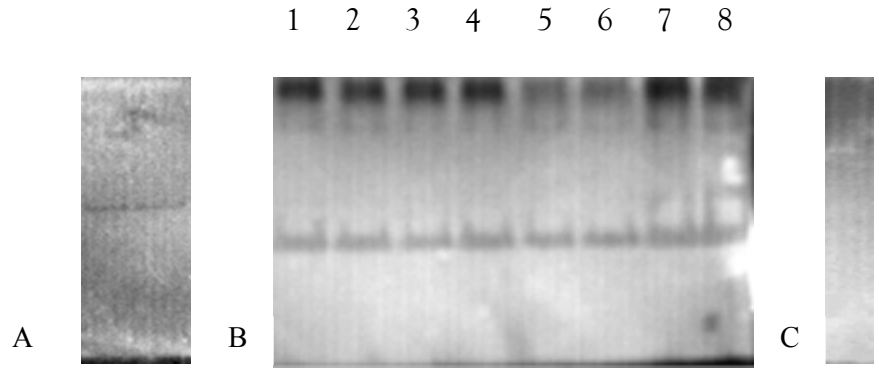

Figure 4.2. Zymograms on the crude cell extract of *L. plantarum* obtained from the exponential growth phase. Image (A) represents the positive control, image (B) cells grown in presence of 10 mM lysine (lanes 1,2 contain 16.3 µg of protein loaded per lane), 10mM threonine (lanes 3,4 contain 14.7 µg protein loaded per lane), 10mM methionine (lanes 5,6 contain 13.12 µg of protein loaded per lane) and 10mM isoleucine (lanes 7,8 contain 14.7 µg of protein loaded per lane). Image (C) represents the negative control.

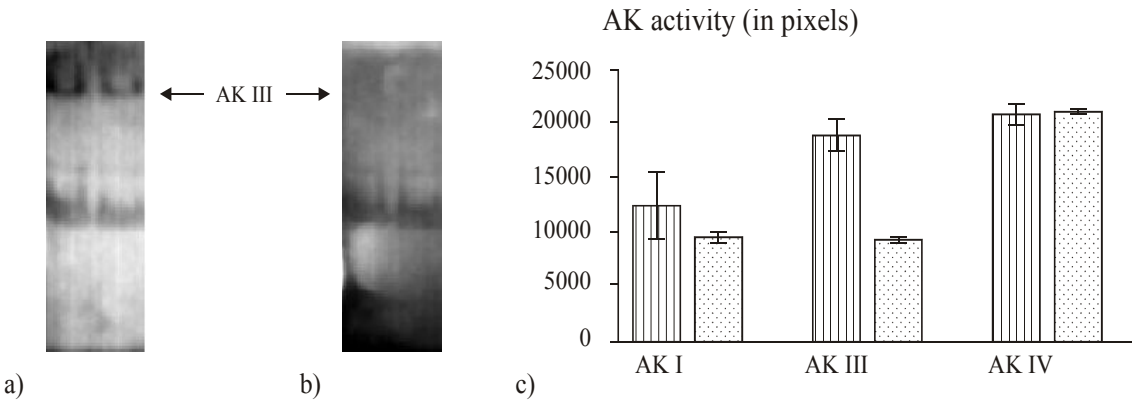

Figure 4.3. The sensitivity of AK III to lysine present in the stationary phase. Image (A) represents zymogram incubated in the absence of lysine. Image (B) represents the same sample incubated in a buffer containing 10 mM lysine. Image (C) represents the results of spot densitometry as recommended by Qazi and Khachatourians (2007) performed on a native zymograms incubated in the buffer containing no lysine (▥) and with 10 mM lysine (⁘).

The AK IV was strongly inhibited (74 %) by threonine, and was strongly repressed by lysine or threonine and to some extent by isoleucine (Table 4.2). Methionine was the only amino acid of the aspartate family amino acids with no inhibitory or repressing effect on AK IV. Zymography confirmed that, among the aspartate family amino acids, methionine alone does not have its repressing effect on the threonine sensitive aspartokinase. Figure 4.2B, lanes 5 and 6 showed AK IV as a light colored band when the cells were grown in the presence of methionine. Consequently, the activity of AK IV (22.18 μmol $min^{-1}mg^{-1}$) was comparable to that of control (25.44 μmol $min^{-1}mg^{-1}$) as opposed to AK IV activities when the cells were exposed to 10 mM lysine, threonine and isoleucine (Table 4.2). AK IV was active in both, the exponential and stationary phase, which suggests that the main purpose of AK IV is obtaining threonine for protein synthesis.

Another interesting phenomenon in AK's of *L. plantarum* was observed: not only that AK was regulated differently in a different growth stages, but AK also lost sensitivity to its inhibitors in the stationary phase (Figure 4.4). The similar phenomenon has been reported in maize, where homoserine dehydrogenase regulation is growth stage dependant, tissue dependant and insensitive to its inhibitor threonine in older tissue (Matthews et al. 1975).

In order to determine the size and the number of AK subunits, the bands were cut, eluted and ran on SDS PAGE (Figure 4.5) followed by Blue silver staining because of its greater sensitivity (Candiano et al. 2004). SDS PAGE revealed that each isozyme exists as a trimer with Mw of subunits ranging 63 534, 75 299 and 86 027 Da respectively, generating a total molecular mass of 224 860 Da.

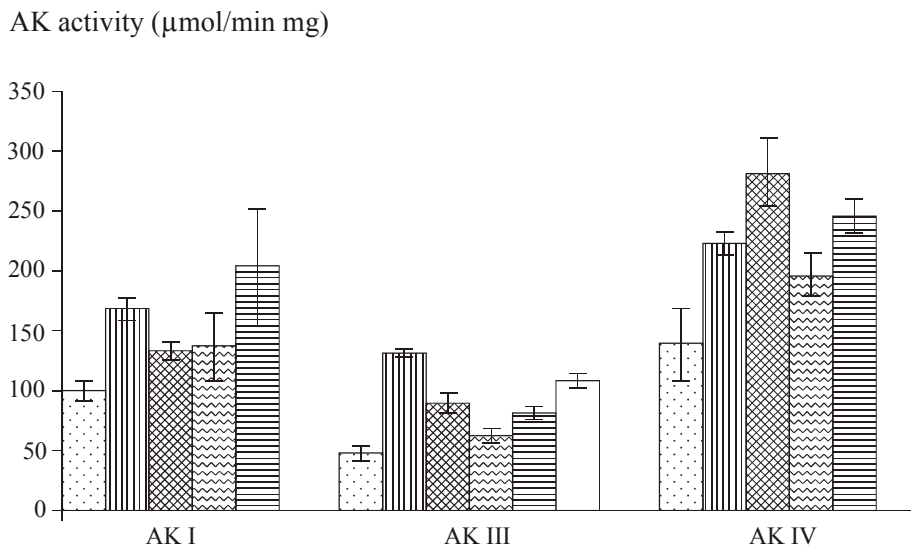

Figure 4.4. Amino acid sensitivities of AK isozymes of *L. plantarum* in stationary phase. Assays were performed on band eluents of zymograms of concentrated crude cell extracts. Amino acids at a final concentration of 10 mM were added to the reaction mixture. Bars represent as follows:( ) control, ( ) lysine, ( ) threonine, ( ) methionine, ( ) isoleucine and ( ) lysine + threonine. The AK activity is expressed as a mean ± standard deviation of two independent experiments each performed in duplicate.

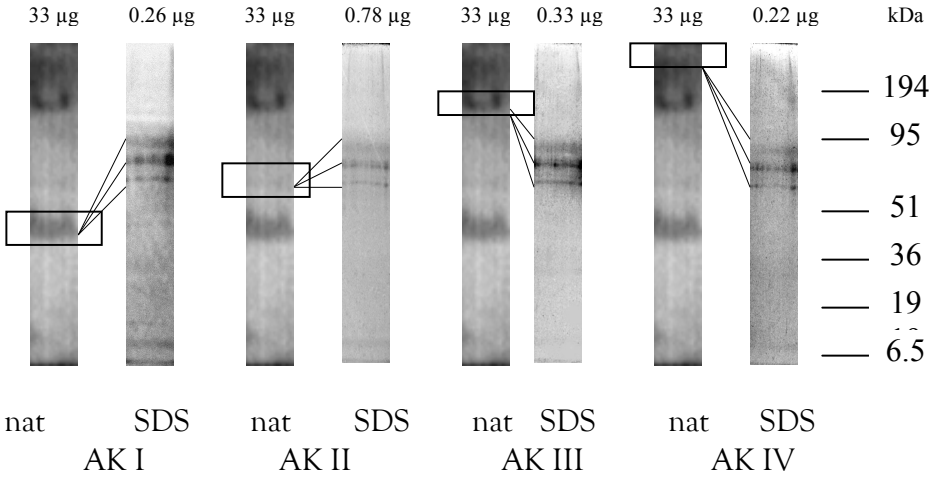

Figure 4.5. Subunit molecular weight determinations of AK isozymes in *L. plantarum.* Isozymes were eluted from the zymogram obtained from the crude cell extract of the cells during stationary growth stage and run on SDS PAGE. Molecular weight standards are myosin (Mr 194 kDa), bovine serum albumin (Mr 95 kDa), ovalbumin (Mr 51 kDa), carbonic anhydrase (Mr 36 kDa), lysozyme (Mr 19 kDa) and aprotinin (Mr 6.5 kDa).

An AK of a similar size was reported in *Bacillus subtilis*, 250 000 Da (Zhang et al. 1990), maize 255 000 Da (Dotson et al. 1989) and carrot 253 000 Da (Relton et al. 1988). In *C. glutamicum*, AK is a medium sized protein, 65 000 Da (Kalinowski et al. 1991).

Zymographic technique, in spite of its high resolution, is a technique infrequently used for separation of isozymes. On the other hand, partial purification of a crude cell extract with ammonium sulfate followed by additional purification by ion exchange chromatography, affinity chromatography or isoelectric focusing are those used frequently.

In this thesis, partial purification of the crude cell extract with ammonium sulfate was performed in order to compare the isozymes obtained by the zymographic technique. It was shown that purification yielded higher specific activity with the higher fraction, meaning that the process itself was successful (Table 4.3). Relton et al. (1988) showed that AK activity could only be detected by partial purification by ammonium sulfate if lysine was added to the precipitation buffer. This has been confirmed in this thesis, as if lysine was omitted from the precipitation buffer, AK activity was not observed.

However, spectrophotometric enzyme assay performed on the fractions gave different results from those obtained on the zymograms. Threonine sensitive AK isozyme precipitated at 30 % and 60 %, methionine sensitive isozyme precipitated at 40 % and lysine sensitive isozyme precipitated at 60 % of ammonium sulfate saturation (Table 4.4). At first glance, these results suggested the conclusion that AK in *L. plantarum* has four isozymes, two of which were threonine sensitive. Based on zymograms, AK in *L. plantarum* has four isozymes indeed, two of which were lysine sensitive.

Table 4.3. Partial purification of the crude cell extract of *L. plantarum* by ammonium sulfate.

Extract or $(NH_4)_2SO_4$ fraction	Volume (mL)	Protein conc.(mg mL^{-1})	Specific activity (µmol $min^{-1}mg^{-1}$)	Purification (%)
Crude extract	67.0	0.806	2.77±0.09	100
$(NH_4)_2SO_4$ 30 %	2.4	0.662	3.31±0.17	119.27
40 %	2.0	0.709	3.36±0.04	121.08
50 %	1.8	0.807	3.84±0.10	138.37
60 %	2.3	0.875	3.98±0.10	143.42

Specific activity is expressed as a mean ± standard deviation of two independent experiments each performed in triplicate.

Table 4.4. The inhibition of aspartokinase by the aspartate family amino acids upon partial purification ammonium sulfate.

Amino acid added	Ammonium sulfate saturation / aspartokinase activity (μmol $min^{-1}mg^{-1}$)				
	Crude cell extract	30%	40 %	50 %	60 %
-	2.75±0.09 [a]	3.31±0.17 [a]	3.36±0.04 [d]	3.84±0.10 [a]	3.98±0.11 [a]
Lysine	3.45±0.01 [a]	2.36±0.04 [b]	6.1 ± 0.08 [b]	3.21±0.71 [a]	2.86±0.08 [b]
Threonine	0.96±0.04 [b]	1.28±0.03 [c]	6.73 ± 0.15 [a]	3.93±0.03 [a]	2.83±0.07 [b]
Methionine	3.52±0.40 [a]	3.95±0.33 [a]	2.54 ± 0.05 [e]	4.08±0 [a]	4.11±0 [a]
Isoleucine	2.93±0.42 [a]	3.13±0.21 [ab]	4.07 ± 0.2 [c]	3.12±0.07 [a]	3.96±0.11 [a]

The AK activity is expressed as a mean ± standard deviation of two independent experiments each performed in triplicate. Means followed by the same letter in the column do not differ significantly ($p \leq 0.05$).

It is possible that the same isozyme precipitates at more than one ammonium sulfate fraction (Stadtman et al. 1961) that must be confirmed by methods that are more specific for an isozymal entity. In some cases, isozymes are hard to separate.

Only a few tools can distinguish one isozyme from another: pH optima, susceptibility to cofactors, kinetic parameters and different substrate concentration optima (Markert 1977). In order to distinguish isozymes from each other, partial purification by ammonium sulfate is just a first step and it is necessary to continue further purification in order to perform kinetic studies. This is labor and time consuming. In this thesis, concentrating the sample by freeze-drying followed by the zymographic technique has proven the best choice for studies on biosynthesis and regulation with respect to isozymes.

Studies on the growth of *L. plantarum* and *L. delbrueckii* in relation to regulation of DAP pathway were undertaken to complement the above results obtained by enzyme assay. Cahyanto et al. (2006) found that the results of simple growth studies of *L. plantarum* were in concordance with AK enzyme assay using the crude cell extract. However, Adebawo et al. (1997) found discrepancies of results when these two methods were used. Study of growth can be used as a first choice in order to identify the phenomenon, but one has to be aware of its limitations.

The studies of growth in *L. plantarum* have confirmed that lysine and threonine affected the regulation of AK (Figure 4.6A). These findings agreed with the enzyme assay performed using the crude cell extract from stationary phase cultures. It is worth mentioning that the above-mentioned growth studies represent end-point data that correspond to the stationary growth phase. The same could be said about the influence of methionine on AK

(Figure 4.6B) and its concordance with the data obtained on activation, inhibition and repression of specific isozymes in *L. plantarum*.

In *L. delbrueckii* AK was regulated in the same manner as in *L. plantarum*. It can be appreciated from Figure 4.6C that lysine and threonine exerted inhibition in AK of *L. delbrueckii*, similar to *L. plantarum*. Unlike *L. plantarum*, in *L. delbrueckii* methionine de-repressed or activated the inhibition brought about by both lysine and threonine (Figure 4.6D). The phenomenon was more prominent than that in *L. plantarum*. However, one must keep in mind that, although the growth studies are precise enough to allow observation of the general phenomenon, they do not allow one to conclude whether the influence of threonine on AK in *L. delbrueckii* is due to inhibition or repression. Additional studies on enzyme kinetics of isozymes are needed in order to address that question.

In some bacterial species, AK is synergistically inhibited by two amino acids. In this thesis, that possibility was also explored during the growth of *L. plantarum* and *L. delbrueckii*. As lysine, threonine and methionine play the main role in regulation in *L. plantarum*, therefore synergistic influence of these three amino acids was explored. However, as already shown by Cahyatno et al. (2006), no synergistic inhibitory effect of aspartate family amino acids was expected nor found here (Figure 4.6E and F).

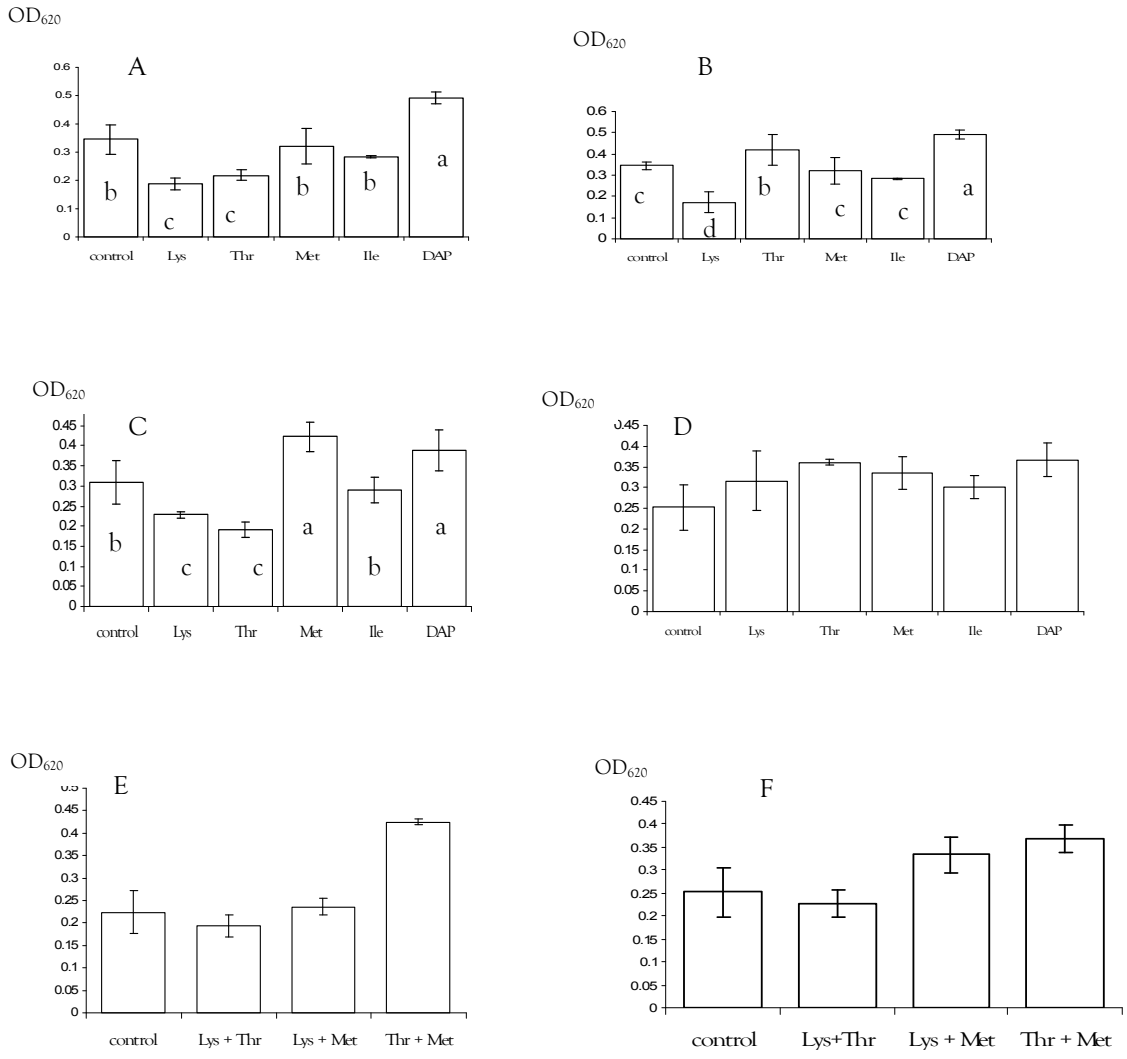

Figure 4.6. The influence of the aspartate family amino acids on the growth of *L. plantarum* and *L. delbrueckii*. Panels A, B and E show the growth response of *L. plantarum*. Panels C, D and F show the growth response of *L. delbrueckii*. The axis X shows amino acids added. Panels A and C show growth in the absence of methionine and B and D the presence of methionine (0.1 g/L) in the tested media. Tested amino acids were added in concentration of 1 g/L. The control is growth in M3. Data are presented as an average of four replicates with standard deviations.

