INTERACTION OF *LACTOCOCCUS LACTIS* AND *STAPHYLOCOCCUS XYLOXUS* IN MIXED CULTURE

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STAPHYLOCOCCUS XYLOXUS IN MIXED CULTURE

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ปนัดดา โหน่งบึ้ง : การศึกษาอัตรกิริยาของ *LACTOCOCCUS LACTIS* และ *STAPHYLOCOCCUS XYLOXUS* ในสภาวะการเพาะเลี้ยงร่วม (INTERACTION OF *LACTOCOCCUS LACTIS* AND *STAPHYLOCOCCUS XYLOXUS* IN MIXED CULTURE) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.สุนทร กาญจนทวี และอาจารย์ ดร.Pascal Loubière, 98 หน้า

การศึกษาอันตรกิริยาของ *Lactococcus lactis* และ *Staphylococcus xylosus* ในสภาวะการเพาะเลี้ยงร่วมในถังหมักที่ควบคุมสภาวะ เพื่อศึกษาผลของออกซิเจนที่มีต่ออันตรกิริยาของแบคทีเรียทั้งสองชนิด ซึ่งได้แก่ การควบคุมปริมาณออกซิเจนที่ละลายได้ในถังหมักร้อยละ 80 และสภาวะที่ไม่มีการควบคุมออกซิเจนในถังหมัก การศึกษาสภาวะโดยแบคทีเรียทั้ง 3 ได้แก่ *L. lactis* บริสุทธิ์, *S. xylosus* บริสุทธ์ และการเลี้ยงเชื้อผสม จากการศึกษาพบว่าภายใต้สภาวะที่มีการควบคุมออกซิเจน ในสภาวะการเลี้ยงเชื้อผสมแสดงการหมักแบบไม่ใช้ออกซิเจน ภายหลังจากออกซิเจนถูกใช้หมดไป ปริมาณน้ำหนักแห้งของแบคทีเรีย *L. lactis* และการเลี้ยงเชื้อผสม ไม่มีความแตกต่างกล่าวคือ 2.0 และ 2.3 $g.L^{-1}$ ตามลำดับ อัตราการใช้กลูโคสของ *L. lactis* บริสุทธิ์ , *S. xylosus* บริสุทธิ์ และการเลี้ยงเชื้อผสม คือ 38.6, 10.3 และ 36.8 $mmol.g^{-1}.h^{-1}$ ตามลำดับ ผลิตภัณฑ์ที่เกิดขึ้นของการเลี้ยงเชื้อผสมและ *L. lactis* บริสุทธิ์ ไม่มีความแตกต่างกัน ภายใต้สภาวะออกซิเจนร้อยละ 80 ปริมาณน้ำหนักแห้งของ *S. xylosus* บริสุทธิ์และการเลี้ยงเชื้อผสมเพิ่มขึ้น (3.7 และ 3.5 $g.L^{-1}$ ตามลำดับ) เช่นเดียวกับอัตราการใช้กลูโคสจำเพาะ 37.3, 30.9 และ 38.6 $mmol.\ g^{-1}.h^{-1}$ ตามลำดับ *S. xylosus* ในสภาวะการเลี้ยงเชื้อผสมมีการใช้แลคแตทเพื่อการคงสภาวะของเซลล์ ภายหลังจากกลูโคสถูกใช้หมดไป

การศึกษาความเป็นไปได้ในการวิเคราะห์ การแสดงออกยีนของ *L. lactis* ภายใต้เพาะเลี้ยงร่วมกับ *S. xylosus* โดยใช้ *L. lactis* chip พบว่า มีการเกิด cross-hybridization ของกรดนิวคลีอิก จาก *S. xylosus* ในปริมาณมาก ดังนั้นการศึกษาการลดการเกิด cross-hybridization โดยการเติม genomic DNA ของ *S. xylosus* ในปริมาณ 50 และ 400 μg นั้นไม่สามารถลดการเกิด cross-hybridization บน *L. lactis* chip ได้ ดังนั้นการศึกษาการแสดงออกยีนของ *L. lactis* ในสภาวะนี้ไม่สามารถเป็นไปได้

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2551

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PANADDA NONGBEUNG : INTERACTION OF *LACTOCOCCUS LACTIS* AND *STAPHYLOCOCCUS XYLOXUS* IN MIXED CULTURE. THESIS ADVISOR : ASST. PROF. SUNTHORN KANCHANATAWEE, Ph.D., AND PASCAL LOUBIERE, Ph.D., 98 PP.