4.5. DISCUSSION

The amino acid biosynthesis pathway studied here plays a vital role in providing precursors for protein and bacterial cell wall synthesis. As such, amino acid biosynthesis is subjected to a tight and complex regulation where all of the amino acids that are end- products are involved in the regulation in some manner (Table 4.5).

In most bacterial and plant species, AK exists in a form of two or three isozymes and in a few cases as a single form. In this study, four isozymes were found in *L. plantarum*. The DAP pathway is crucial in *L. plantarum* for two reasons: it is the source of four amino acids, some essential for bacterial growth (Chapter 3) and it is a source of DAP, the cell wall constituent. The cell wall of *L. plantarum* contains peptidoglycan of meso-DAP type (Perkins 1963). The branch of the pathway for the synthesis of the DAP leads only to lysine where DAP is the direct precursor. During the exponential growth phase, lysine had no inhibitory, but rather stimulatory effect on AK, likely because DAP synthesis is highly important for the cell wall formation and cell proliferation.

The zymographic technique was considered as the most appropriate approach of demonstrating the AK activity as opposed to the partial purification with ammonium sulfate because it can (i) be customized to substrate-enzyme pair, (ii) be performed with a high degree of sensitivity allowing quantification of activity for nano grams of enzyme, (iii) permit quantitative assessment with respect to activity, and (iv) make convenient future use of isozymes separated and immobilized on gel matrix convenient.

Table 4.5. The summary of the regulation of aspartokinase isozymes by the aspartate family amino acids in *L. plantarum* NRRL B 4496.

Isozyme	Inhibitor	Inducer	Repressor	Presence in a growth phase
AK I	Lys	Thr	not repressed by AF	Stationary
AK II	Met	–	Lys or Thr or Met	Stationary and exponential
AK III	Lys	Thr	Lys or Met or Ile	Stationary
AK IV	Thr	–	Lys or Thr or Ile	Stationary and exponential

Lys = lysine, Thr = threonine, Met = methionine, Ile = isoleucine, AF = aspartate family amino acids

It was found that concentration of the sample by freeze-drying as opposed to purification (Relton et al. 1987, Dotson et al. 1989) made determination of isozymes easier.

In this study was found that two isozymes of *L. plantarum,* AK II and AK IV, were active during the exponential phase suggesting that the main purpose was to ensure synthesis of methionine and threonine for growth. In *Bacillus subtilis,* AK II and AK III showed similar activity; their level declined at the end of the exponential growth (Zhang et al. 1990). However, in *Bacillus* species, post-exponential growth and entry into sporulation is a unique event, which is absent in *L. plantarum.* Unlike AK II and AK III in *Bacillus*, AK II and AK IV in *L. plantarum* are present in the stationary phase as well, but are unresponsive to their inhibitors. This type of regulation was reported in maize (Matthews et al. 1975). Knowing that *L. plantarum* is, in terms of its habitat often associated with plants, it is tempting to suggest gene transfer between the two groups.

Unique in terms of regulation is the existence of two AK isoforms, active only in the stationary phase of growth and/or if cells are exposed to an excess of the aspartate family amino acids. In all likelihood, the excess of amino acids triggers the biosynthesis of AK I and AK III as a high, but underutilized amino acid pool can be expected in the stationary stage. Interestingly, both isozymes were lysine sensitive, although repression must occur in a different manner. Aspartokinase I was active only when any of the amino acids of the aspartate family amino acids were present in excess in the growth environment. It was inhibited by lysine, signaling that cell wall synthesis was no longer needed as DAP was synthesized only in the branch that leads solely to lysine. Threonine appeared to be the inducer of AK III, but unlike AK I, AK III is repressed by lysine or methionine or isoleucine. Sensitivity to lysine, induction

solely by threonine and repressibility by lysine, methionine or isoleucine dedicate AK III isozyme action to be dedicated only to balancing the pool of lysine and threonine in conditions when bacterium is exposed to an excess of amino acids. Two lysine sensitive AKs, both active in the stationary phase, explain the overall inhibition by lysine, in spite of the fact that threonine and methionine sensitive isozymes were still active. The presence of two lysine sensitive isozymes is similar to that in barley plants (Rognes et al. 1983). Once again, there is similarity to the plants in terms of AK regulation. The simultaneous presence of two similar entities suggests that elimination of one by mutation would leave the cell with the means to compensate for the deficiency by the utilization of the remaining gene product. One must agree that this evolutionary choice is convenient for the members of this genus that harbor high numbers of point mutations responsible for complex nutritional requirements (Morishita et al. 1981).

High genomic plasticity and adaptability of *L. plantarum* was established and discussed from different aspects. This study showed that *L. plantarum*, by changing the level of activity of four AK isozymes, adjusted internal amino acid pools according to the cell's need in metabolically different growth stages: exponential and stationary. As a result, the DAP pathway regulation differed between these two stages (Figure 4.7). Interestingly enough, this thesis found that the regulation of AK in the crude cell extracts is due to the presence of single isozymes in each growth phase studied.

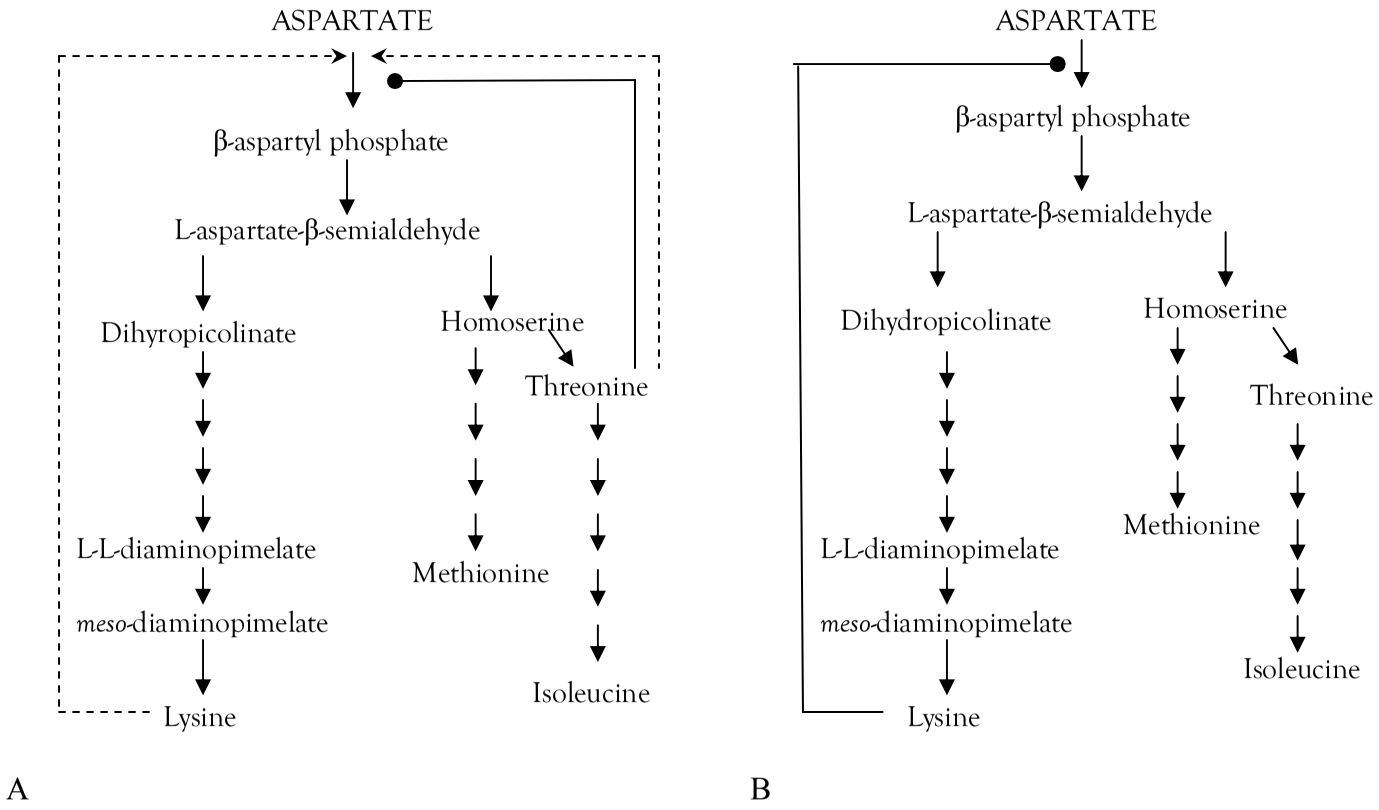

Figure 4.7. The biosynthetic pathway and the regulation of the aspartate family amino acids in *L. plantarum*. Figures represent the regulation of AK in the crude cell extract in (A) exponential phase and (B) stationary phase. The inhibition is represented by (——●) and the repression by (- - - -➤) symbol.

Examination of AK I-IV activity in crude cell extracts in exponential phase revealed that the greatest single isozyme contributing to the AK activity is AK IV. It responded to Thr (inhibitor), Lys and Thr (repressors), which was in concordance to regulation AK exerts in crude cell extract. Figure 4.1 showed the evidence that AK IV was the most active isozyme in the exponential phase. The expression of precipitation was referred as a measure of activity, which in this case is white, i.e., and significantly higher levels than that of AK II. In stationary phase, however, the greatest single isozyme contributing to the AK activity in crude cell extract was AK I. Table 4.2 shows that AK I was inhibited by lysine and not repressed by the aspartic acid family of amino acids, which corresponded to the regulation of AK in crude cell extract (Table 4.1). It can be appreciated from Figure 4.4 that activity of AK I was greater than that of AK III in stationary phase. SDS PAGE revealed that AK isozymes in *L. plantarum* NRRL B 4496 were trimetric and of an identical molecular weight suggesting recent gene duplication.

Binding of subunits into multimeric form may produce more uniform structures than the individual subunits would have as monomers. These facts point out that AK in *L. plantarum* has evolved to ensure evolutionary stability and flexibility, necessary to survive in a broad range of environments and frequent exposure to stress. Lactobacilli in general are adapted to a diversity of environments rich in protein and carbohydrate breakdown products, thus relying on the uptake of peptides and free amino acids from the environment. Due to this, they have evolved as multiple auxotrophs, one of their most striking features. Since the absence or presence of biosynthetic pathways does affect metabolism, it is tempting to believe that auxotrophy has an impact on the regulation of a vital biosynthetic pathway like the DAP. In *L. plantarum* NRRL B 4496, either lysine or threonine is necessary in the medium. Indeed,

the regulation of three aspartokinase isozymes, AK I, AK II and AK IV is shared by these two amino acids. Methionine is stimulatory in terms of requirements, inhibits only AK II and represses AK II and AK III. Isoleucine is essential in *L. plantarum* and in most other lactobacilli (Bringel and Hubert 2003); none of the AK isozymes is sensitive to isoleucine that represses only AK III and AK IV. It seems that emphasis is given to amino acids for which functional biosynthetic pathways exist as opposed to amino acids that are abundant in the environment.

Unlike *L. plantarum*, *Streptococcus bovis* has only two AKs, one inhibited and repressed by lysine, the other inhibited by DAP, while its repressor is unknown (Kalcheva et al. 1994). *Streptococcus faecalis* has a single aspartokinase inhibited and repressed by lysine (Gilboe et al. 1968). Note that in the genera lactobacillus certain *Streptococcus* species have the simplest requirements as opposed to *L. plantarum* NRRL B 4496 which requires 14 amino acids (Chapter 3) and *L. plantarum* NCIMB 8826 (Cahyanto et al. 2006) that requires 9 amino acids (Morishita et al. 1981).

The above reports on the regulation of DAP in *Streptococcus* and *L. plantarum* NCIMB 8826, force the conclusion that it is species-dependant in lactobacilli. However, according to the comparative studies on growth, *L. delbrueckii* and *L. plantarum*, share similarities with respect to the regulation of DAP pathway where both lysine and threonine seemed to play an important role in the regulation. These two species are distantly related on the phylogenic tree in spite of the fact that they do have common nutritional requirements (Chapter 3).

In conclusion, this study gave sufficient indication that members of the lactobacillus genus synthesize the aspartate family of amino acids via DAP pathway as in many other

bacteria and in plants, but because of the highly specialized nutritional requirements, its regulation differs compared to other genera.

4.6. RELEVANCE OF CHAPTER 4 TO THE THESIS

Lack of information about the regulation of lysine biosynthesis in lactobacilli made it necessary to investigate this complex pathway and its regulation. The results obtained in Chapter 5 confirmed that AK is pivotal to the synthesis of lysine and the regulation of the biosynthetic pathway. The knowledge gained helped in the task of isolating lysine over-producing mutants of *L. plantarum*. It was shown again that applied microbiology could be based only on the strong basic knowledge of physiology of microorganisms. Viewing the findings from that prospective, Chapter 4 is the most valuable contribution of this thesis to general understanding of the genus *Lactobacilli*.

5. ISOLATION OF SPONTANEOUS OR INDUCED *L. PLANTARUM* NRRL B 4496 LYSINE OVER-PRODUCING MUTANTS

5.1. ABSTRACT

The aim of this study was to mutant *L. plantarum* resistant to lysine analog(s) and therefore select a lysine over-producer. *Lactobacillus plantarum* cells were challenged by four analogs: *S*-aminoethyl-L-cysteine (AEC), ß-flouropyruvic acid (FPA), DL-aspartic acid ß-hydroxamate (ASP) and L-lysine hydroxamate (LHX). The former analog was found to be the most potent; although, the bacterium tolerated all of the remaining analogs well. Two methods were employed in order to get the most significant inhibition of the growth (i) challenging the bacterium with the analog after depletion of the endogenous lysine and (ii) modifying the chemically defined medium by removing nutrients. The second method provided better results. The minimal inhibitory concentration of LHX in the modified M3, designated as M17, was 2 mM as opposed to the minimal inhibitory concentration of 20 mM in M3. While the wild type did not excrete detectable amounts of lysine, the mutant resistant to 2 mM LHX consistently secreted 4.52 µM of lysine into M3. Surprisingly, the lysine content could not be further improved by isolation of secondary mutants after UV irradiation. This suggested that lysine over-producers in lactobacilli are rare and that larger searches or site-directed mutagenesis would be a better choice.

5.2. INTRODUCTION

The effect of the metabolite analogs (anti-metabolites, antagonists) on biological systems has received and continues to receive a great deal of attention. This phenomenon has been studied from different venues according to their applications. In pharmacology, analogs are interesting as inhibitors of enzymes responsible for the development of tumors and/or cancer. Furthermore, in this field the analog-enzyme relationship has been studied in more detail than in microbiology. Current research in microbiology has shifted towards molecular biology; if a certain enzyme is not desirable, the idea is to use a gene knockout strategy instead of a strategy isolating traditional spontaneous mutants. This approach, however, is neither applicable nor practical when it comes to more complex human genetics. Lysine analogs in particular are seen often as antimicrobial agents effective for Gram+ bacterial pathogens, but harmless to mammals due to their inability to synthesize lysine (Hutton et al. 2003).

The purpose of this thesis is to mutant lactobacillus regulatory mutants with a deregulated DAP pathway that can over-produce lysine. Fast and efficient selection of this class of mutants can be obtained using lysine analog(s) as a screening tool. The first observation that a bacterium resistant to anti-metabolites excretes metabolites came from Oakberg and Luria (1947), who reported that *Staphyolcoccus aureus* resistant to sulfonamide excretes *p*-aminobenzoic acid. Extensive studies of *Escherichia coli* resistant to various anti-metabolites showed that this phenomenon could be extended to more bacterial species and anti-metabolites (Adelberg 1958). More importantly, these findings opened the door for studies on the regulation of various biosynthetic pathways. The biochemical basis for an anti-metabolite as a screening tool is that chemical similarity to the metabolites allows them to interfere with the

active site of the enzyme. At the same time, anti-metabolites are sufficiently different, which prevent them from functioning in the place of metabolites, causing toxicity to normal cells. Cells harboring spontaneous mutation(s) in genes encoding an analog binding enzyme, can survive. In the case of enzymes subject to feedback inhibition, the analogs will select for mutant enzymes. Subsequently, the metabolite should accumulate, as the key enzyme cannot respond to the feedback inhibition. Indeed, much of the work done in the isolation of amino acids over-producers using anti-metabolites. The first successes were reported with *Corynebacterium glutamicum* as a lysine over-producer due to resistance to *S*-aminoethyl-L-cysteine (AEC). Sands and Hankin (1974, 1976) reported lysine excreting *L. plantarum* and *Leuconostoc mesenteroides* resistant to AEC. *Escherichia coli* AEC^R mutant was reported by Di Girolamo et al. (1988). Sen (1991) obtained *Arthrobacter globiformis* lysine excreting mutants resistant to AEC. Satiawihardja et al. (1993) reported β-flouropyruvic acid (FPA) and AEC resistant lysine over-producers of *Brevibacterim lactofermentum*. Aside from bacteria, a few attempts have been made to mutant yeast lysine over-producers resistant to the lysine antagonist AEC (Haidaris and Bhattacharjee 1978, Gasent-Ramirez and Benítez 1997, Odunfa et al. 2001).