BACTERIAL INTERACTION/*LACTOCOCCUS LACTIS*

/*STAPHYLOCOCCUS XYLOSUS*

The study of interaction of *L. lactis* and *S. xylosus* which were grown in mixed culture in a controlled bioreactor under well-defined conditions was conducted in order to understand the impact of oxygen on the interaction of the two types of bacteria, that is, the control of 80% oxygen quantity dissolved in the bioreactor, and under uncontrolled state of oxygen. From the study of the three bacteria conditions*:* pure *L. lactis*, pure *S. xylosus*, and mixed culture, it was found that under controlled state of oxygen in mixed culture that expressed anaerobic fermentation after oxygen was completely exhausted, biomass of pure *L. lactis* and mixed culture was not different, about 2.0 and 2.3 g/l, respectively. Specific glucose consumption rates ($q_{glucose}$) in pure *L. lactis, S. xylosus* and mixed culture, were 38.6, 10.3 and 36.8 $mmol.g^{-1}.h^{-1}$, respectively. Products produced under both conditions were also not different. Under 80% oxygen condition, biomass of pure *S. xylosus* and mixed cultures was increased (3.7 and 3.5 $g.L^{-1}$, respectively). The same was true of specific glucose consumption rates($q_{glucose}$) which were 37.3, 30.9 and 38.6 $mmol.\ g^{-1}.h^{-1}$, respectively. The results showed that *S. xylosus* in mixed culture consumed lactate for cell maintenance after glucose was exhausted.

After the transcriptome analysis of *L. lactis* and *S. xylosus* in mixed culture was performed on specific *L. lactis* DNA chip, it was discovered that massive cross-hybridization of *S. xylosus* nucleic acid on *L. lactis* DNA chip occurred. The possible reduction of cross-hybridization by adding partner unlabeled genomic DNA was conducted in different concentrations, but the results showed that this strategy could not be accomplished.

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Panadda Nongbeung

CONTENTS

Page

CONTENTS (Continued)

Page

CHAPTER

LIST OF TABLES

LIST OF TABLES (Continued)

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

μ_{max}	=	Specific growth rate
$q_{glucose}$	=	Specific glucose consumption
$\nu_{lactate}$	=	Specific acetate production
$\nu_{acetate}$	=	Specific acetate production
$\nu_{formate}$	=	Specific formate production
$\nu_{ethanol}$	=	Specific ethanol production
$\nu_{acetoin}$	=	Specific acetoin production
$Y_{cell, glucose}$	=	Biomass yield relative to glucose
$Y_{lactate, glucose}$	=	Lactate yield relative to glucose
$Y_{acetate, glucose}$	=	Acetate yield relative to glucose
$Y_{ethanol, glucose}$	=	Ethanol yield relative to glucose
$Y_{formate, glucose}$	=	Formate yield relative to glucose
$Y_{acetate, lactate}$	=	Acetate yield relative to lactate
$Y_{acetoin, glucose}$	=	Acetoin yield relative to glucose

CHAPTER I

INTRODUCTION

1.1 Background

The microbial ecosystems associated with fermented foods are often complex, with several species acting in interaction, between themselves and with the food matrix. Most of the time, microbial ecosystems involved in food processes are considered as a black box because of their complexity. In the past fermented foods were carried out spontaneously by action of the natural microflora present in the employed ingredients or in the manufacturing environment. Many countries have traditional fermented food products and the consumers prefer to keep their typical characteristics. However, traditionally fermented foods products are made by the use of uncontrolled microbial processes which may sometimes lead to either inferior or even unsafe final products. Therefore, the utilization of starter cultures for food products to improve and guarantee safety is generalised. Nowadays, microbial product formations, flavour profiles, enzyme activities and growth influencing factors have sometimes been individually studied but rarely in cultures containing more than one characterised microorganism**.** Among fermented foods, cheese and sausages have a place of choice. Among the starters used in these products, lactic acid bacteria (LAB) and coagulase-negative staphylococci (CNS) are widely employed. *Lactococcus lactis* is the LAB model and used as starter in most cheese and sausages manufacturing. The other main starter microorganism used for sausages.

manufacturing is the CNS, *Staphylococcus xylosus* which can also be present in some cheese as a minor flora. In cheese manufacturing this leads to the coagulation of soluble proteins and development of the texture and organoleptic properties of the final product. In fermented food, acidification is also employed to reduce the development of undesired acid-sensitive bacteria, especially pathogenic bacteria, which increases food safety.

As these two bacteria can share the same environment, the objective of this research was to determine the interaction that can exist between two different species. The first part consisted of the precise quantification of the evolution of different parameters when *L. lactis* and *S. xylosus* were grown in mixed culture, compared to pure cultures, in a model environment. Cultures were grown in bioreactor under well-defined conditions. Their growth and the product formation monitoring in defined synthetic media and static control fermentation offer understanding of the participation of each present microbial organisms in the evolution of the environment.

The second part of the project consisted of the feasibility to perform a transcriptomic analysis of the *L. lactis* physiology in mixed culture with *S. xylosus*. The availability of whole-genome sequence and DNA microarray of *L. lactis* offers the potential to monitor and compare the global gene expression pattern. This technology provides the way to discover cell responses to environmental changes at the transcription level. Analysis of gene expression is important, since the physiology and metabolism of organism are most often the consequences of changes in their expression pattern. With the availability of an increasing number of genome sequences from microorganism in food ecosystems such as dairy products and

fermented meat, strategies using mixed genome microarray can be now careful.

1.2 Research objectives

The aim of this project was to analyze the behaviour of *L. lactis* and *S. xylosus* in mixed cultures. The objectives of this research are as follow.

1. To analyze the mode of interactions of *L. lactis* and *S. xylosus* in term of growth, fermentation products and substrate consumption in chemically defined medium (CDM).

2. To study the feasibility of transcriptome analysis of *L. lactis* when grown in mixed culture with *S. xylosus*.

1.3 Research hypothesis

1. When *L. lactis* and *S. xylosus* grow together, they could influence the growth of each other in the mixed culture compare to the pure culture. This interaction could be either direct or indirect. Some products from one species could influence, positively or negatively the growth of the other partner. One can speculate that the modification of the environment due to one micro-organism may not have influences on the macro-kinetic behaviour of the other micro-organism but can have some repercussions on the metabolism of the latter. Such differences were pointed out by comparing global metabolic profiles of mixed culture to the pure ones. Differences were most likely resulting from interactions of the two micoorganisms.

2. Total RNA extracted from mixed culture was composed of a *L. lactis* RNA and *S. xylosus* mix. cDNA from *S. xylosus* may interfere with the transcriptomic response of *L. lactis* cRNA on the array (*i.e*. cross-hybridization). According to

results obtained in another study in the labolatory (Maligoy *et al.*, 2008), the potency of *S. xylosus* genomic DNA addition in reducing cross-hybridizations was evaluated. If cross-hybridization were reduced enough to enable trancriptomic analysis of real mixed culture, the transcriptome analysis of. *L. lactis* in mixed culture with *S. xylosus* in cheese matrix will be performed. A *L. lactis* DNA microarray made of PCR fragments designed on the *L. lactis* IL1403 strain genome sequence was employed.

1.4 Possible limitations to the research achievement

1. Interactions of *L. lactis* and *S. xylosus* in bioreactor

a) Growth interactions could not be visible at the macrokinetic level. No growth inhibition or growth advantage was visible.

b) Interactions were nutritional without any growth effect. The molecule(s) involved in the interaction was (were) not part of those quantified.

c) None interaction, neither macrokinetic nor metabolic, exist between the two species in mixed culture in CDM.