Metabolites and metabolite analogs are competitive inhibitors (Ghislain et al. 1990). Their affinity of binding to the active site and of enzyme depends on the structural chemistry of the active site as well as the inhibitor itself. Such a compound must exist in similarly charged ionic species at all points within the physiological pH. In fact, anti-metabolites were referred to as 'isosters' to stress their stereochemical similarities (Fowden et al. 1967). However, natural high tolerance to anti-metabolites is possible in bacteria (Kyriacou et al. 1997). For this reason, the following analogs were screened in this thesis: *S*-aminoethyl-L-cysteine (AEC) as the most

frequently used ß-flouropyruvic acid (FPA), DL-aspartic acid ß-hydorxamate (DL-ASP) and L-lysine hydorxamate (LHX).

In order to find lysine over-producing bacteria, two approaches were taken:

1. Isolation of spontaneous analog resistant mutants, and
2. Isolation of UV induced analog resistant mutants.

The spontaneous mutants have the advantage of a low reversion rate because they harbor stable mutations. The disadvantage is that spontaneous mutations occur infrequently. On the other hand, induced mutants occur more frequently, but they will also harbor multiple mutations, resulting in auxotrophy, low growth rate and reversion (secondary mutants such as suppressors). In order to combine favorable characteristics of both, isolation of spontaneous mutant(s) and improving the probability of selection, the isolation of induced mutants approach was employed.

5.3. MATERIALS AND METHODS

5.3.1. Bacteria, media and culture conditions. The study was performed with *L. plantarum* NRRL B 4496 and *L. delbrueckii* ATCC 9649. The strains were cultivated as described in Chapter 3. The chemically defined minimal media used in this study are named M7 and M17 and are obtained by omitting non essential and stimulatory amino acids and vitamins from M3. The ingredients of all media are listed in Table 5.2.

5.3.2. Study of inhibition. Lysine analogs (AEC, FPA, DL-ASP and LHX) were obtained from Sigma Aldrich. The inoculum was obtained as described in Chapter 3. Inoculum size was

optimized to the sensitivity of the spectrophotometer and the lowest cell number detected was 10^5 cell/mL, which was used as inoculum in all experiments with 150 µL of total culture growth volume. The cultures were incubated for 72 h at 30°C. Bacterial growth was monitored as OD_{620} on Titertek Multiscan plate reader using U-bottom 96 well plate. In the cases when experiments were performed in a higher volume i.e. 3 mL, the growth was monitored as OD_{600} on Baush and Lomb Spectronic 20 spectrophotometer.

5.3.3. Isolation of spontaneous mutants resistant to LHX. *Lactobacillus plantarum* NRRL B 4496 was grown in MRS and washed twice in 0.9 % (w/v) NaCl as described before. Approximately 10^6 cells/mL were introduced to M17 plates supplemented with 2 mM of LHX. Plates without analog were used as the control. Cultures were incubated for 5 days at 30°C. The microscopic image (Figure 5.4) was obtained using a Zeiss, Jena, III RS microscope with 10x objective lens coupled to CCD camera (Sensys model 14016, Photometrics, Tuscon AZ) and processed by PC-based RS Image version 1.7.3. software (Roper, Scientific Inc, Trenton, New York). Mutants tentatively identified as LHX^R were propagated in M3 until mid log phase was reached, washed in 0.9 % (w/v) NaCl, re-suspended to 1 mL and refrigerated (Kropinski 1975). For long-term preservation, mutants grown in M3 until stationary phase were kept in 25 % (v/v) glycerol at -30°C.

5.3.4. Screening of lysine over-producers. Lysine secretion was screened using a microbiological assay with *Pediococcus acidilactici* ATCC 8042 according to AOAC (Official method 960.47) with the exception that the assay was scaled down to 150 µL of the total volume instead of the originally proposed 10 mL. *Lactobacillus plantarum* was grown in MRS, washed and transferred to M3. The M3 supernatant's pH was brought to 6.5 with 1 M NaOH,

filter sterilized (Millipore, 0.22 μm pore size), serially diluted and added to lysine assay medium (LAM, Sigma Aldrich) supplemented with 12 μg/mL of lysine making a total volume of 150 μL. Positive control was LAM without the supernatant. The optimal volume of *L. plantarum* supernatant was shown to be 20 μL, which was used in a screening of lysine over-producers. The standard curve for growth vs. lysine concentration was performed in the LAM with every experiment using L-lysine HCl as a standard. All experiments were performed in a U-bottom 96 well plate. Optical density was read at 620 nm on Titertek plate reader (Titertek Multiscan).

5.3.5. UV survival curve. Cells taken from the exponential phase (for wild type 5 h at 30°C in MRS and mutant #34 24 h at 30°C in M3) were washed twice and re-suspended in 20 mL of saline solution in a Petri plate. UV irradiation was carried out by exposing the saline suspension of the cells (6×10^8 cells/mL for the wild type and 5×10^8 cells/mL for mutant # 34) to UV germicide lamp (UVP Minerlaight lamp, Model R52G, 600 Hz) with an irradiation rate 0.4 W/m^2s in increasing 5 s intervals. The irradiation dose was calculated by the equation:

$$\text{Dose} = \text{Irradiation rate} \times \text{time}$$

Irradiated cells were plated on MRS plates and incubated in the dark for 48 h at 30°C. The cultures were transferred to selective media for survivor mutant isolation.

5.4. RESULTS

5.4.1. Study of bacterial growth inhibition by lysine analogs. The first experiments done in this study were performed in M3 using both *L. plantarum* and *L. delbrueckii*. These strains were chosen because of the highest growth rate in M3 of the initially tested nine strains of lactobacilli (Chapter 3). It was found that both strains are resistant to FPA; AEC showed minor effect on bacterial growth, while DL-ASP and LHX inhibited the growth significantly (Figure 5.1). It was possible that *L. plantarum* and *L. delbrueckii* tolerated FPA and AEC due to their enzymatic system capable of utilizing the compounds (Thomson and Donkersloot 1992).

While FPA resistant lysine over-producing mutants were reported in *B. lactofrermentum* (Satiawihardja et al. 1993), ASP and LHX were not frequently used. In this thesis, LHX caused the most potent growth inhibition in both *L. delbrueckii* and *L. plantarum*. Still, the highest 0.5 mM concentration of LHX used in the initial study, inhibited the growth only 60 % in both *L. plantarum* and *L. delbrueckii* (Figure 5.1 A and B). Complete inhibition (more than 90 %) was obtained with a higher concentration (20 mM) of LHX (Figure 5.1C). In order to make this competition tighter by making the growth conditions even more stringent, the following experiments were considered. First, suppressing the growth by influencing the DAP pathway followed by exposure of cells to LHX after depletion of endogenous lysine and second, omitting nonessential and stimulatory amino acids and vitamins from the growth medium.

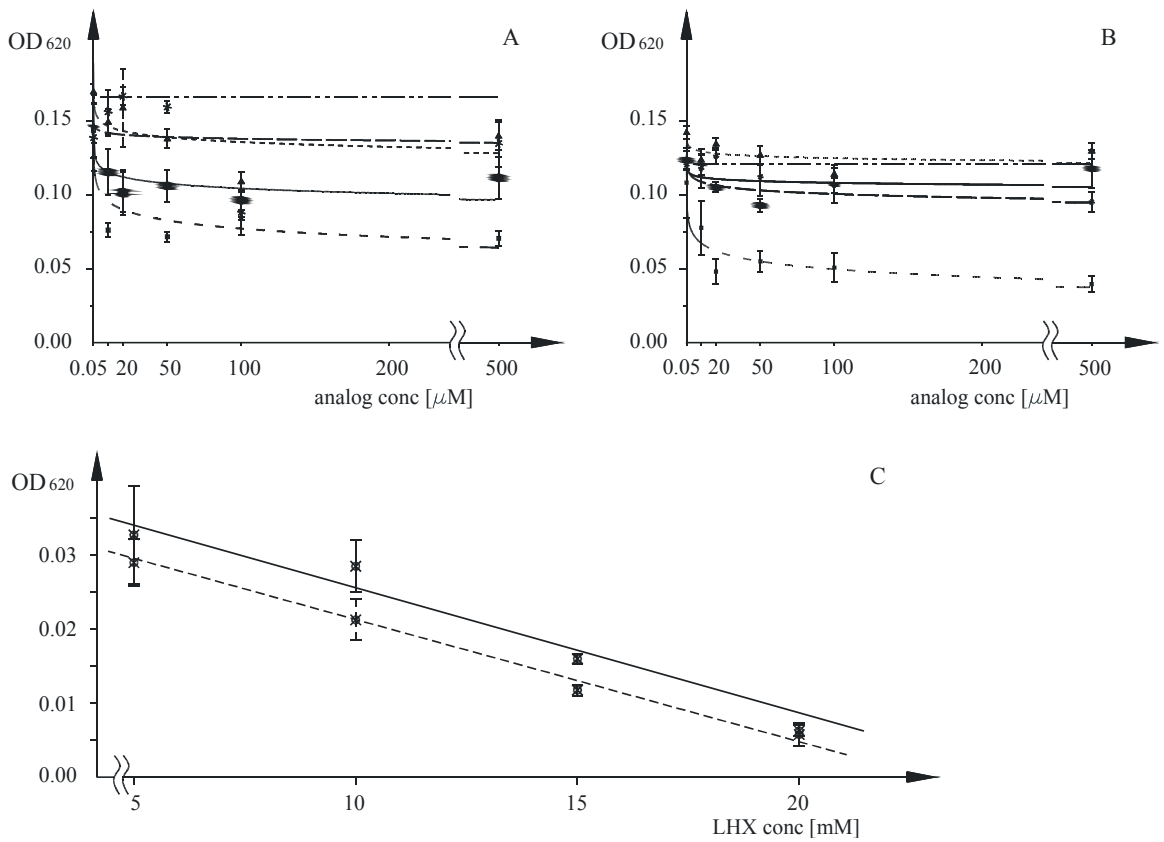

Figure 5.1. Inhibition of the growth of *L. delbrueckii* and *L. plantarum* in M3 by lysine analogs. Figures represent influence of four analogs on *L. delbrueckii* (A) and on *L. plantarum* (B). Analogs are presented as follows: FPA (….), AEC (-), DL-ASP (- - -), LHX (- - -) and control (- ¨ -). Data are an average of six replicates ± standard deviation. Figure (C) represents influence of LHX on *L. delbrueckii* (—) and *L. plantarum* (- - -). Data are an average of four replicates ± standard deviation. Curves are intrapolated according to equations calculated in MATLAB program, version 6.

5.4.1.1. Influencing the DAP pathway. Results obtained in Chapter 4 provided an insight in the regulation of the DAP pathway. The medium containing 0.5 g/L L-threonine and 0.1 g/L of L-isoleucine seemingly provided the highest rate of growth inhibition. Interestingly, challenging strains for this study with LHX in this formulation revealed different responses in these two LAB species.

Experiments were performed in 3 mL of a total volume of medium supplemented with 0.5 mM LHX. The cells were grown in MRS, washed with saline solution and starved for 8h at ambient temperature (22±2°C) to deplete endogenous lysine (Ledesma et al. 1977, Li et al. 1999). The optical density, which is the function of both cell number and the cell size, was measured. Decreased OD_{600} in *L. delbrueckii* was observed although there was a similar total cell count in the control and the treatment. This result implied that this lactobacillus responded to these particular stresses by decreasing the cell size. On the other hand, *L. plantarum*, clearly showed decreased total cell count when exposed to the above-mentioned stresses (Table 5.1). Microscopic examination confirmed this finding. Further, LHX caused stronger growth inhibition in *L. plantarum* than in *L. delbrueckii*. From this point on, work continued with *L. plantarum.*

5.4.1.2. Modification of M3. Chapter 3 provided detailed information on the nutritional needs of *L. plantarum*. In order to simplify the medium, all nonessential, stimulatory and some essential amino acids and vitamins were omitted, which resulted in a decreased final cell number. In part, laws of stochiometry should apply to the inhibition by an anti-metabolite, when the total cell count is decreased, they should be inhibited by a decreased concentration of the anti-metabolite. However, what matters more is the number of the target molecules with

which anti-metabolite reacts. Therefore, it is preferable to work with the minimal medium, as that will provide minimal total cell count and stringent competition (Sands and Hankin 1974, Winston and Bhattacharjje 1982).

Table 5.2 shows the differences in the composition of M3, M7 and M17. The total cell count in these media was 2.4×10^8, 2.5×10^7 and 8×10^6 cells/mL respectively. The absolute minimal requirements were reached in medium M17; if any subsequent ingredient was omitted, the growth was completely aborted.

Omitting vitamins and amino acids did not influence the pH. Depending on the medium, the inhibition study showed differences in MIC of LHX (Figure 5.2). For example, 5 mM of LHX exerted 55.6 % inhibition in M3, 75 % in M7 and 87.5 % in M17. The result confirmed that the decreased total cell count was associated with decreased concentration of the anti-metabolite needed for MIC.

It was found in microassay results that MIC for *L. plantarum* in M17 was 2 mM. In order to confirm that this LHX concentration inhibited the growth by 90 %, the experiment was repeated with 20 fold larger volume (e.g., 3 mL), which yielded the same result. From this point onward, M17 was used as a screening medium and 2 mM of LHX as MIC for the isolation of lysine over-producer mutants.

Table 5.1. Growth of *L. delbrueckii* and *L. plantarum* after depletion of endogenous lysine in modified M3 in response to LHX.

Lactobacilli	Treatment	OD_{600}*	Total cell count (cells/mL)
L. delbrueckii	—	1.600	2.0×10^{8}
	0.5mM LHX	0.850	1.4×10^{8}
L. plantarum	—	1.500	2.4×10^{8}
	0.5mM LHX	0.071	8.5×10^{6}

* Optical density was measured in Baush and Lomb Spectronic 20 spectrophotometer. Modified M3 contained 0.5 g/L L-threonine and 0.1 g/L of L-isoleucine.

Table 5.2. The composition of M17, M7 and M3 media.

Ingredient	Medium (g/L)			Ingredient	Medium (g/L)		
	M17	M7	M3		M17	M7	M3
Glucose	10	10	10	L-phenylalanine	0.1	0.1	0.1
Sodium acetate	15	15	15	L-methionine	0.1	0.1	0.1
Ammonium citrate	4.6	4.6	4.6	L-threonine	0.1	0.1	0.1
Magnesium sulfate heptahydrate	0.1	0.1	0.1	L-tyrosine	-	0.1	0.1
Manganese sulfate hydrate	-	-	0.05	L-proline	-	0.1	0.1
Ferric sulfate heptahydrate	0.02	0.02	0.02	L-arginine	-	-	0.1
Tween 80	5	5	5	L-histidine	-	-	0.1
Di-potassium phosphate dibasic	3	3	3	Pyridoxine HCl	0.02	0.02	0.02
L-glutamine	0.5	0.5	0.5	Ca-panthotenate	-	-	0.021
L-aspartic acid	0.2	0.2	0.2	Riboflavin	-	-	0.001
L-cysteine	0.2	0.2	0.2	Cyanocobalamine	-	-	0.001
L-valine	0.1	0.1	0.1	Niacin	-	-	0.001
L-isoleucine	0.1	0.1	0.1	Folic acid	-	-	0.0001
L-leucine	0.1	0.1	0.1	Thymidine	-	-	0.005
L-tryptophan	0.1	0.1	0.1				

The composition of M7 and M17 was determined by the single omission technique as per Chapter 3.

Lysine and LHX are competitive inhibitors; it was interesting to see how the increased dosage of lysine would affect the inhibition. Addition of equal concentrations of lysine and LHX to the medium did not completely reverse the inhibition regardless of the phase growth. Two conclusions can be made on this; either affinity of aspartokinase for LHX is much higher than that of lysine or growth inhibition did not occur due to the lysine accumulation. In the work of Sinha et al. (1971) and Haidaris and Bhattcharjee (1978), inhibitory effects of anti-metabolites were reversed by the addition of metabolites suggesting that growth inhibition is due to the metabolite accumulation.

Both studies were performed on yeast, which synthesizes lysine via the AAA pathway and lysine is the only amino acid synthesized. Since that DAP pathway was shared by three other amino acids, it was possible that, in addition to lysine, LHX caused accumulation of threonine, methionine, isoleucine or a combination of these amino acids. Chapter 3 showed that lysine is not an essential amino acid in *L. plantarum.* It is a primary metabolite synthesized during the logarithmic growth phase. Accordingly, there should be a difference between lysine concentrations in mid log vs. stationary phase. The differences in the steepness of the curves shown in Figure 5.3 allowed the hypothesis that there was a difference in the level of lysine in the exponential (Figure 5.3A) versus the stationary phase of growth (Figure 5.3B).

Addition of lysine to the culture in the exponential phase did not reverse the inhibition, while in the stationary phase its effect was more prominent, probably due to the higher lysine pool.

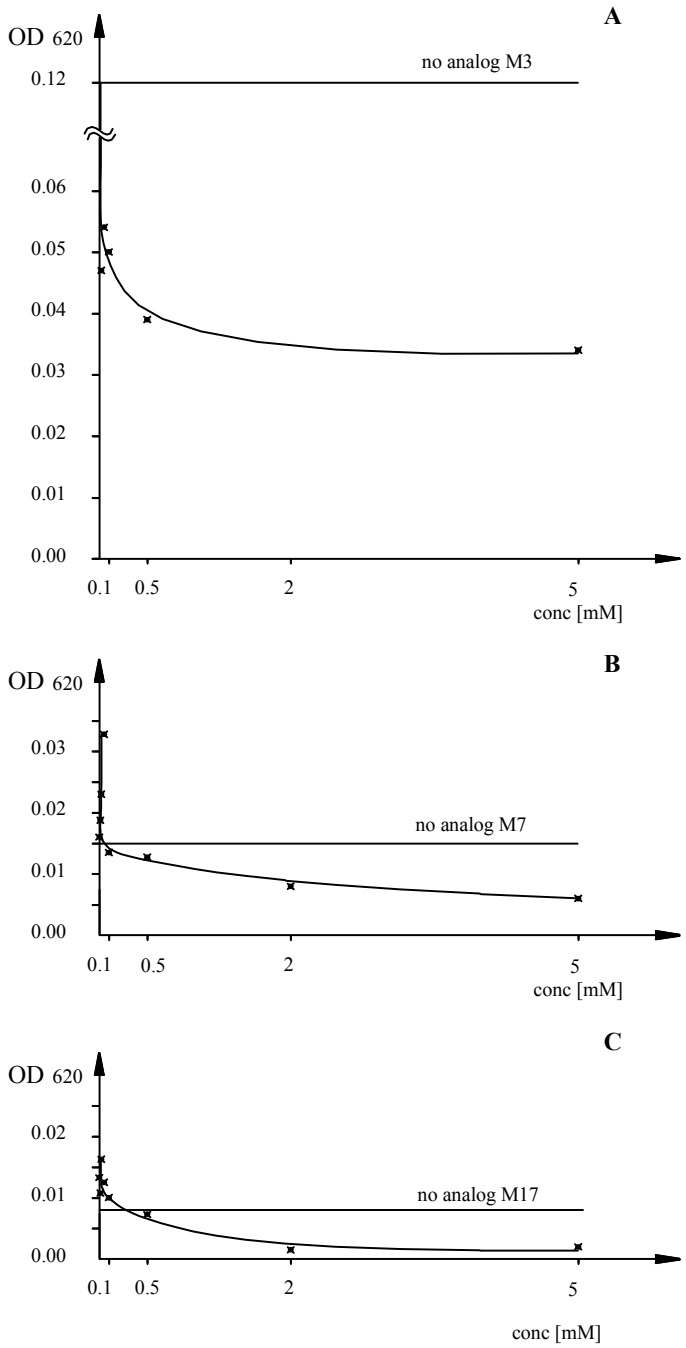

Figure 5.2. Growth inhibition of *L. plantarum* by LHX in different media. Figures present inhibition of culture OD_{620} in response to LHX in media (A) M3, (B) M7 and (C) M17. The study was performed with the inoculum concentration 10^5 cells/mL. Data presents an average of four replicates at end-point reading.