2. Transcriptomic analysis of *L. lactis* in mixed culture with *S. xylosus*.

L. lactis and *S. xylosus* genomes are too closely related and the DNA microarray employed is not specific enough to measure only *L. lactis* cDNA. High cross-hybridization frequency will be encountered. The addition of *S. xylosus* genomic DNA did not reduce adequately cross-hybridization to enable trancriptomic analysis of mixed cultures.

CHAPTER II

LITERATURE REVIEW

2.1 Lactic acid bacteria

The term LAB was then used synonymously with “milk-souring organism.” Important progress in the classification of these bacteria was made when the similarity between milk-souring bacteria and other lactic acid producing bacteria of other habitats was recognized (Salminen and Wright, 1993). Traditionally, the LAB is defined by the formation of lactic acid as a sole or main end-product from carbohydrate metabolism. The fact that bacilli may fulfill this criterion has been recognized for a long time, although they have never been recognized as belonging to the LAB. This will be simplifled the practical handling of, for example, ‘food-grade’ strains and may be supportive of considerations and legislative procedures towards their approval for application. On the other hand, phylogenertic evidence has clearly separated the bifidobacteria (with >50mol % G+C in the DNA) from other LAB, all containing < 50 mol% G+C.

LAB comprises a diverse group of Gram-positive, non spore-forming bacteria, with shapes of cocci or rods and generally lack of catalase, although pseudo-catalase can be found in rare cases. They are chemo-organotropic and grown only in complex media. Fermentable carbohydrates are used as energy source. Hexoses are degraded mainly to lactate, ethanol, CO_2, formate or succinate (heterofermentative).

2.1.1 The genus *Lactococci*

The genus *Lactococcus* was proposed by Schelifer *et al*., (1985) to accommodate the non-motile, mesophilic streptococci carrying a group N antigen. *Lactococci* are cocci, Gram-positive, anaerobic bacteria which produce L(+)-lactic acid form lactose in spontaneously fermented raw milk which is left at ambient temperatures (around 20-30 ºC) for 10-20 h. They are commonly called mesophilic lactic streptococci. It is temping to suggest that the first isolation, identification and description of the chemical entity lactic acid by Carl Wielhelm Scheel from sour milk in Sweden in the year 1780, was actually L(+)-lactic acid produced by lactococci. The microbial nature of lactic fermentation was recognized in 1857 by Louis Pasteur. The first bacterial pure culture on earth, obtained and scientifically described by Joseph Lister (1983) was *Lactococcus lactis*, at that time called: *'Bacterium lactis'*.

The taxonomic confusion was generated by the fact that some unrelated bacteria were put and maintained in the genus *Streptococcus* on merely morphological criteria ended finally when modern methods of chemical taxonomy were successfully applied. Base on nucleic acid hybridization studies and immunological relationships of superoxide dismutase, the mesophilic lactic acid streptococci were separated from the true streptococci (genus *Streptococcus*) and the enterococci (genus *Enterococcus*) and generated the new genus *Lactococcus* (Schleifer *et al*., 1985). This name is an ingenious description of the function and morphology of these bacteria were obviously influenced by the already existing genus *Lactobacillus* which contain most of the rod-shape LAB (Tueber, 1993). A basic phylogenetic positioning of the lactococci as an own cluster within the *Clostridium* subvision of bacterial evolution (low GC Gram-positive bacteria) became evident from the analysis of sequence

similarities of ribosomal RNAs (Stackebrand and Tueber, 1988). The historical development of commercially avaible starter cultures for the diary industry started with the fresh liquid culture, continued with the invention of viable freeze-dried cultures after World War II, and stands today at a peak with the offer of concentrate deep-frozen cultures in cans or in pellets which contain up to 10^{12} living bacteria per ml and are suitable for direct vast inoculation (Tueber, 1993).