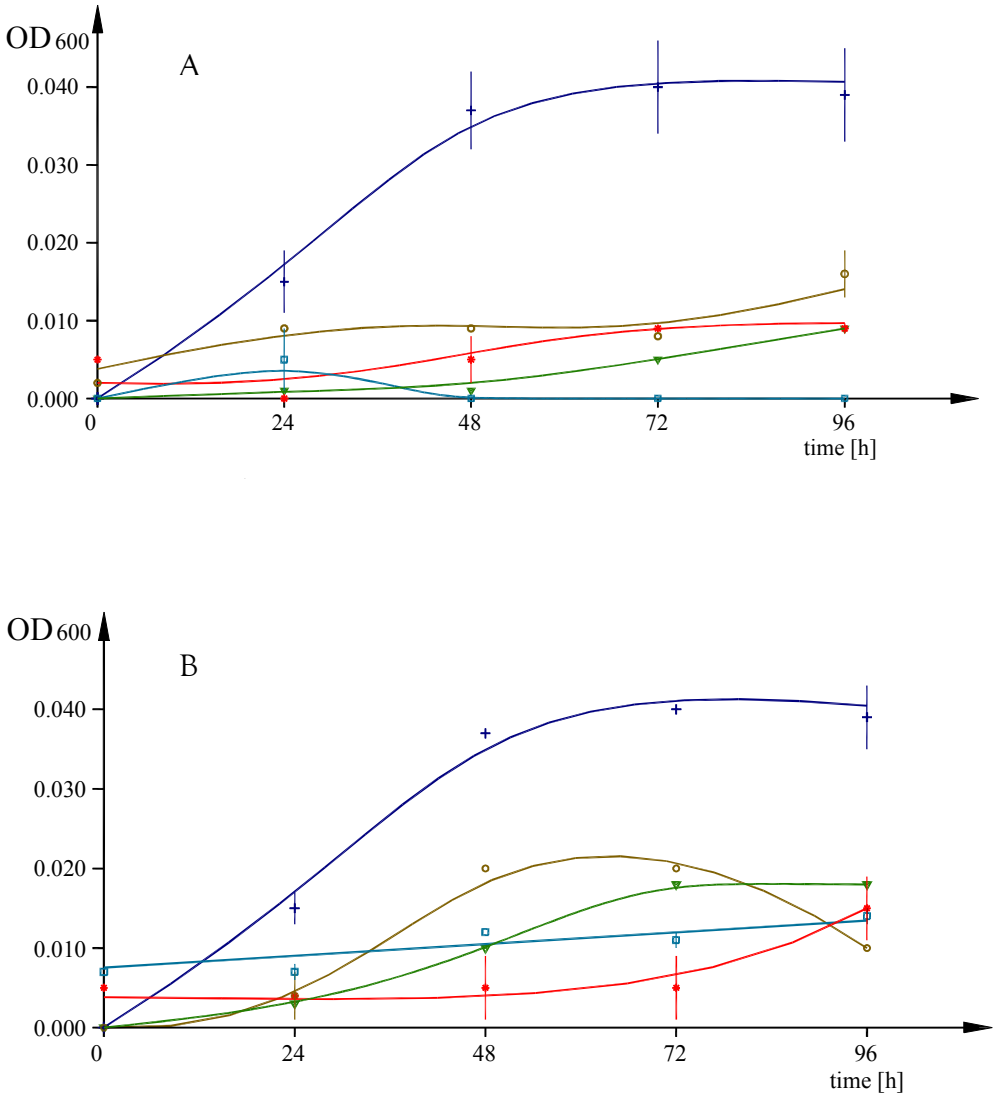

Figure 5.3. Titration of LHX by lysine in the different growth stages of *L. plantarum*. Figures show changes in OD_{600} as a function of time where (A) represents exponential and (B) stationary phase. Concentrations of lysine added are as follows: (○) 2 mM, (▼) 0.2 mM and (□) 0.01 mM. Controls are marked as (•) M17 +2 mM LHX and (+) M17. Data for lysine added in concentration 1 mM, 0.4 mM and 0.1 mM are not shown for clarity, but fall within presented data.

5.4.2. Isolation of spontaneous *L. plantarum* LHX^R. Observation of colonies on M17 plates vs. those supplemented with 2 mM of LHX showed two different morphologies (Figure 5.4). Colonies on plates without LHX were several fold smaller in size (Figure 5.4.B) compared to the colonies supplemented with 2 mM LHX (Figure 5.4.A). The flow chart depicting the isolation of the spontaneous lysine over-producer is shown in Figure 5.5. In order to confirm that larger colonies, tentatively designated as LHX^R, were indeed resistant to LHX, mutants were re-cultured in 1 mL of M17 supplemented with 2 mM LHX and grown at 30°C for 7 days.

Mutants that grew in the presence of the antagonist were washed in saline solution and transferred to M3 instead of M17, as it could support the attainment of higher density than M17 (Table 5.3). All mutants except #3, #16 and #25 showed reproducible growth yields, hence optical densities in two independent experiments. It is important to mention that requirements of lactobacilli may differ depending on their growth in broth or on agar (Morishita et al. 1981). For *L. plantarum* NRRL 4496, threonine is essential in broth cultures (Chapter 3), but stimulatory on solid agar plates (this Chapter), while requirements for methionine remained the same in either broth or agar plate. Auxotrophy for threonine and methionine of a spontaneous LHX^R mutants on agar plates were determined because these two amino acids were synthesized in the DAP pathway along with lysine. Mutants #6, #16, #27, #33, #34, #35 and #36 were different from the others with respect to threonine and methionine requirements (Table 5.3).

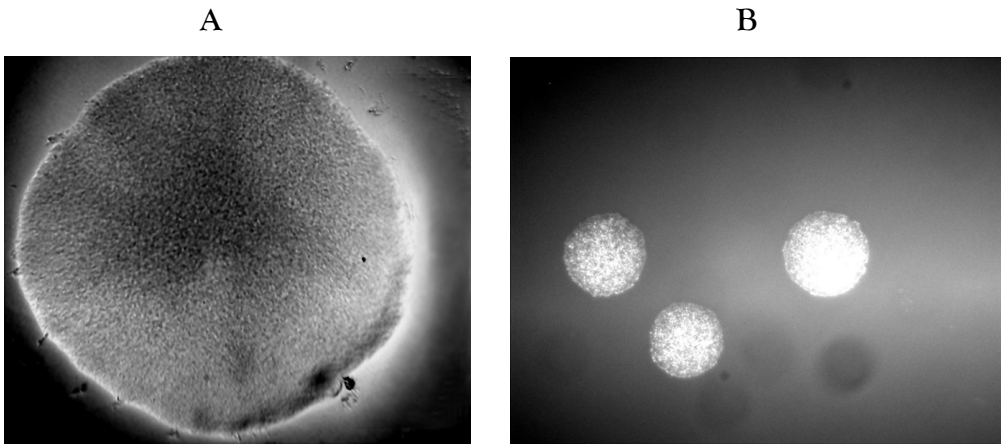

Figure 5.4. Colony morphologies of *L. plantarum* caused by LHX on M17 plates. Figure (A) shows colony on a plate supplemented with 2 mM LHX and (B) control containing no LHX. Images were taken on Zeiss, Jena, III RS microscope with 10× objective lens.

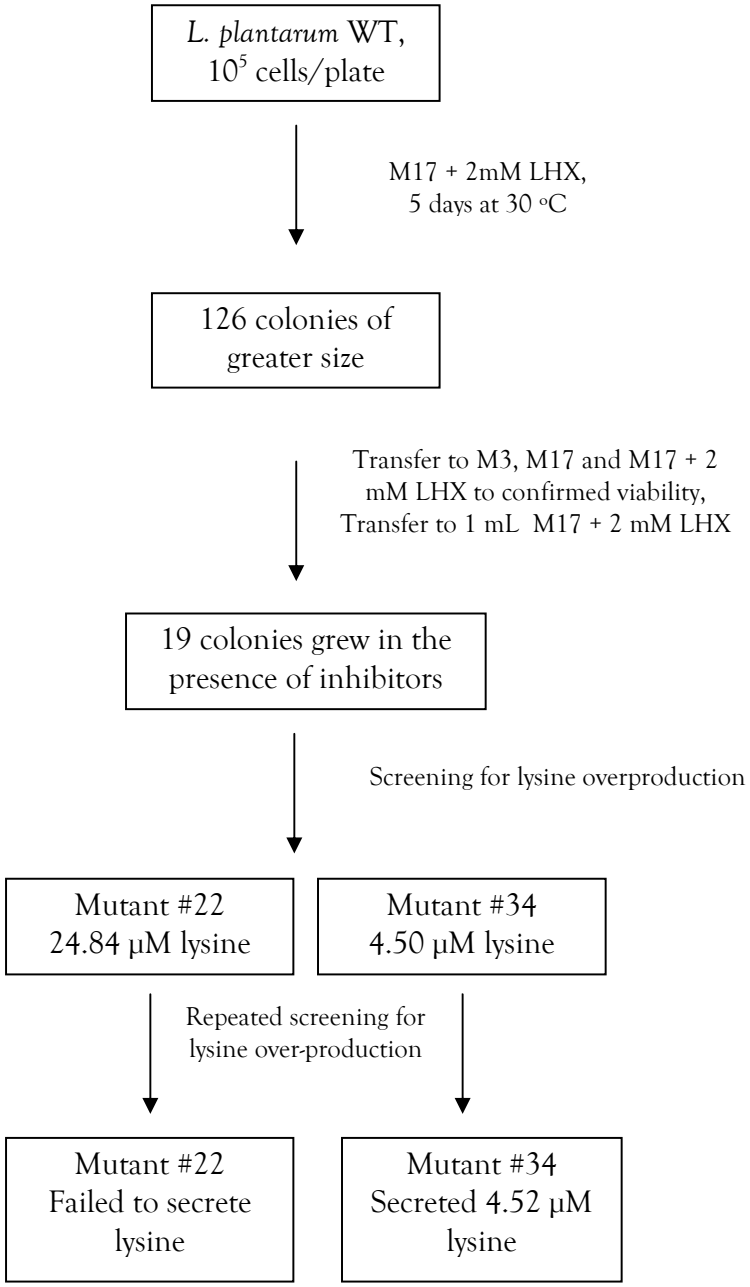

Figure 5.5. Scheme of isolation of spontaneous lysine over-producers in *L. plantarum*.

Table 5.3. Growth characteristics of *L. plantarum* LHX^R mutants on –Thr, –Met or –Thr–Met M3 plates.

LHX^R mutant #	Growth rate in M3 (h^{-1})	OD_{600} [a]		Qualitative growth on M3 plates [b]		
		M3	M17	–Thr	–Met	–Thr–Met
WT	0.18	2.90 ± 0.00	0.04	+ +	+ +	-
25	0.16	1.50 ± 0.22	0.01	+ + +	-	-
31	0.14	1.45 ± 0.04	0.02	+ + +	-	-
35	0.13	1.10 ± 0.63	0.01	+ +	-	-
26		1.35 ± 0.04	0.01	+ + +	-	-
33		1.45 ± 0.04	0.01	+ +	-	-
7	0.13	1.30 ± 0.00	0.02	+ + +	-	-
38	0.13	0.87 ± 0.10	0.03	+ + +	-	-
6	0.12	1.60 ± 0.08	0.02	+ + +	+	-
27	0.12	0.85 ± 0.06	0.01	+ +	-	-
22	0.11	1.60 ± 0.03	0.01	+ + +	-	-
16	0.11	1.45 ± 0.19	0.01	+ +	-	-
3	0.10	1.60 ± 0.24	0.01	+ + +	-	-
41	0.10	1.50 ± 0.00	0.02	+ + +	-	-
34	0.10	1.40 ± 0.08	0.02	+ + +	+ +	+ +
28	0.09	1.25 ± 0.20	0.03	+ + +	-	-
37	0.09	0.90 ± 0.08	0.03	+ + +	-	-
17	0.08	1.59±0.06	0.01	+ + +	-	-
4	0.08	1.30 ± 0.07	0.04	+ + +	-	-
36	0.08	1.22 ± 0.06	0.01	+ +	-	-

(a) Absorbency in M3 represents an average from two independent experiments ± standard deviation.

(b) The scores are; 100 % + + + + (growth of WT on M3); 75 % + + + ; 50 % + +; 25 % +; no growth.

Growth of mutants or their absence on plates lacking Thr, Met, or both (Table 5.3) lead to the conclusion that mutants #16, #27, #33, #35 and #36 were similar to the wild type in terms of threonine requirements. The rest of the mutants probably over-produce Thr, since WT was able to grow in the absence of Thr to up 50 % of the yield with Thr. Omission of Met resulted in a 50 % reduction of the growth in WT and 100 % in all mutants except for #6 and #34. Over-production of Thr may completely inhibit the bi-functional AK isozyme, which results in starvation for Met.

5.4.3. UV irradiation, survival and yield of induced lysine over-producer mutants. The flow chart of the isolation of induced lysine over-producing mutants is shown in Figure 5.6. In attempts to mutant induced stable lysine over-producing mutants, a strategy similar to that of the isolation of spontaneous lysine over-producer was used. Resistant mutants were tested for viability to eliminate potential mutant(s) that have impaired cell division (Winston and Bhattacharjee 1982).

The desirable percentage of survival values for isolating colonies with point mutations often mentioned in the literature are 1-10 %. However, the survival itself is not the only important parameter. Note the yield of the mutants obtained from different irradiation doses. A 0.1 % survival was obtained by an irradiation dose of 16 W/m^2s for the wild type and 0.3 % survival obtained by an irradiation dose of 10 W/m^2s for mutant #34 (Figure 5.4). In order to estimate the yield of the mutants, M3 as a control and M3 without threonine, methionine or both were used. Cultures were incubated at 30°C and 37°C. Ideally, mutants should have survival curves similar to WT.

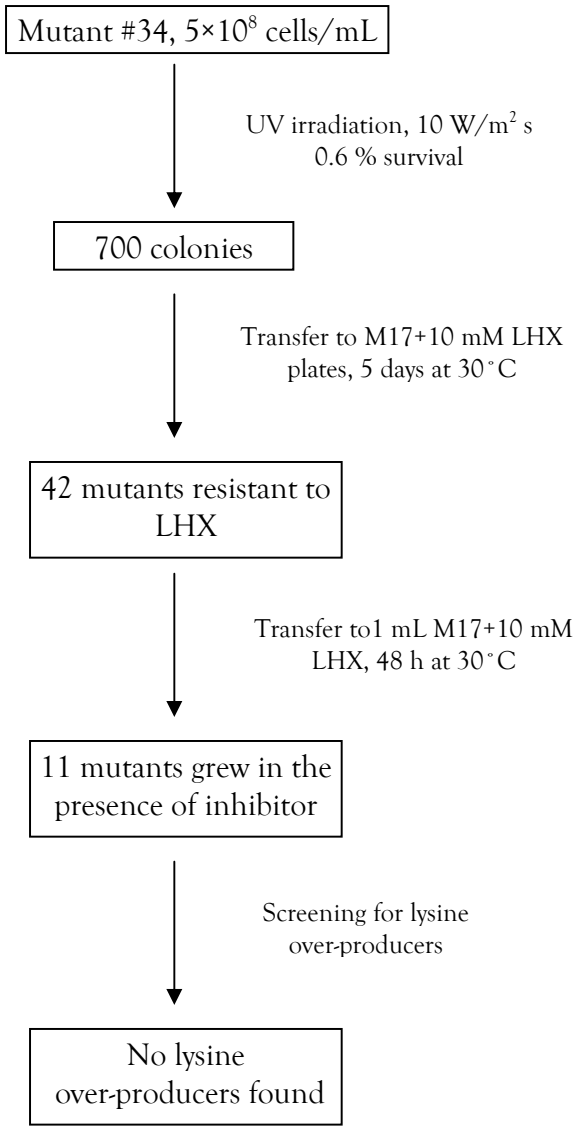

Figure 5.6. Scheme of isolation of UV induced lysine over-producer mutants.

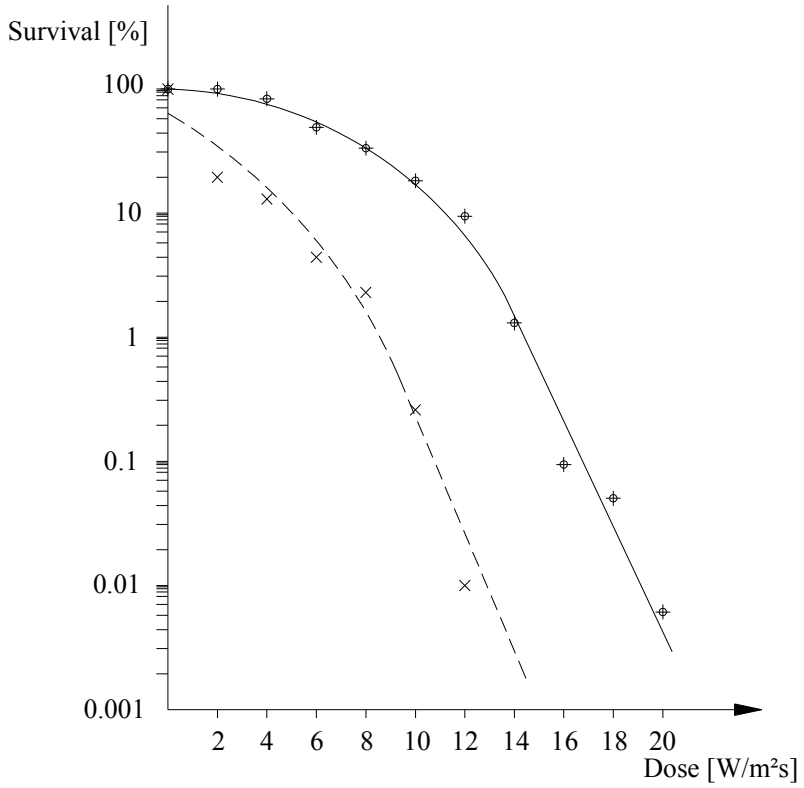

Figure 5.7. The survival curves of *L. plantarum* WT and *L. plantarum* #34 exposed to UV irradiation. Line (——) represents wild type and line (- - -) represents mutant #34.

Table 5.4. Phenotypes of mutants obtained by UV irradiation of WT and mutant #34.

Class	Percentage of total pick (%)	
	L. plantarum WT	# 34
Auxotroph* for nutrient(s) not in M3	3	5
Thr⁻	3	9
Met⁻	3	9
Thr⁻, Met⁻	3	9
Temperature sensitive	6	NA**
Temperature sensitive, Thr⁻, Met⁻, Thr⁻Met⁻	24	NA
Temperature sensitive, Thr⁻Met⁻, Auxotroph for nutrient not in M3	3	NA
Other classes of mutants plus wild type phenotype	55	68

* Auxotrophs were assayed on agar plates.

** Not assayed (NA).

In this case, #34 killing rate was high and there was no shoulder (Figure 5.7). Interestingly, UV irradiation of #34 yielded roughly three-fold higher survival value of 0.3 % and 5-9 % (of the total pick) of the mutants, compared to WT with 0.1 % of survival and a 3 % of the mutants of the same class (Table 5.4).

5.4.4. Screening of the spontaneous and induced LHX^R mutants for lysine overproduction. Due to much suppressed growth of *L. plantarum* in M17 comparing to M3 (Table 3.3), M17 was used for a screening, while M3 was used as a maintaining and secretion studies. Haidaris and Bhattacharjee (1978) used a similar approach, i.e. screening medium and maintenance medium for *S. cerevisiae*. *Pediococcus aciditlactici* was used as a bioassay microorganism. The minimal amount of *L. plantarum* supernatant sufficient to screen lysine secreted without inhibiting its growth was identified. The addition of the supernatant in amounts less than 20 µL did not decrease the growth of *P. acidilactici* as opposed to volumes greater than 20 µL (Figure 5.8). Addition of 50 µL of the supernatant caused 37 % growth inhibition in the assay bacterium. Therefore, 20 µL of the supernatant was a sufficient volume to measure lysine secretion.

From spontaneous LHX^R mutants #3 to #41, only mutants #22 and #34 secreted 24.84µM and 4.5µM of lysine respectively. On the repeated experiment, mutant #22 failed to excrete lysine, while #34 secreted 4.52 µM of lysine. Mutant # 34, due to consistent level of lysine production, was irradiated and survivors were challenged with five fold MIC of LHX on M17 plates. It was interesting to compare the percentage of lysine over-producers obtained as spontaneous and induced mutants.

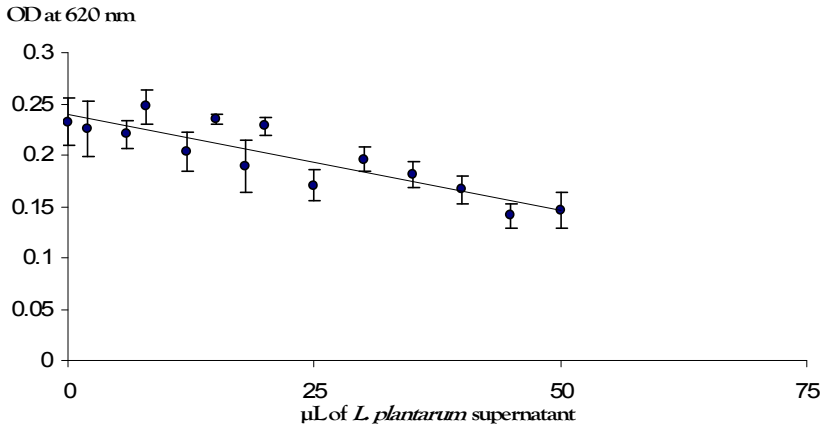

Figure 5.8. Growth response of *P. acidilactici* in LAM. Growth is assessed in relation to the addition of *L. plantarum* M3 supernatant to LAM.

When *L. plantarum* WT was exposed to MIC of LHX, only 0.12 % of the population showed different morphology than that of WT; 15 % of which was confirmed to be resistant to LHX. Two mutants were indeed lysine over-producers, representing 10.5 % of those confirmed to be resistant to LHX. Still, only one mutant showed consistent lysine secretion accounting for 50 % of the lysine over-producers. However, when the induced mutants were examined, 6 % of its survivors were resistant to LHX (as opposed to 0.12 % in spontaneous mutations) and 26.6 % were confirmed to be resistant to LHX (as opposed to 15 % in spontaneous mutations). Surprisingly, in spite of this relatively high percentage of LHX^R mutants obtained, lysine over-producer(s) was not found.

5.5. DISCUSSION

Several obstacles were encountered while attempting to mutant lysine over-producer(s) of lactobacilli. Studies with amino acid (or another metabolite) over-production must be performed in a chemically defined medium in order to have accurate measurements of the metabolite concentration. After screening nine strains, two (*L. plantarum* and *L. delbrueckii)* were chosen due to their simple nutritional requirements. Unexpectedly, both *L. plantarum* and *L. delbrueckii* tolerated all four anti-metabolites well. Indeed, in *Lactococcus lactis* species N^2-(carboxyethyl)-amino acids [N^5-(CE)-amino acids] were found which had not been suspected to be present in either prokaryotic or eukaryotic cells (Depicker et al. 1982, Bevan et al. 1983). More importantly, the enzymes involved in N^2-(carboxyethyl)-amino acids metabolism were identified. N^5-(CE)-ornithine synthase, for example, exhibits a narrow specificity with respect to

substrate. Only L-ornithine and to a lesser degree AEC serve as amino acid substrates (Thompson and Donkersloot 1992).

One mode of action of growth-inhibitory amino acid analogs is that they may be incorporated into proteins specifically and stoichiometrically replace the corresponding amino acid (Fowden et al. 1967). Yoshida (1958) showed that the analog could incorporate into the enzyme without impairing normal enzyme function. Moreover, the cells grew in the presence of the analog. This allowed the conclusion that LHX could indeed incorporate into protein(s) in *L. plantarum*. However, the replacement of the residues of the normal protein amino acid by an analog is random. There is an equal chance of replacement of any of the residues, irrespective of their position in the polypeptide chain (Fowden et al. 1967). In the system used in this thesis, AEC, FPA and DL-ASP did not cause significant growth inhibition. In fact, lysine analog resistant mutants in lactobacilli were only obtained after exposure to high concentrations of any of the analogs. Adebawo et al. (1997) obtained lysine overproducers at 12.5 mM of AEC and Cahayanto et al. (2006) at 100 mM of AEC. As these bacterial cells must have endogenous precursor pools, one can expect an anti-metabolite to compete with the metabolite for binding to an active site. Therefore, depletion of endogenous lysine was employed. Cells exposed to LHX after depletion of endogenous lysine showed different defense mechanisms in *L. plantarum* and *L. delbrueckii*. *Lactobacillus plantarum*, during such regimen, responded by decreasing the cell number, while *L. delbrueckii* responded by decreasing the cell size. Keeping in mind that lactobacilli, to begin with, have a small cell size even when grown in a rich medium (0.5-1.1 × 2-8 μm), a further decrease in size may create difficulties for the isolation and/or purification of prospective lysine over-producing mutants. The

continuation of work was therefore with *L. plantarum* alone; although, a better strategy for obtaining strong inhibition of the growth of *L. plantarum* by LHX was modifying the medium. Omitting nonessential and stimulatory constituents from M3 resulted in the development of minimal medium M17. This was successfully employed as a screening medium: MIC of growth by LHX in M17 was 2 mM comparing to 20 mM MIC in M3. When *L. plantarum* WT was challenged by LHX on plates, two types of colony morphologies arose: pin-point size colonies which were observed on control plates and larger colonies (1-2 mm). Larger colonies were challenged by LHX again and only mutants that grew in the presence of the inhibitor were further analyzed. Mutants with LHX^R phenotype had different growth rates and requirements with respect to threonine and methionine than the wild type. These selective features justified the use of LHX as a screening tool for this class of mutants. Furthermore, the fact that inhibition caused by LHX could not be reversed by lysine implied that resistance to LHX cannot be due to lysine accumulation. Indeed, this pathway is shared by threonine, methionine and isoleucine. Deregulation of the key enzyme synthesis may result in accumulation of any other amino acid or their combination. From 19 mutants LHX^R, only mutant #34 secreted a small amount (4.52 μM) of lysine into M3. This result indirectly confirmed that LHX probably affected enzymes in the threonine, methionine or isoleucine pathway. In fact, mutant #34 grew on the plates lacking Thr to a higher degree than the wild type. This obviously meant that the DAP pathway was deregulated and threonine biosynthesis was promoted in mutant #34. Surprisingly, exposure of mutant #34 to UV did not yield new mutants with improved lysine secretion. UV light as a mutagen creates random and multiple mutations: (i) possibly some of the survivors resistant to LHX are secretion defective mutant(s), (ii) secondary mutants are

much rarer, requiring large number to be screened or (iii) there are no other genes involved. Therefore, lysine accumulation occurred due to resistance, but not its secretion into the medium and lysine over-production could not be assayed. Mutagenesis of mutant #34 caused a higher killing rate, a higher percentage of the LHX^R mutants, but none of these mutants was suitable for this thesis. On the other hand, spontaneous mutation, in spite of a low mutation rate, yielded one lysine over-producer. There are no precedents in the literature that confirm that in isolation of this class of mutants, spontaneous mutations are more selective, as lysine over-producers have been obtained using both spontaneous and induced mutagenesis.

In conclusion, it is clear that lysine over-producing mutants are rare in lactobacilli if random mutagenesis was applied. Obviously, larger searches or site-directed mutagenesis could be a better choice for the isolation of *L. plantarum*, lysine over-producers.

5.6. RELEVANCE OF CHAPTER 5 TO THE THESIS

Given the objective of this thesis, which is to enhance lysine content in canola meal by bacterial fermentation, Chapter 5 contains studies that are dedicated to that goal. Here it is documented the relevant features of employing *L. plantarum* as a lysine over-producer.

6. ISOLATION OF *ASPERGILLUS FICUUM* NRRL B 3135 LYSINE OVER-PRODUCERS RESISTANT TO LYSINE ANALOG

6.1. ABSTRACT

Aspergillus ficuum was challenged by five lysine analogs in order to mutant regulatory mutants that excrete lysine. The plate assay showed that DL-aspartic acid β-hydroxamate (DL-ASP) was the most potent inhibitor. The minimal inhibitory concentration determined in broth assay showed close to 90 % growth inhibition by a concentration of 2 mM on the minimal medium for *Aspergillus*. Optimal inoculum was found to be $2x10^4$ conidia/mL. It was established that the nitrogen source influenced lysine secretion and pellet morphology in *Aspergillus*. Among various nitrogen sources, ammonium phosphate promoted lysine secretion to a higher extent than ammonium chloride, sodium nitrate, urea, ammonium citrate or ammonium nitrate. Ammonium nitrate suppressed the growth as well as lysine production in the wild type. Mutant 5-10, resistant to 5 mM DL-ASP, secreted the highest amount of lysine in the medium, 29.25 μM that was approximately 30 % higher than the lysine secretion of the wild type under the same growth conditions. It was established that the maintenance of this class on minimal medium (SMMA) ensured higher and more consistent lysine secretion as opposed to maintenance on rich, potato dextrose agar plates (PDA).

6.2. INTRODUCTION

While the world's lysine production relies on bacteria, the other option, largely neglected, is fungi as lysine over-producers. Fungi synthesize lysine via the α-aminoadipic acid (AAA)

pathway (Vogel 1964), which involves eight steps, but leads solely to lysine in terms of amino acids. The AAA is a branching point that divides biosynthetic pathway in directions, L-lysine and penicillin G. Due to greater commercial importance, more effort has been placed to understand the biosynthesis of penicillin and its regulation. Thus, all of the genes involved in the branch specifically leading to penicillin have been characterized in *Aspergillus nidulans* (Ramon et al. 1987, MacCabe et al. 1990, Brakhage 1998). The repressing influence of L-lysine on penicillin biosynthesis is also well-established (Brakhage and Turner 1992).

On the other hand, only the *lysF* gene, encoding homoaconitase (Weinder et al. 1997) and *lysA* encoding saccharopyne dehydrogenase (Zabriskie and Jackson 2000) that lead to lysine biosynthesis have been characterized in *A. nidulans*. Natural fungal lysine production, but not over-production, has been somewhat explored. Richards and Haskins (1957) tested various fungal genera for lysine secretion and found that *Ustilago maydis* PRL 1092 and 1229 were the most successful and consistent producers of this essential amino acid. Tauro et al. (1963) screened five *Ustilaginales* species and reported that modification of the medium can improve the lysine secretion reasonably well. To date, there is only one report of a spontaneous lysine over-producer in *Penicillium chrysogenum* (Masurekar and Demain 1974) which was selected by using lysine analogs. In fact, the authors, while hoping to mutant penicillin over-producers, did not consider lysine over-producers useful for industrial purposes. Success that is more significant was obtained with *Ascomycetes*, mainly *Saccharomyces cerevisiae* (Haidaris and Bhattacharjee 1971, Gasent-Ramirez and Benítez 1997). The possibilities of finding the lysine over-production in *A. ficuum* by the selection of lysine analogs resistant mutants were explored in this thesis. *Aspergillus* species are known to be among fungi that are penicillin producers, but

lysine over-production with this genus has not been reported. It had been demonstrated that L-lysine inhibits homocitrate synthase, the first enzyme in AAA pathway in *P. chrysogenum* (Masurekar and Demain 1974). Therefore, it was felt that the isolation of *A. ficuum*, lysine over-producers could be advantageous in that lysine is the only amino acid produced by the AAA pathway and the deregulated mutants should accumulate lysine alone. Studies have shown that mutants of *P. chrysogenum* resistant to lysine analogs exert deregulation of lysine biosynthesis (Friedrich and Demain 1977). These authors found *P. chrysogenum* resistance to the anti-metabolites, even at the high doses, when conidia were challenged by the analogs on a rich medium. Lysine analogs used in this Chapter were those used in Chapter 5, except DL-DAP was additionally assayed. It was found that young mycelia on the minimal medium are much more susceptible to inhibition. Therefore, mycelia were chosen as more convenient way to screen for the resistant mutants against anti-metabolites. In this study, the parameters for obtaining minimal inhibition concentration (MIC) of the lysine analogs and enhanced lysine production in *A. ficuum* with respect to nitrogen source in fermentation medium were defined.

6.3. MATERIAL AND METHODS

6.3.1. Fungus and the growth condition. *Aspergillus ficuum* NRRL 3135 wild type (WT) was obtained from Northern Regional Research Laboratory (now the National Center for Agricultural Utilization Research). The conidia harvested from fungal growth on potato dextrose agar (PDA) (Difco) plates were washed twice in 0.03 % Tween 80 (v/v) by centrifugation at 10 000×g (IEC Micromax) for 5 min and kept at -30°C in 25 % glycerol as a stock. The strain was maintained on PDA plates incubated at 30°C and refrigerated at +4°C.

6.3.2. Inoculum preparation. Conidia were harvested from PDA plates by flooding the plate with 5 mL of 0.03 % (v/v) Tween 80 and a glass spreader. The suspension was transferred with a Pasteur pipette and filtered through glass wool to remove larger particles and hyphae. The conidia were washed twice by centrifuging for 3 min at 10 000×*g* (IEC Micromax) and re-suspended in 0.03 % (v/v) Tween 80. Inoculum concentration was determined by the haemotocymeter count of conidia. To ensure that contamination by hyphae was negligible and that large conidial aggregates were dispersed, the inoculum was examined microscopically.

6.3.3. Inhibition assay. Five lysine analogs were screened: *S*-aminoethyl-L-cysteine (AEC), ß-flouropyruvic acid (FPA), DL-aspartic acid ß-hydroxamate (DL-ASP), L-lysine hydroxamate (LHX) and DAP. Assays were performed on Minimal Medium for *Aspergillus* (MMA) (Pontecorvo 1953) supplemented with the anti-metabolites. Solid medium contained 1.5 % agar (w/v).

The assay protocol was divided in three stages:

1. Determination of starting conidiation point on MMA when conidia were used as an inoculum. Conidia were inoculated on MMA plate by single point inoculation of a 5 µL sample from the stock inoculum containing approximately 7.4×10^4 conidia. The plate was incubated at 30°C and diameter of mycelial growth was marked on the bottom of the plate every 24 h. The degree of conidiation was observed under a dissecting microscope with magnification 3.5 X. It was determined that the fungus grew approximately 5 mm/24 h and the conidiation started after 72 h.
2. Determination of the starting conidiation point on MMA when pieces of the young mycelia were used as an inoculum. Pieces (4 mm×4 mm) from the edge of 48 h old

colony were cut and transferred to a fresh plate, incubated at 30°C and growth and conidiation were measured as described above. Under these conditions, *A. ficuum* started conidiation after 48 h and the fungus grew approximately 4 mm/24 h.

3. Impact of lysine analogs on the growth of *A. ficuum* NRRL 3135. In MMA plate that contained 20 mL of the medium, a hole 5 mm in diameter was made. Pieces of agar from 48 h (4 mm × 4 mm) old mycelia were placed on the each side of a hole (4 pieces) at a distance of 4 mm (growth in 24 h). The 4 pieces were treated as replicates. Different concentrations of an anti-metabolite in a volume of 80 µL were introduced to the plate. In order to allow an analog to diffuse into the plates, they left on the bench top for about 1.5 h and then incubated at 30°C. The positive control was distilled water and negative control was 3 % sodium hypochlorite (NaClO) instead of the analog. Plates were scanned every 24 h and the resultant growth was documented and analyzed by densitometry using Alpha Easy program, version 5.5.

6.3.4. The determination of MIC. The MIC determinations were performed in MMA broth. Washed conidia were soaked 8 h at the ambient temperature (22±2°C) in 1 mL of 0.03% Tween 80 (v/v), washed once and inoculated as 1×10^3, 2×10^4 and 6×10^4 conidia/mL in 6 mL of the medium in 50 mL volume flasks. The culture was incubated 96 h at 30 °C in water-bath shaker with 150 rpm (New Brunswick Scientific, N.J.). The mycelia were vacuum-filtered through pre-weighed 0.45 µm pore size filter (Millipore) and dried at 70 °C. Viability was determined by estimation of colony forming ability of supernatant of the culture exposed to DL-ASP on MMA plates. Plates were incubated for 96 h at 30°C.