Genetics and genetic engineering of lactococci started with the detection of plasmids in all investigated strains by McKay's Laboratory and others. Nowadays, the basic structure of the chromosome is known, gene transfer by conjugation and electroporation is routinely applied, cloning and expression vectors are available, gene structures and functions are elucidated at levels coming close to well-characterized species like *Escherichai coli* and *Bacillus sustilis* (including genetic engineering and expression of heterologous proteins (Gasson and de Vos, 1994)). Since, biochemical and genetic research on lactococci has a high and continuing priority within several research programs of the European Community/Union. The lactococci used in starter cultures for the dairy industry are listed in Table 2.1.

2.1.1.1 Morphology

The morphology of the Gram-positive lactococci is characterized by sphere of ovoid cells occurring as single cells, pairs or chains, and being often elongated in the direction of the chain. One of the earliest micrographs is represented in Figure 2.1. With the imagination of a chemist, which bacteria was called *Kiel I* and *Kiel II*. The length of the chain is mainly strain dependent, sometimes also influenced by the growth medium.

Table 2.1 Lactococci as component of starter cultures for fermented dairy products

Type of product	Composition of starter culture
1.Cheese type without eye formation	*L. lactis* subsp. *cremoris,* 95-98%; *L.*
2.Cottage cheese, quarg, fermented	*lactis* subsp. *lactis,*2-5%
milk, cheese type with few or small eyes	*L. lactis* subsp. *cremoris,* 95%
3.Culture butter, fermented milk, butter	*L. lactis* subsp. *cremoris*, 20-25%; *L.*
milk	*lactis* subsp. *diacetylactis*, 15-20%
4.Teatte (Scandinavian ropey milk),	*L. lactis* subsp. *cremoris*
casein	*L. lactis* subsp. *lactis*
5.Kefir	

Source : (Salminen and Wright, 1993)

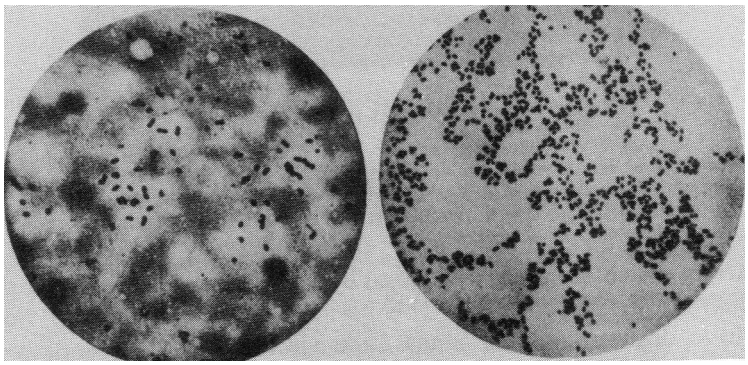

Figure 2.1 Early photomicrograph of mesophilic lactic streptococci isolated by Weigmann from sour milk and sour cream (Salminen and Wright, 1993)

The ovoid shape is shown in the scanning electron micrograph in Figure 2.2. This basic morphology is typical, but not suitable enough to differentiate the genus

Lactococcus from the genera *Streptococcus* or *Enterococcus,* or to differentiate between the five *Lactococcus* species (*L. lactis, L. garviae, L. raffinolactis, L. plantalum, L. piscium*). Lactococci do not form endospore and do not develop flagella. Motile streptococci previously regarded as belonging to the genus *Lactococcus* due to the presence of a group N antigen have been shown to represent a genus on their own (Vagococcus; Collins *et al*., 1989). This genus has a closer relationship with *Enterococcus*.

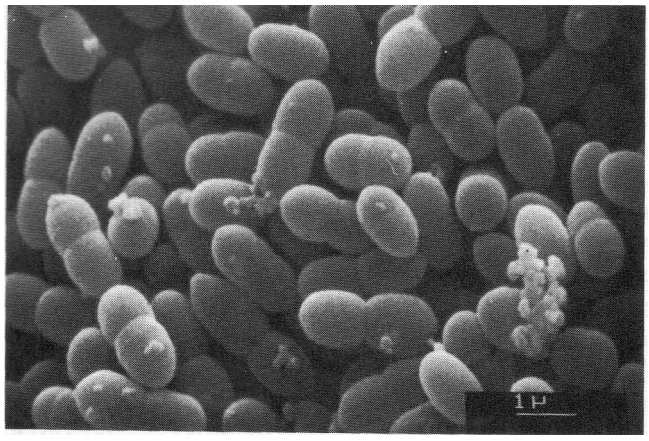

Figure 2.2 Scanning electron micrograph of *L. lactis* subsp. *lactis* Bu2-60 (Lan *et al.*, 2006)

2.1.1.2 Habitats

The lactococci comprise the species *L. lactis, L. garviae, L. plantarum, L. rafinolactis* and *L. pisium. L. lactis* subsp. *lactis* and *diacetylactis* have commonly been detected directly or following enrichment in plant material, including fresh and frozen corn, corn silks, navy beans, cabbage, lettuce, wheat, grass, clover, potatoes, cucumbers and cantaloupe. Lactococci are usually not found in fecal material or soil. Only small number is presented on the surface of cows and in their saliva. Since, raw

cow's milk consistently contains *L. lactis* subsp. *lactis* and to a much less extent *L. lactis* subsp. *diacetylactis* and *L. lactis* subsp. *cremoris*, it is tempting to suggest that lactococci enter the milk from the exterior of the udder during milking and from the feed, which may be the primary source of inoculation. Lactococci are Gram-positive, microaerophilic cocci, which lack the cytochromes of the respiratoty chain. They can be simply differentiated from pediococci and leuconostoc by the main fermentation products produced from glucose. The common morphology consists of the spherical or ovoid cells, 0.5-1µm in diameter, in pair or more or less long chains. The GC content of DNA ranges from 34-43%. The genome sizes of different lactococci were estimated to be 2300-2600 kb (Le Boureois et al., 1993). Since the mesophilic dairy species differ only in a few properties, the GC content can not be employed for this differentiation. The fact that the three mesophilic dairy species are closely related is also implied by the observation that many bacterio-phage can infect strain of all three species. Also, the plasmid patterns investigated so far do not allow species differentiation, but do allow the re-identification of strains. Another approach to differentiate strains is the protein pattern after gel electrophoresis of soluble cell extracts (Jarvis and Wolf, 1979). By this method, classification of closely related strains seems possible. The lactococci contain menaquinone with nine isoprene units as the major component, in contrast to dimethylmenaquinones with nine isoprene units and menaquinones with eight isoprene units in enterococci. To date, it is not possible to assess the number of different lactococcal strains existing in dairies and starter culture throughout the world. The routine differentiation of the dairy lactococci strain is outline in Table 2.4.

2.1.1.3 Biochemistry and physiology

The metabolism of milk ingredients (lactose, caseins, citrate and others) by the two subspecies *lactis* and *cremoris* provide the basis for the spontaneous and industrial fermentations of milk to sour milk, sour cream and many type of cheeses. Although lactococci do not process a citric acid cycle and a complete respiratory chain, they are able to grow in the presence of oxygen partially due to some oxygen metabolizing enzyme like superoxide dismutase or NADH oxidases, the resulting micoraerophilic phenotype does not require a fully free oxygen substrate (e.g. milk) which facilitates the large scale of industrial application of lactococci.

1) Carbohydrate metabolism

The metabolism of lactic acid bacteria is efficient carbohydrate fermentation couple to substrate level phosphorylation. The generated ATP is subsequently used for biosynthetic purpose. Glycolysis is used by all bacteria. Hexose is converted to pyruvate in a metabolic sequence including substrate level phosphorylation. Under limited accesse to oxygen, pyruvate is reduced to lactic acid by NAD^+ dependent lactate dehydrogenase, thereby reoxidizing the NADH formed during the earlier glycolytic step. Pyruvate has a key position in much fermentation though its role of electron acceptor for the reaction of NADH regeneration. The alternative fates of pyruvate are represented in Figure 2.3. The diacetyl and acetoin pathway occur in aerobically condition and low pH via catabolic, anabolic, 2-acetolactate synthase and acetolactate decarboxylase. Another pathway of pyruvate metabolism consists of formate lyase system. This enzyme catalyze the reaction of pyruvate and CoA to formate and acetyl CoA, resulting ultimately to ethanol formation (Sedewitz *et al.*, 1984; Oliveira *et al.*, 2005a)**.**

2) Nitrogen metabolism

Generally, lactococci are auxotrophic for the amino acids isoleucine, valine, leucine, histidine, methionine, arginine and proline. A detailed list for different strain is given in Table 2.3 and 2.4, which clearly shows that additional amino acids may be necessary for growth. For exponential growth, *L. lactis* does need that the medium contain 19 different amino acids (in addition to seven vitamins; Jensen and Hammer, 1993).