6.3.5. The isolation of *A. ficuum* DL-ASP^R mutants. The conidia were challenged with DL-ASP at the concentrations higher than MIC in 5 mL of MMA broth with the inoculum concentration of 2×10^4 conidia/mL. Conidia were pre-soaked for 8h in 0.03 % Tween 80 (v/v) at room temperature (22±2°C), washed and introduced to MMA broth. The cultivation parameters were as described above. Mutants obtained from the determination of viability on MMA plates were transferred to MMA that enhances sporulation, SMMA (Pontecorvo 1953). SMMA contained 2 % glucose and 0.1 % sodium nitrate instead of 1 % glucose and 0.6 % sodium nitrate in MMA medium. The conidia were picked and transferred by sterile wire touch on SMMA plates and incubated 7 days at 30°C. The conidia were harvested as described and kept in 25 % glycerol at -30°C.

6.3.6. Lysine secretion of *A. ficuum* WT. In order to test the influence of the nitrogen source on lysine secretion, the following nitrogen sources were examined: urea 0.06 % (w/v), ammonium phosphate monobasic 0.24 %, (w/v), ammonium citrate 0.24 % (w/v), ammonium chloride 0.1 % (w/v), ammonium nitrate 0.016 % (w/v) and sodium nitrate, 0.18 % (w/v). The study was performed in the minimal medium described by Tauro et al. (1963) except that trace elements (Liebman et al. 2004) and 1 % (w/v) of glucose instead of the originally proposed 10 % were added. The pH was adjusted to 6.5 with 10 M KOH. The inoculum was prepared as described and 10^6 conidia/mL was introduced to 5 mL of broth in 25 mL flask. Culture was incubated 72 h at 30°C in water-bath shaker with 160 rpm (New Brunswick Scientific, N.J.). The fungal biomass was separated by centrifugation, 3 000×g, 10 min (Jouan), supernatant was decanted, filtrated through filter paper 0.22 μm pore size (Millipore) and used for lysine assay.

6.3.7. The determination of the mycelial dry weight. The mycelia were filtrated through pre-weighed filter paper 0.45 µm pore size (Millipore) by vacuum filtration and washed thoroughly with distilled water. Filtered mycelia were dried at 70°C until the constant weight was obtained. The weight of the filter paper was subtracted from the weight of the dry mycelia and expressed as mg dry weight.

6.3.8. Lysine assay. Secretion of lysine by designated regulatory mutants DL-ASP^{R} of *A. ficuum* was assayed in modified minimal medium containing ammonium phosphate monobasic as a nitrogen source with prescribed inoculum described by Tauro et al. (1963). Lysine secreted into the medium was measured in duplicates by the chemical assay (Vogel and Shimura 1971) with L-lysine as a standard. The standard curve intrapolation, regression and equation were calculated in MATLAB software, version 6. Concentration of L-lysine secreted was derived from the standard curve.

6.3.9. Statistical analysis. These analysis were performed using CoStat software, version 6.0. Analysis of variance (ANOVA) was performed using one-way completely randomized experimental design. The level of significance (a, b, c, d) was determined by Duncan's Multiple Range test where $p \leq 0.05$.

6.4. RESULTS

To determine the most potent lysine analog, the mycelia obtained from exponential growth were challenged with five analogs (Figure 6.1).

Unlike most prokaryotes and plants, eukaryotes synthesize lysine via AAA pathway where DAP is not involved (Vogel 1964). Still, the similar chemical structures of L-lysine and DAP allowed their use in this system. Initial attempts to use conidia on a plate assay were unsuccessful. Changing the strategy and employing exponentially growing mycelia however, indicated exclusion of AEC, FPA, DAP and LHX from further work and continuation with DL-ASP only (Table 6.1). Data presented in Table 6.1 were obtained by spot densitometry in Alpha Easy program, version 5.5. The relationship between the growth and the inhibition was expressed according to Alpha Imager TM User Manual where:

AVG = IDV/AREA

AVG: average value of the pixels after background subtraction

IDV: sum of all the pixel values after background correction

AREA: size of the region measured enclosed by box

In the assay for determination of the MIC, conidia were subjected to two treatments: (i) soaking in 0.03 % (v/v) Tween 80 at ambient temperature (22±2 ^{o}C) for 8 h prior to anti-metabolite exposure and (ii) immediate exposure to the analog (Sinha 1967). Soaking treatment was more effective; there was a decrease in the dry weight according to the higher concentrations of analog (Figure 6.2).

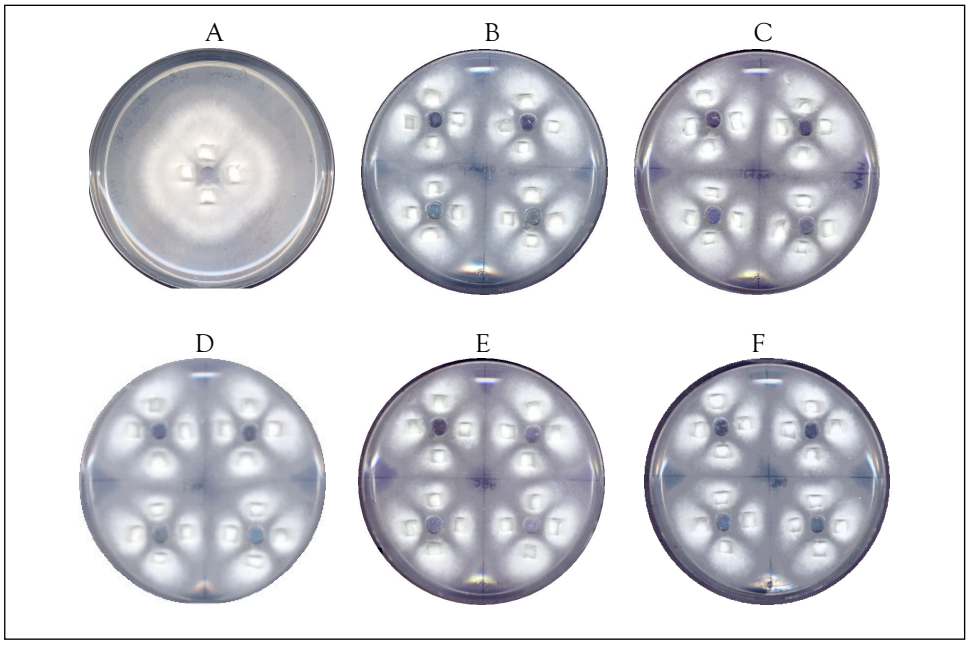

Figure 6.1. Impact of lysine analogs on the mycelial growth of *A. ficuum* NRRL 3135. Images represented are as follows: (A) positive control, (B) DL-ASP, (C) FPA, (D) LHX, (E) AEC and (F) DAP. In each plate the quadrants contained 0.5 mM (upper left), 0.1 mM (upper right), 50 µM (lower left) and 20 µM (lower right) of an analog concentration.

Table 6.1. Inhibition of *A. ficuum* mycelial growth by lysine analogs.

Analog	Concentration (μM) and growth (pixel value)			
	20	50	100	500
DAP	73.75 ± 7.46^{c}	67.00 ± 7.82^{bc}	66.25 ± 4.11^{bc}	71.00 ± 5.17^{b}
LHX	77.50 ± 5.98^{bc}	65.75 ± 4.53^{bc}	71.75 ± 7.41^{bc}	66.25 ± 5.3^{bc}
AEC	87.50 ± 3.92^{b}	69.75 ± 9.80^{b}	77.25 ± 6.46^{b}	58.00 ± 4.75^{c}
FPA	81.00 ± 8.83^{bc}	60.25 ± 0.74^{bc}	65.75 ± 8.20^{c}	64.00 ± 4.89^{bc}
DL-ASP	61.25 ± 6.49^{d}	56.25 ± 5.07^{c}	50.25 ± 4.07^{d}	45.75 ± 6.52^{d}

The positive control was the growth of *A. ficuum* w/o analog produced the pixel value of 102.50 ± 2.86^{a}.

Means followed by the same letter in a column do not differ significantly ($p \leq 0.05$).

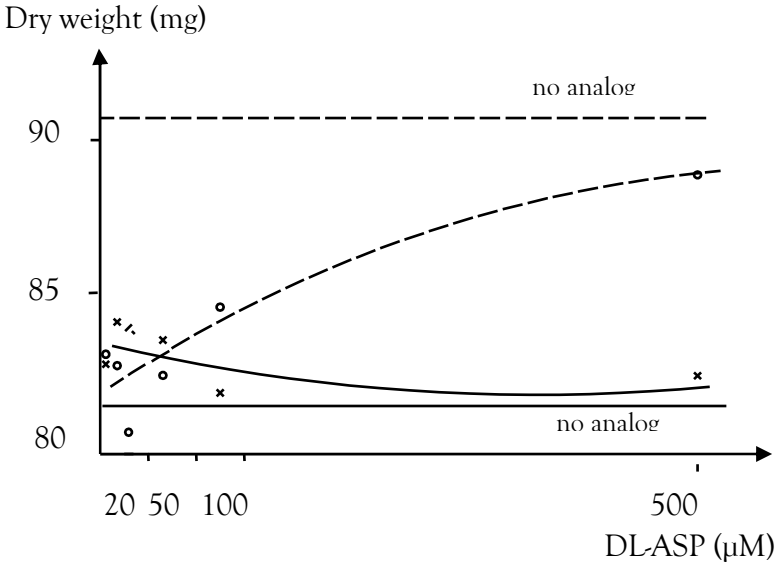

Figure 6.2. Influence of DL-ASP on conidia of *A. ficuum* in MMA broth. Weight of the filter is included. The treatments are presented as follows: (___) soaking 8 h in 0.03 % Tween 80 (v/v) and (_ _ _) w/o soaking. Experiment was performed with 10^3 conidia/mL in 6 mL total volume. Data present an average of duplicates. All standard errors are less than 10 %.

6.4.1. The relationship between inoculum size and MIC. It is known that inoculum size accompanied with mechanism of action of inhibitors has profound effect on inhibition by anti-metabolite or fungicides on growth of fungi (Moore et al. 2000, Antachopoulos et al. 2006).

As shown on Figure 6.3A and B, when 10^3conidia/mL was used in MMA broth both, growth and viability were inhibited by 2 mM of DL-ASP. There is a linear relationship between the inhibitor concentration, the growth inhibition and the viability. With an inoculum of 2×10^4 conidia/mL, however, DL-ASP had no effect on biomass, but viability was affected by all the concentrations of the inhibitor tested (Figure 6.3C and D). Using an inoculum of 6×10^4 conidia/mL abolished the inhibition and viability remained that of control (Figure 4.3E and F). In the system used in this thesis, inoculum 6×10^4 conidia/mL was large enough to overcome inhibitory effect of DL-ASP even at high concentration i.e. 20 mM. In contrast, all concentrations of DL-ASP tested were effective with 1×10^3conidia/mL. Interestingly, if 2×10^4 conidia/mL were inoculated, the growth occurred, but the mycelia could not conidiate. It is worth mentioning that conidia were not observed in the controls as well as at any concentration of inhibitor and inoculum. Lass-Flörl et al. (1998) showed that increase in inoculum size results in increase in MIC in fungal hyphae. As expected, the same principle is applicable to conidia challenged with lysine analog, DL-ASP.

In addition to the inoculum concentration, the inhibitor concentration is an important factor. Generally, increase in concentration should result in decreased growth.

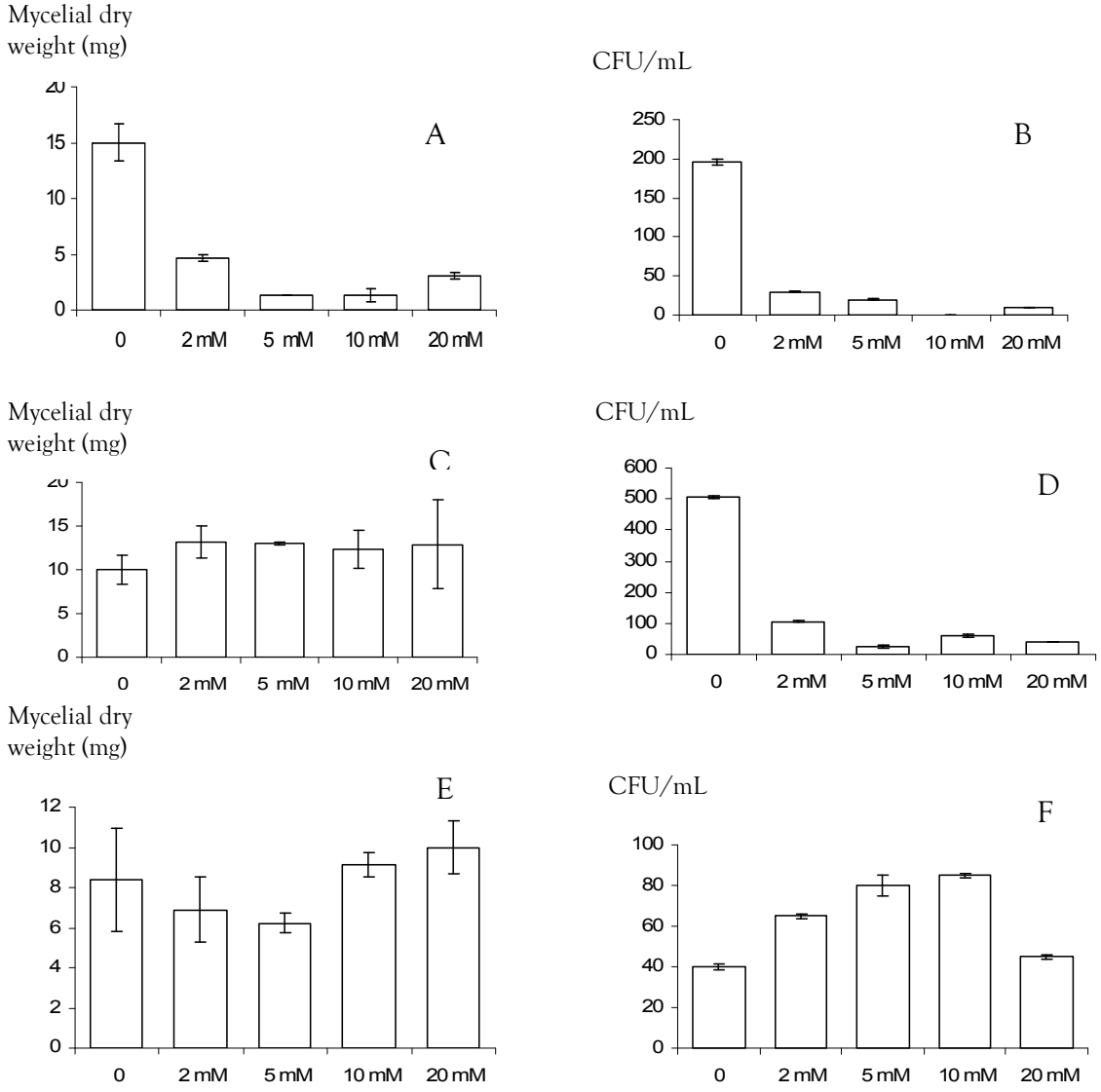

Figure 6.3. The relationship between inoculum size, MIC and viability of *A. ficuum* exposed to DL-ASP. Figures (A) and (B) represent inoculum 10^3 conidia/mL, (C) and (D) represent 2×10^4 conidia/mL, (E) and (F) represent 6×10^4 conidia/mL. Figures (A), (C) and (E) show inhibition. Axis X represents DL-ASP concentration. Figures (B), (D) and (F) show viability at corresponding inoculum where X-axis represents DL-ASP concentration to which mutants are resistant. Data present an average of duplicates with standard errors.

It has been reported that increased inhibitor concentration, namely itraconazole, terbinafine and amphotericin B, prolonged lag phase and delayed germination, while elongation rates of the hyphae remained that of control in *Aspergillus flavus* and *A. fumigatus* (Meletiadis et al. 2003). Note that design of this study was partially influenced by clinical studies on pathogenic strains of Aspergilli where killing curve was needed (Moore et al. 2000). The study of inhibition performed in this thesis was more subtle; the goal was inhibition of the growth, but survivors were necessary.

6.4.2. The lysine secretion of *A. ficuum* WT in MMA with respect to the nitrogen source. It is well known that fungi are able to utilize a number of nitrogenous organic and inorganic compounds. It had been shown by Richards and Haskins (1957) and Tauro et al. (1963) that nitrogen sources influenced lysine secretion in different fungi.

In *A. ficuum* WT a differences in lysine secretion with respect to the nitrogen source were observed. As shown in Table 6.2, ammonium phosphate promoted lysine secretion at a greater amount than other nitrogen sources. This was in concordance with the finding of Tauro et al. (1963), who reported that ammonium phosphate was a favorable nitrogen source for lysine secretion in *Ustilago* species. In contrast, ammonium chloride was the second best nitrogen source, while *Ustilago* species secreted approximately ten-fold lower concentrations of lysine on ammonium chloride compared to ammonium phosphate.

Table 6.2. Influence of nitrogen sources on lysine secretion, growth and pellet in *A. ficuum* NRRL 3135.

Nitrogen source	Lysine secreted (μM)	Dry weight of mycelium (mg)	Pellet morphology
Amm. Phosphate	21.94 ± 0.43 [a]	9.25 ± 0.45 [cd]	Amorphous
Amm. Chloride	19.15 ± 0.71[b]	10.30 ± 0.32 [c]	Slightly irregular, vary in size
Sodium nitrate	18.28 ± 0.76 [bc]	13.70 ± 0.32 [b]	d≈2 mm, slightly irregular
Urea	17.40 ± 0.21 [cd]	14.30 ± 0.92 [b]	d≈2 mm, distinct, round
Amm. Citrate	15.91 ± 0.09 [d]	18.95 ± 0.04 [a]	d≈ 5 mm, tend to agglomerate
Amm. Nitrate	Not detected	7.15 ± 0.12 [d]	Slightly irregular, agglomerates

Results represent a mean of two independent experiments, each performed in duplicate. Means followed by the same letter do not differ significantly ($p \leq 0.05$).

Having in mind that lysine biosynthesis in *A. ficuum* and *Ustilago* species occur via same AAA pathway, differences in the lysine secretion with respect to nitrogen source reflect to differences in primary metabolism and/or lysine secretion system. The comparison between sodium nitrate and ammonium nitrate was interesting.