Concerning nucleotide metabolism, *L. lactis* has the ability to metabolise uracil, uridine, deoxyuridine, cytidine, and deoxycytidine, but not cytosine as sole pyrimidine source. Cytosine can not be used as *L. lactis* lacks the enzymes cytidine deaninase in addition to deoxycytidine kinase and dCMP deaminase (Marinussen *et al*., 1994)

3) Vitamin and mineral requirements

The maximum growth of *L. lactis* requires biotin, pyridoxal, folic acid, riboflavin, niacianmine and pantothenate. Niacin, pantothenate and biotin are essential. Micro nutrients should include molybdate, borate, cobalt, copper, manganese and zinc. The defined medium named SA and BL are used for this bacterial group as represented in Table 2.2 (Jensen and Hammer, 1993).

4) Reaction to oxygen

Oxygen metabolizing enzyme have been divided into 6 systems for LAB; 1) NADH: H_2O_2 oxidase; 2) NADH: H_2O oxidase; 3) pyruvate oxidase; 4) glycerophosphate oxidase; 5) superoxide dismutase (SOD); and 6) NADH peroxydase (Codon, 1987). The investigated lactococci demonstrated high level of enzymatic superoxide dimutase activities in the order of 5.4-11.8 units/mg protein.

The H_2O_2 (about 10-20 mg/liter) generated by SOD and NADH oxidase in *L. lactis* accumulated in the medium since no catalase or NADH peroxidase is presented in this bacteria. Inhibitory levels of H_2O_2 in starter culture production can be reduced by addition of catalase to the growth medium. The only other enzyme activity of oxygen metabolism reported in lactococci is NADH peroxidase in *L. raffinolactis* (Wood and Holzapfel, 1995).

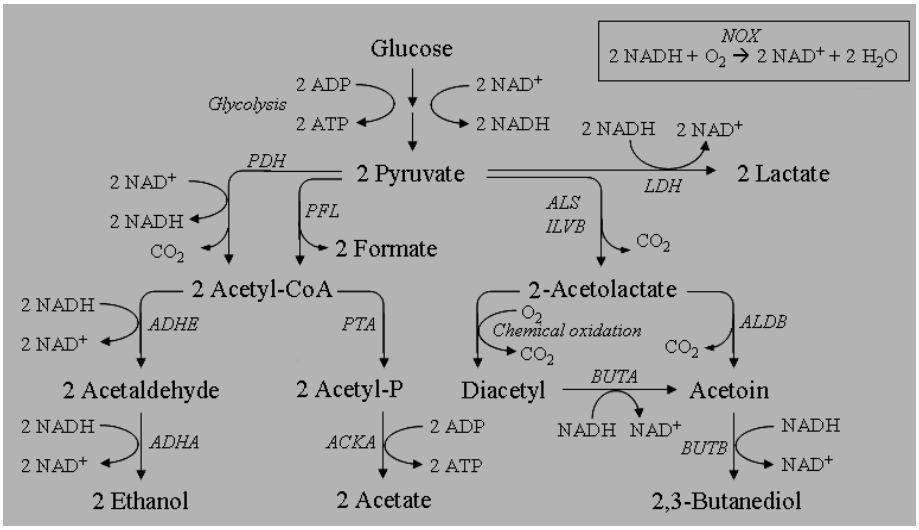

Figure 2.3 The pyruvate metabolism LDH: lactate dehydrogenase; PDH: pyruvate dehydrogenase complex; PFL: pyruvate formate-lyase; ADHE: acetaldehyde dehydrogenase ; ADHA: alcohol dehydrogenase; PTA: phosphotransacetylase; ACKA: acetate kinase, ALS/ILV B: catabolic and anabolic 2-acetolactate synthase; ALDB-acetolactate decarboxylase; BUTA-diacetyl reductase; BUTB: acetoin reductase; NOX: NADH – oxidase (Oliveira *et al.*, 2005b).