Sodium nitrate promoted reasonably high lysine production, 18.28 μM, while ammonium nitrate was the only nitrogen source on which *A. ficuum* failed to secrete lysine. One can speculate that this significant difference is not due to cation NO_3^- rather to anions NH_4^+ and Na^+.

On the other hand, if the biomass and dry weight of mycelia were taken into account, it was clear that ammonium nitrate had an inhibitory effect on the growth (mycelial dry weight 7.15 mg) comparing to the other nitrogen sources 10.30-18.95 mg. Interestingly, when *A. ficuum* utilized ammonium phosphate as a nitrogen source, low biomass yielded high lysine concentration; ammonium citrate yielded the highest biomass and relatively low lysine content. Nitrogen source not only had an effect on lysine biosynthesis and secretion, but also post-centrifugal pellet morphology. This feature, which could be valuable for isolation of the over-producers to date, has not been reported. *Aspregillus ficuum* grown in MMA, urea, ammonium chloride and sodium nitrate promoted the formation of small, compact and round pellets, while the fungus grown on ammonium phosphate as a nitrogen source produced an amorphous pellet (Table 6.2).

6.4.3. The lysine secretion of *A. ficuum* DL-ASP^R mutants. Mutants resistant to 5 mM, 10 mM and 20 mM of DL-ASP were examined for lysine production as described.

As shown on Figure 6.4, DL-ASP^R mutants showed strikingly different morphologies on the minimal vs. rich medium. Mutants resistant to 5 mM DL-ASP differed in a rate of conidiation ranging from complete absence to colonies completely covered by conidia. Interestingly, mutants resistant to 10 mM and 20 mM of inhibitor did not show the variation as did mutants resistant to 5 mM. *Aspergillus ficuum* exposed to 10 mM and 20 mM yielded colonies with centers covered by conidia; peripheral mycelia were white and wooly, but without conidia. Culturing mutants on SMMA plates promoted higher conidiation (Figure 6.4) that made it the obvious choice of medium for maintenance.

As shown in Table 6.3, mutant 5-10 (colony pick #10 resistant to 5 mM DL-ASP) secreted significantly higher amounts of lysine in the medium. Other mutants tested secreted amount similar to the wild type, except mutant 20-2, which failed to secrete lysine. Mutants 5-4 and 5-5 had similar dry weight and lysine content to the wild type (Table 6.2 and 6.3). Mutant 20-2 failed to secrete lysine and had significantly lower biomass (Table 6.3).

Mutant 5-10 was challenged by 12 mM and 19 mM of the analog in MMA broth. The survivors appearance is shown on Figure 6.4A on SMMA plates; all were of the same morphology. From the 33 survivors, 11 randomly picked colonies were transferred to PDA and SMMA plates in order to further ensure their culture purity. These mutants were screened for lysine secretion as described.

A B

Figure 6.4. Appearance of *A. ficuum* 5-10 mutant on different solid media. Figure (A) shows mutant on SMMA plate and (B) on PDA plate.

Table 6.3. Lysine secretion of *A. ficuum* DL-ASP^R mutants.

Mutant #	Lysine conc. (μM)	Dry weight (mg)
5-10	29.25 ± 1.92 [a]	15.60 ± 0.57 [a]
10-1	21.80 ± 1.87 [b]	16.00 ± 0.73 [a]
5-1	20.93 ± 1.17 [bc]	15.60 ± 1.55 [a]
5-5	20.77 ± 0.48 [bc]	8.30 ± 0.36 [b]
10-2	19.78 ± 1.20 [bcd]	13.55 ± 1.10 [a]
10-4	19.17 ± 0.35 [bcd]	15.10 ± 0.65 [a]
20-4	18.25 ± 0.29 [bcd]	15.00 ± 1.06 [a]
5-4	17.44 ± 0.36 [cd]	8.80 ± 1.51 [b]
20-11	17.09 ± 0 [cd]	16.50 ± 0.73 [a]
10-3	16.32 ± 0 [d]	16.60 ± 1.46 [a]
20-2	Not detectable	3.25 ± 0.53 [c]

Results represent a mean of duplicates ± standard deviation. Means followed by the same letter do not differ significantly ($p \leq 0.05$).

Table 6.4. Lysine secretion of *A. ficuum* DL-ASP^{R} mutants resistant to 12 mM DL-ASP maintained on SMMA plates.

Mutant #	Lysine conc. (μM)	Dry weight (mg)
12-10	26.50 ± 0.55 [a]	7.96 ± 0.89
12-5	23.85± 0.78 [b]	13.14 ± 0.53
12-3	23.22 ± 0.37 [b]	11.50 ± 0.92
12-8	23.09 ± 0.26 [b]	8.62 ± 0.66
12-11	20.71 ± 0.33 [c]	10.50 ± 1.20
12-9	20.31 ± 0.66 [c]	7.70 ± 0.45
12-6	16.75± 0.11 [d]	17.90 ± 1.10
12-4	Not detectable	19.73 ± 1.07

Results represent a mean of duplicates ± standard deviation. Means followed by the same letter do not differ significantly ($p \leq 0.05$).

Mutants 12-1, 12-2 and 12-7 failed to form conidia on SMMA plates and therefore were not assayed for lysine secretion. It was obvious that no further improvement in lysine secretion of any significance was gained (Table 6.4). Mutant 12-10 secreted significantly higher lysine, 26.50 μM, compared to other mutants resistant to 12 mM DL- ASP, but less than mutant 5-10, which secreted 29.25 μM of lysine. An interesting relationship between the growth and lysine secretion was observed.

Mutants resistant to 5, 10 and 20 mM of DL-ASP could be roughly divided in a three clusters: (i) mutants that show reasonable high biomass and lysine secretion (Figure 6.5Aa), (ii) those of moderate biomass and lysine secretion (Figure 6.5.Ab) and (iii) mutant with the lowest biomass and no secreted lysine (Figure 6.5.Ac). Mutants resistant to 12 mM of DL-ASP could be divided into (i) mutants of reasonable high biomass and lysine secretion (Figure 6.5.Bd), (ii) mutant showing relatively high biomass and moderate lysine secretion (Figure 6.5 Be) and (iii) mutant of a high biomass, but failed to secrete lysine (Figure 6.5 Bf). It is well established that mutants resistant to a given concentration of anti-metabolite may not produce derivatives. They reach the plateau at the certain concentration and no further resistance and over-production can be gained. We believe that with *A. ficuum* DL-ASP^{R} mutant 5-10 has reached maximum lysine over-production, a 30 % increase compared to the wild type.

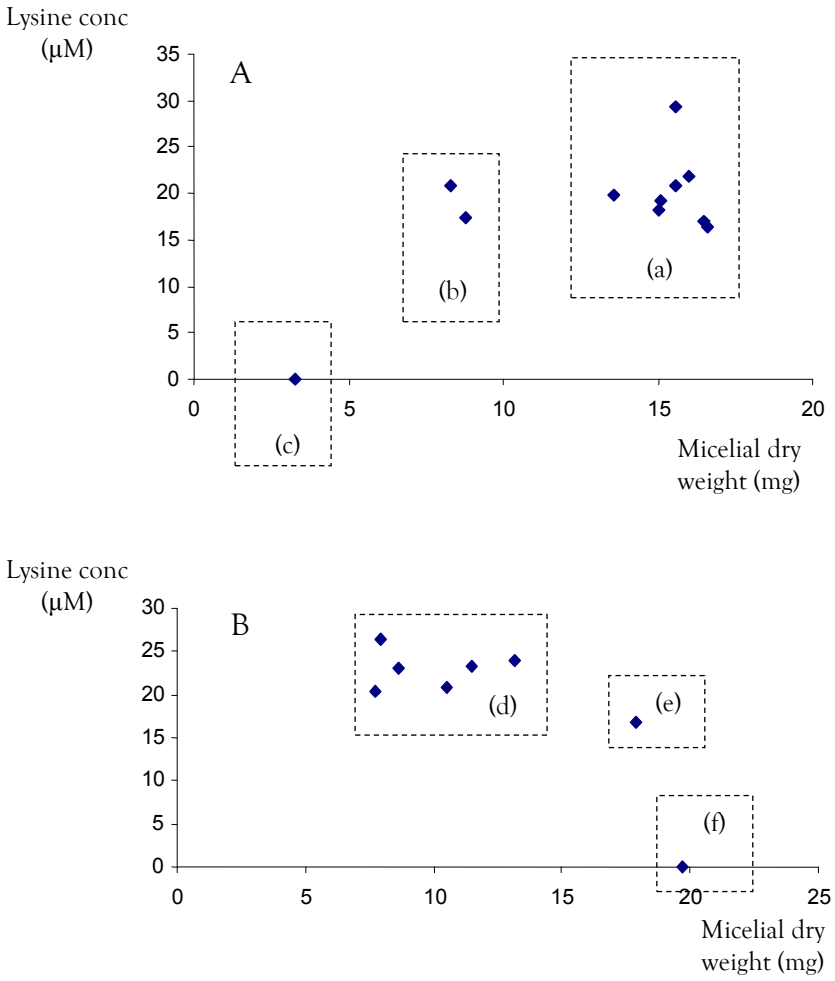

Figure 6.5. Relationship of the mycelial dry weight and lysine secretion in *A. ficuum* DL-ASP^{R} mutants. Figure (A) shows mutants resistant to 5, 10 and 20 mM of DL-ASP and corresponds to the Table 6.3. Figure (B) shows mutants resistant to 12 mM of DL-ASP and corresponds to the Table 6.4. For (a), (b), (c), (d), (e) and (f) see the text.

Table 6.5. Lysine secretion of *A. ficuum* DL-ASP^{R} mutants maintained on SMMA and PDA plates.

Mutant #	Maintenance medium			
	SMMA		PDA	
	Lysine (μM)	Dry weight (mg)	Lysine (μM)	Dry weight (mg)
12-10	26.50 ± 0.55	7.96 ± 0.89	18.42 ± 0.33	18.3 ± 1.97
12-9	20.31 ± 0.66	7.70 ± 0.45	17.89 ± 0.17	18.4 ± 1.45

Results are expressed as an average of duplicates ± standard deviation.

The mutants maintained on SMMA plates and PDA plates with respect to the lysine production were tested. Beside different morphologies (Figure 6.4), they differed in the concentration of lysine secreted. It was clear that minimal medium SMMA was more suitable for maintenance of spontaneous regulatory mutants of *A. ficuum* DL-ASP^{R} (Table 6.5). Interestingly, dry mycelium weight of mutants obtained on rich medium, PDA, was significantly higher, while lysine content secreted was reasonably lower than that of mutants maintained on minimal medium SMMA.

6.5. DISCUSSION

In the study of growth inhibition using fungi, one has to consider the choice of either of mycelia or conidia as inoculum. Mycelia respond to changes in the environment more drastically than conidia. More importantly, filamentous fungi compared by yeast, grow by apical elongation, leaving the major portion of the hyphae, which are away from the tips, metabolically inactive. For this reason, the mycelia were used in a plate assay for comparative study of five lysine analogs. The advantage of this assay was not only that provided an answer, but also allowed the replicates under minimal costs. However, the disadvantage of the plate assay is that at critical concentration of the inhibitor, results (clearing zones) do not fall into linear relationship, thus there is no correlation between clearing zone and concentration. Therefore, the plate assay was sufficient to define the strongest analog, but not to define the MIC as well. Following this thought, the broth assay using conidia as an inoculum and two

treatments were implemented. Another advantage of use of conidia instead of the hyphae in a broth assay is that the argument of apical growth is excluded.

Subjecting the conidia to the DL-ASP immediately after harvesting, did not inhibit the growth, but rather promoted it. Soaking conidia, in order to deprive them of internal amino acid pool, was shown to be a better choice. In this particular study, amino acid deprivation is desirable, as metabolic flux is directed towards primary, instead of secondary metabolites (Busch et al. 2003). Soaking 8 h in 0.03 % Tween 80 hydrated conidia and made them partially swollen, but germination did not occur. However, DL-ASP inhibited the growth without complete killing, which led to the conclusion that DL-ASP did not inhibit germination *per se*. At this point, one can speculate that the mechanism of action of DL-ASP is not linked with the inhibition of nucleic acid or RNA synthesis, but probably with the biosynthesis of malfunctional proteins or growth inhibition due to metabolite accumulation. Since *Aspergillus* species are saprophytes, which become common contaminants or pathogens, the relationship between inoculum size, biomass and viability with respect to susceptibility to fungicides and growth inhibitors have been studied in detail. It is shown that due to cell signaling and communication that occurs when conidia are exposed to stress, fungal conidia are able to survive (Ward et al. 2006). It was found that DL-ASP causes significant inhibition, but the MIC varied depending on the inoculum size. Inoculum sizes tested in this thesis were in a close range; 10^3, 2×10^4 and 6×10^4 conidia /mL. It was shown that at the low inoculum, 10^3 conidia/mL, even 5 mM DL-ASP was effective to suppress the growth as well as the viability. At 2×10^4 conidia/mL, conidia initially grew, resulting in overcoming the influence of the inhibitor where biomass, but no spores were observed. However, viability was strongly

impaired and the inhibition was strong enough to disallow the fungus conidia production. At three fold higher inoculum size than the former case, 6×10^4 conidia /mL, the inhibitory effect of the anti-metabolite was completely abolished, which was true even at high dose. It is worth to compare the differences in sensitivity of young mycelia vs. conidia. On the plate assay, the most potent concentration of inhibitor was established using concentrations 20 µM, 50 µM, 0.1 mM and 0.5 mM on the exponentially growing mycelia. Here 0.5 mM inhibited the growth by more than 50 %. However, an inoculum as low as 6×10^4 conidia /mL, was large enough to overcome the same inhibitor at concentration of 20 mM in spite of previous conidial depletion of amino acids. This finding is in concordance with Moore et al. (2000), who reported a 64-fold increase in MIC with 100-fold increase in inoculum from 4×10^3 conidia/mL in *A. fumigatus*. In contrast, Antachopoulus et al. (2006) showed that high inoculum, 10^5-10^6 conidia/mL, allowed susceptibility of *A. fumigatus*, *A. flavus* and *A. terreus* to antifungal agents in the first 4-6 h. In this thesis, the focus was not only on determination of susceptibility, but also on the obtaining the survivors for selection of DL-ASP^R. Therefore, low inoculum concentration and end-point measurements were applied.

Different nitrogen sources in the medium influenced the lysine secretion in *A. ficuum*. *Aspergillus* can utilize a number of amino acids as a nitrogen source that was especially explored in the penicillin production. It was reported that upon amino acid starvation, the cross-pathway control overrules secondary metabolite biosynthesis and favors the metabolic flux towards amino acids instead of penicillin in *A. nidulans* (Busch et al. 2003). Due to this, amino acids as a sole nitrogen source were not used in this study. It was observed that nitrogen source influences the pellet morphology in *A. ficuum*. Pellet morphology is an important

consideration for pilot plant and/or large-scale fermentation. During submerged fermentation, a fungus grows by hyphal extension and branching that affects the rheology and the viscosity of the cultivation in the medium. This has further impact on the accessibility of oxygen, which is crucial for *Aspergillus* as strict aerobes. Unfortunately, the relationship between branching and pellet formation is still not well understood. It is reported, however, that disruption of the *chs*B gene, which encodes for chitin synthase, results in altered morphology and pellets less prone to form inseparable clumps in *Aspergillus oryzae* (Müller et al. 2002).

In the concordance with previous findings (Richards and Haskins 1957, Tauro et al. 1963), ammonium phosphate as a nitrogen source promotes enhanced level of lysine secretion in a various fungal species. In order to address this question, one has to take into consideration fungal physiology and the central metabolism. It is well established that cross-pathway-control (CPC) system presents the global regulation of the biosynthetic routes in fungi (Crasiotis and Jones 1974). It acts as a response to the environmental stresses, mainly the amino acids deprivation. When amino acid starvation occurs, uncharged tRNA molecules accumulate, which are bound by the HisRS domain of an eIF2α kinase. The phosphorylation of this subunit leads to down-regulation of general translation, accompanied by increased expression of the activator to generate transcriptional read-out, in order to counteract the starvation conditions (Krappmann and Braus 2005). On the other hand, ammonium is a primary and favorable nitrogen source in fungi. In the system used here, *A. ficuum* conidia were exposed to the minimal medium, where amino acid starvation occurred. Ammonium phosphate in these conditions acts as a donor of NH_4^+, which is easily utilized nitrogen source

and as a donor of PO_4^{3-}, thus helping in triggering the CPC system and enhancing amino acid biosynthesis and secretion.

Mutants *A. ficuum* DL-ASP^R resistant to 5 mM, 10 mM and 12 mM of an analog, secreted lysine in various concentrations. At the low end were mutants 12-6 and 10-3 excreting 16.75 µM and 16.32 µM of lysine respectively. At the high end were mutants 5-10 and 12-10 excreting 29.25 µM and 26.50 µM of lysine respectively. Interestingly, it was not possible to obtain higher secretion by exposing to higher anti-metabolite concentration. However, the best lysine over-producer (mutant 5-10) secreted roughly a 30 % higher amount of lysine than the wild type. It was shown that the preferred maintenance of the mutants is on SMMA medium as opposed to the rich, PDA medium. Even if a 30 % of increase in lysine content in the fermentation broth while a modest value does prove that *A. ficuum* NRRL B 3135 mutants deregulated for lysine production are possible and can be employed as a lysine enhancer in feed. More significant over-production would assume defining and resolving the bottlenecks in biosynthesis and secretion.

6.6. RELEVANCE OF CHAPTER 6 TO THE THESIS

In concordance to the objectives proposed in the research program of the thesis and reflected in the title, Chapter 6 is dedicated to the isolation of *Aspergillus ficuum* lysine over-producing mutant. This fungus is chosen because of its high level of phytase production, which enhances its possible application in biotransformation of canola meal in addition to enhancing lysine content.

7. GENERAL DISCUSSION

The goal of this thesis was to obtain regulatory mutants of lactobacilli and/or *Aspergillus* that over-produce lysine. These mutants can be used for the enrichment of lysine content in canola meal and other. The choice of microorganisms was deliberate; lactobacilli have a reputation as 'friendly' bacteria that contribute to the microbiological and nutritional value of feed and food. *Aspergillus ficuum* has a commercial value as a source of phytase that is important for decreasing the phytic acid content, an undesirable component of canola meal (Espitia 2004, Hindra 2006).

It is well established that LAB are highly adaptable. Their complex nutritional requirements are not solely the result of the adaptation to the environments rich in protein and carbohydrate breakdowns, but to dynamic horizontal and vertical gene transfer as well. Starting with these known and well-established facts, the first step in the thesis was to determine the requirements for lysine in LAB. This thesis took the approach of enhancing lysine production by de-regulation of the active biosynthetic pathway, instead of reconstructing the pathway that is non-existing or impaired, which is usual in LAB. The choice of strains was their availability and use in a previous thesis (Espitia 2004). Nine LAB strains, originating from a versatile environment were tested. This, at first glance broad range of strains, was necessary due to the evidence that the nutritional requirements of LAB may not be highly connected to the environment (Molenaar et al. 2005). Chapter 3 showed that lysine was the least required

amino acid, which theoretically permitted the use of any LAB tested. On the other hand, these bacterial strains displayed different growth rates and cell yields in the chosen growth medium. This became the second criterion in the selection of working bacterium. *Lactobacillus delbrueckii* and *L. plantarum* showed growth rates higher then other LAB, 0.2 h^{-1} and 0.18 h^{-1} respectively and had reasonably simple requirements. This outcome decided the direction of the work with *L. plantarum* and *L. delbrueckii* in order to obtain lysine over-producers. Unexpectedly, both strains were resilient to lysine analogs to be used for selection of the mutants. Some studies have shown that slowly growing microorganisms survive adverse conditions better than faster ones (Brown et al. 1990). It has been noted that AEC caused complete inhibition of growth in *S. cerevisiae* at a concentration of 20 µM (Gasent-Ramírez and Benítez 1997), 0.3 mM inhibited the growth of *Arthrobacter globiformis* (Samanta and Bhattacharyya 1991), while in *L. plantarum* and *L. delbrueckii* 0.5 mM caused only 60 % of growth inhibition. *Saccharomyces cerevisiae* is a fast growing eukaryote having generation time of 1.5 h in a rich medium, while *Arthrobacter globiformis* and *L. plantarum* had generation times of 1 h (Streit et al. 1991) and 5-6 h (this thesis) respectively. Lactobacilli are certainly considered the slower growing bacteria. In spite of the seeming correspondence between the low growth rate and the high tolerance to the lysine analogs, inhibition of the growth by anti-microbial agents or susceptibility to them is a complex phenomenon. Therefore, the statement that low growth rate is the cause of anti-metabolite resistance should be taken cautiously.

Exposure to the anti-metabolites following the depletion of endogenous lysine revealed that *L. delbrueckii* responded by significantly decreasing the cell size instead of decreasing the cell yield. While the phenomenon itself is known (Brown et al. 1990), in this thesis, *L.*

delbrueckii displayed it to a higher degree than expected. Interestingly, in *L. plantarum* the same phenomenon was not observed. However, lack of knowledge on the details of the basic metabolism and stress responses in *L. plantarum* NRRL B 4496 and *L. delbrueckii* ATCC 9649 does not allow a firm conclusion, but rather speculations.

According to Chapter 3, lactobacilli showed absolute requirements for seven amino acids, one vitamin and one mineral salt. Requirements for other amino acids, vitamins and minerals tested were scattered among the strains showing overall auxotrophy for a high number of ingredients and therefore high dependency on the vagaries of the environment or growth medium. We can conclude that due to this, lactobacilli have seemingly simple metabolism where biosynthetic pathways for essential nutrients are either obviously impaired or completely absent. But, how does this genus relate or cope with its high dependency on the environment, which makes it fragile? Chapter 4 provided the answer to this question: some pathways were absent, but those that were active were tightly regulated and in a complex manner.

In this thesis, the connection between AK regulation and auxotrophy of *L. plantarum* was observed (Table 7.1). The family of amino acids derived from this biosynthetic pathway had an interesting relationship to growth. Isoleucine was essential, but had a little effect on AK. Methionine did not play the major role in AK regulation and had no major impact on growth. Regulation of AK in *L. plantarum* was shared by lysine and threonine and at least one of these amino acids was necessary for the cells to sustain their growth. Obviously, in *L. plantarum* AK regulation has evolved in a way that growth was not compromised even in lysine or threonine deficient environments. Considering the diversity of the genus, one can

confidently hypothesize that AK regulation is not uniform within the genus. Therefore, it is tempting to believe that there is correlation between specific requirements for the aspartate family amino acids and AK regulation among different species. The study of growth performed on *L. delbrueckii* ATCC 9649 indicated that *L. plantarum* and *L. delbrueckii* share similarities in requirements (Chapter 3) and AK regulation (Chapter 4), in spite of the taxonomic distance separating them on phylogenic tree. Lack of information on this subject in other species and biosynthetic pathways prevents generalization. However, it is clear that the DAP pathway is conserved in bacteria as well as its important features, such as high involvement of lysine and threonine, less involvement of methionine and no involvement of isoleucine in the DAP pathway regulation.

Lactobacillus plantarum obviously must share such features with other bacteria, regardless of its origin or requirements. What seems to be the result of specific adaptation of *L. plantarum* as a multiple auxotroph is the regulation of the DAP pathway that is more complex and stringent than that in other bacteria.

This thesis also showed that activity and regulation of AK in *L. plantarum* depended on the growth stage, in order to serve better the cells' need in the exponential versus stationary phases, the two metabolically different growth stages. The regulatory feature of AK was shown to be under control of four isozymes that, in concordance with the previous statement, exerted different activities throughout the cells' growth. Interestingly, regulation of AK isozymes in the exponential growth stage was typical feed-back inhibition, while they were insensitive to the same in stationary stage. Another complex finding is that all four amino acids of the aspartate family amino acids were involved in the regulation of the AK isozymes. Further more, lysine

sensitive AK I, was active only when bacteria were exposed to an excess of aspartate family amino acids, while AK III (also lysine sensitive) was active only if bacteria was exposed to an excess amount of threonine.

Table 7.1. Summary of the influence of aspartate family amino acids on *L. plantarum* growth and AK activity.

Amino acid	Influence on growth	Influence on AK activity with respect to the growth stage
Lysine	Not essential if threonine is present	Repression in exponential Inhibition in stationary
Threonine	Not essential if lysine is present	Inhibition and repression in exponential No effect in stationary
Methionine	Stimulatory	Stimulatory in both phases
Isoleucine	Essential	Stimulatory in exponential No effect in stationary

This clearly showed that the regulatory role of AK of amino acids supplied in the growth environments, or the broad range of habitats in which *L. plantarum* was found. From the perspective of finding lysine over-production, complex regulation of the biosynthesis did not go in our favor. The system with two lysine sensitive isozymes both active in stationary phase, but insensitive to their inhibitor lysine, does not leave much hope to find that any lysine analog will de-regulate them. Indeed, as shown on Figure 5.3 (Chapter 5), addition of lysine to the medium while it did not reverse inhibition caused by LHX in the exponential phase, but it did to some extent in the stationary culture. Chapter 4 showed that lysine sensitive isozymes were not present in exponential, but only in stationary growth phase, which confirmed the results of inhibition study with respect to growth stages.

Another obstacle in obtaining lactobacilli lysine over-producer with high lysine yield was that lysine sensitive AK III has multiple repressors; in fact, lysine served as an enzyme activity inhibitor and synthesis repressor at the same time. One can speculate that modest lysine over-production obtained in mutant #34 was due to deregulation of AK I as it was not repressed by the aspartate family amino acids.

In the experiments with *A. ficuum*, this thesis faced better prospects then those with *L. plantarum*. *Aspergillus ficuum*, due to its ubiquitous nature, is able to utilize many carbon and nitrogen sources and has simple growth requirements. Therefore, there was no need for screening for lysine as a growth requirement. On the other hand, high adaptability of *A. ficuum* sensitive gave room for optimizing the medium making it more suitable for lysine production. This led to the observation that ammonium phosphate is the choice ingredient for nitrogen source, which promotes lysine secretion into the medium. Surprisingly, nitrogen sources also influenced the pellet formation in *Aspergillus*. Unlike in prokaryotes, the lysine biosynthetic pathway in eukaryotes is linear and leads to lysine only. Consequently, the regulation of the pathway is simple. Indeed, regulatory mutants were obtained with *A. ficuum* and the best lysine producing mutant, #5-10, secreted approximately 30 % more lysine than the wild type.

Despite the fact that eukaryotic systems are more complex, in this thesis, higher lysine over-production was obtained by *A. ficuum* as opposed to *L. plantarum*.

8. CONCLUSIONS AND FUTURE PROSPECTS

1. The tight connection between origin of LAB and their nutritional requirements as proposed by Morishita et al. (1981) was not observed in this thesis.

2. The regulation of the DAP biosynthetic pathway is more complex in *L. plantarum* than in other bacteria and plants. The key enzyme, AK exists as a complex of four isozymes whose activity depends on bacterial growth stage. All of the aspartate family amino acids are involved in its regulation, as inhibitors, repressors or activators. Existence of two lysine sensitive isozymes insensitive to their inhibitors in the stationary growth stage that showed similarity with plants implies a shared ancestry through possible horizontal gene transfer.

3. This thesis found a connection between LAB nutritional requirements and AK regulation. Emphasis in regulatory role was given to the amino acids for which functional biosynthetic pathways exist as opposed to amino acids that are abundant in the environment.

4. Lysine over-producing mutants in *L. plantarum* are rare, due to complex regulation of the biosynthesis. Since that methionine and isoleucine are repressors of lysine sensitive

AK, higher lysine over-production assumes insensitivity to methionine and isoleucine as well.

5. An *A. ficuum* mutant providing 30% higher lysine content was obtained, which could be used for enrichment in feed.

6. Ammonium phosphate as a nitrogen source promoted lysine secretion in the minimal medium by *A. ficuum*. It was observed that nitrogen source influenced pellet morphology in *A. ficuum* by the mechanisms that has not been reported nor well understood.

As for the future prospects, according to Chapter 4, high lysine yield in *L. plantarum* requires AK that is insensitive to lysine, threonine and methionine at the same time, because all of these amino acids are involved in the regulation of AK. This thesis showed that zymography can have larger and innovative uses than it was historically proposed- strictly to establish the number of isozymes. Here, a complete regulation of AK using zymograms was established. Conventionally, purified isozymes are used for kinetic and genetic studies. The knowledge of zymography could also be used for isolation of mutant isozymes. Such a system needs to be developed.

Aspergillus ficuum showed promising results that can be improved with a larger search of spontaneous mutants that over-produce lysine. Further, it is possible to enhance the yield by selection for over-producers and optimizing the fermentation parameters.

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10. APPENDICES

APPENDIX 1

Table 10.1. Growth of lactobacilli upon omission of amino acids from M3.

Omitted L-amino acid	Lactobacillus species and OD_{620} ($\times 10^{-1}$)								
	L. delbrueckii	*L. plantarum*	*L. rhamnosus*	*W. confusa*	*L. zeae*	*L. frigidus*	*L. agilis*	*L. casei*	*L.acidophilus*
Glu	0.77 ± 0.12	0.33 ± 0.03	0.03 ± 0.01	0.13 ± 0.03	0.21 ± 0.02	0.32 ± 0.02	0.14 ± 0.01	0.15 ± 0.00	0.13 ± 0.04
Pro	1.32 ± 0.05	1.44 ± 0.09	3.01 ± 0.04	0.16 ± 0.01	0.24 ± 0.01	0.29 ± 0.01	0. 15± 0.02	0.15 ± 0.02	0.15 ± 0.01
Arg	1.47 ± 0.11	1.50 ± 0.19	2.49 ± 0.09	0.14 ± 0.01	0.17 ± 0.04	0.37 ± 0.03	0.20 ± 0.04	0.14 ± 0.01	0.16 ± 0.01
Asp	0.67 ± 0.08	0.66 ± 0.04	0.46 ± 0.10	0.18 ± 0.01	0.38 ± 0.03	0.34 ± 0.00	0.17 ± 0.01	0.12 ± 0.00	0.16 ± 0.01
Ile	0.66 ± 0.11	0.48 ± 0.13	0.65 ± 0.20	0.64 ± 0.07	0.59 ± 0.07	0.34 ± 0.04	0.54 ± 0.04	0.27 ± 0.04	0.41 ± 0.02
Thr	0.83 ± 0.01	0.73 ± 0.04	0.40 ± 0.02	0.16 ± 0.01	0.26 ± 0.03	0.29 ± 0.02	0.18 ± 0.02	0.12 ± 0.01	0.16 ± 0.00
Met	1.55 ± 0.11	1.80 ± 0.18	2.09 ± 0.42	0.17 ± 0.01	0.19 ± 0.03	0.37 ± 0.03	0.28 ± 0.08	0.10 ± 0.03	0.15 ± 0.00
Val	0.72 ± 0.06	1.07 ± 0.32	0.71 ± 0.19	0.18 ± 0.01	0.25 ± 0.01	0.32 ± 0.01	0.18 ± 0.02	0.14 ± 0.00	0.14 ± 0.00
Leu	0.42 ± 0.04	0.55 ± 0.11	0.27 ± 0.04	0.18 ± 0.01	0.36 ± 0.06	0.27 ± 0.04	0.20 ± 0.04	0.12 ± 0.01	0.15 ± 0.00
Cys	1.22 ± 0.11	0.50 ± 0.08	1.18 ± 0.03	0.11 ± 0.01	0.18 ± 0.06	0.33 ± 0.01	0.25 ± 0.11	0.13 ± 0.01	0.15 ± 0.00
His	2.34 ± 0.12	2.19 ± 0.14	2.52 ± 0.03	0.14 ± 0.02	0.27 ± 0.03	0.27 ± 0.12	0.18 ± 0.03	0.16 ± 0.01	0.15 ± 0.00
Phe	0.69 ± 0.08	0.45 ± 0.06	1.17 ± 0.16	0.17 ± 0.01	0.23 ± 0.04	0.30 ± 0.04	0.21 ± 0.01	0.11 ± 0.05	0.14 ± 0.01
Tyr	0.86 ± 0.09	0.86 ± 0.07	0.85 ± 0.10	0.75 ± 0.19	0.54 ± 0.04	0.39 ± 0.05	0.51 ± 0.12	0.26 ± 0.06	0.43 ± 0.04
Trp	1.51 ± 0.12	1.16 ± 0.09	2.31 ± 0.30	0.14 ± 0.01	0.20 ± 0.05	0.32 ± 0.05	0.19 ± 0.02	0.12 ± 0.04	0.14 ± 0.00

Table supports the data shown in Table 3.3, Chapter 3. Experiment was performed as described in Materials and Methods of the same Chapter. The end point of growth was expressed an average OD_{620} ± standard deviation.

APPENDIX 2

Table 10.2. Growth of lactobacilli upon omission of vitamins, minerals and buffer components from M3.

Omitted constituent	Lactobacillus species and OD_{620} ($\times 10^{-1}$)								
	L. delbrueckii	*L. plantarum*	*L. rhamnosus*	*W. confusa*	*L. zeae*	*L. frigidus*	*L. agilis*	*L. casei*	*L. acidophilus*
Tween 80	3.47 ± 0.21	2.83 ± 0.15	1.82 ± 0.14	0.57 ± 0.02	0.87 ± 0.03	0.60 ± 0.04	0.40 ± 0.01	0.28 ± 0.01	0.23 ± 0.03
Amm. citrate	2.18 ± 0.21	2.22 ± 0.13	2.67 ± 0.06	1.63 ± 0.06	1.29 ± 0.13	0.21 ± 0.04	0.81 ± 0.04	0.51 ± 0.03	0.53 ± 0.02
Sodium acetate	2.76 ± 0.08	2.64 ± 0.24	3.49 ± 0.11	1.61 ± 0.03	1.28 ± 0.011	0.52 ± 0.04	0.65 ± 0.07	0.54 ± 0.06	0.61 ± 0.03
K_2HPO_4	0.97± 0.17	1.00 ± 0.06	0.10 ± 0.04	0.21 ± 0.06	0.45 ± 0.05	0.08 ± 0.04	0.21 ± 0.02	0.16 ± 0.04	0.19 ± 0.03
$MgSO_4 \times 7H_2O$	2.91± 0.07	2.51 ± 0.27	2.33 ± 0.08	0.23 ± 0.03	0.45 ± 0.04	0.29 ± 0.03	0.19 ± 0.04	0.21 ± 0.01	0.15 ± 0.04
$MnSO_4 \times H_2O$	4.32 ± 0.09	3.59 ± 0.26	3.25 ± 0.13	1.12 ± 0.06	1.14 ± 0.09	0.22 ± 0.04	0.30 ± 0.03	0.20 ± 0.02	0.18 ± 0.02
$FeSO_4 \times 7\ H_2O$	1.76 ± 0.06	1.95 ± 0.02	2.93 ± 0.17	0.91 ± 0.07	0.85 ± 0.03	0.41 ± 0.04	0.38 ± 0.01	0.26 ± 0.03	0.27 ± 0.04
Ca pantothen.	2.19 ± 0.21	2.23 ± 0.08	3.12 ± 0.16	0.98 ± 0.09	0.97 ± 0.07	0.34 ± 0.07	0.36 ± 0.04	0.20 ± 0.02	0.25 ± 0.02
B6	1.17 ± 0.01	1.16 ± 0.26	1.50 ± 0.07	0.29 ± 0.06	0.41 ± 0.10	0.34 ± 0.01	0.22 ± 0.02	0.12 ± 0.02	0.16 ± 0.04
B12	2.19 ± 0.05	2.41 ± 0.01	2.70 ± 0.09	1.10 ± 0.02	1.21 ± 0.07	0.46 ± 0.04	0.35 ± 0.03	0.25 ± 0.02	0.28 ± 0.02
B3	2.10 ± 0.08	2.00 ± 0.10	2.60 ± 0.25	1.03 ± 0.03	0.93 ± 0.05	0.27 ± 0.03	0.33 ± 0.03	0.21 ± 0.01	0.25 ± 0.01
B2	3.06 ± 0.13	3.10 ± 0.24	2.97 ± 0.17	0.94 ± 0.11	1.01 ± 0.07	0.39 ± 0.04	0.32 ± 0.04	0.25 ± 0.04	0.21 ± 0.06
Folic acid	2.23 ± 0.05	2.24 ± 0.09	3.13 ± 0.25	0.50 ± 0.03	0.74 ± 0.08	0.30 ± 0.05	0.32 ± 0.03	0.21 ± 0.06	0.31 ± 0.00
Thymidine	2.39 ± 0.05	2.32 ± 0.26	2.83 ± 0.07	1.03 ± 0.01	1.00 ± 0.07	0.41 ± 0.05	0.33 ± 0.06	0.28 ± 0.03	0.23 ± 0.05

Table supports the data shown in Table 3.4, Chapter 3. Experiment was performed as described in Materials and Methods of the same Chapter. The end point of growth was expressed an average OD_{620} ± standard deviation.