2.1.1.4 *L. lactis*

L. lactis is a gram positive bacterium, classified as LAB, a facultative anaerobe, which oxygen having a strong regulation effect. *L. lactis* consists of two subspecies, *lactis* and *cremoris*, both subspecies being widely found in dairy starter cultures. These species contribute substantially to human nutrition and well-being.

Economically and technically, they are outmost importance as a starter for the production of fermented meat and milk. About one third of the annual world output of milk is transformed it fermented products. They can also be isolated from a variety of environment sources. LAB has complex nutrient requirements, limited ability to synthesize B vitamins complex and amino acids. For this reason they are naturally found in nutrient-rich environments such as milk and in some plant products. It was shown that growth can be improved by supplementing the culture media with peptone, yeast extract or amino acids (Law *et al.*, 1976; Benthin *et al.*, 1996). *L. lactis* conducts homolactic fermentation under anaerobic conditions and switches to aerobic heterolactic fermentation under aerobic conditions in response to oxygen stress (Cocaign-Bousquet *et al.*, 2002; Nuves *et al.*, 2005). LAB are responsible for the rapid fermentation of the product, leading to pH decrease which prevent spoilage by undesired microorganisms (Chevallier *et al.*, 2006). In fermented meat products fermentation this acidification below the isoelectric point of muscle proteins causes water-binding-capacity leading to coagulation of the soluble proteins giving the desired texture (Lerbert *et al.*, 2007). This starter cultures play a key role in the quality of fermented products with respect to shelf-life, preservation and sensory quality.

Table 2.2 Define media which support exponential growth of *L. lactis.*

Constituent	Concentration (mM) in medium	
	SA	BL
L- Alanine	3.4	
L-Arginine	1.1	0.8
L-Asparagine	0.8	
L-Cysteine	0.8	
L-Glutamate	2.1	21
L-Glutamine	0.7	0.7
Glycine	2.7	
L-Histidine	0.3	0.3
L-Isoleucine	0.8	0.8
L-Leusine	0.8	1.5
L-Lysine-HCl	1.4	
L-Methionine	0.7	0.5
L-Phenylalanine	1.2	
L-Proline	2.6	
L-Serine	2.9	
L-Threonine	1.7	
L-Tryptophan	0.5	
L-Tryrosine	0.3	
L-Valine	0.9	2.6

BL is Blood-Liver medium and SA is starch agar medium

Table 2.2 Define media which support exponential growth of *L. lactis* (Cont.).

Constituent	Concentration (mM) in medium	
	SA	BL
KH_2PO_4	1.3	1.3
Na-acetate	15	15
Glucose	50	50
MOPS	40	40
Tricine	4	4
$CaCl_2$	0.0005	0.0005
$MgCl_2$	0.52	0.52
$FeSO_4$	0.001	0.001
NaCl	50	50
Vitamins †	+	+
Micronutrient ‡	+	+

Source; (Jensen and Hammer, 1993)

†Vitamins: 0.4 µM biotin, 10 µM pyridoxal-HCl, 2.3 µM folic acid, 2.6 µM riboflavin, 8 µM niacinamide, 3 µM thiamine-HCl, and 2 µM phethothenate. ‡Micronutrients: 0.003 µM $(NH_4)_6(MO_7)_{24}$, 0.4 µM H_3BO_3, 0.03 µM $CoCl_2$, 0.01 µM $CuSO_4$, 0.08 µM $MnCl_2$, and 0.01 µM $ZnSO_4$.

This bacteria is also used for lactic acid production in the fine chemical industries and for the production of diacetyl as flavour compound, exo-polysaccharide for texture development and bacteriocins (Vlieg *et al.*, 2006). Moreover, *L. lactis* itself and a number of products produced by this bacterium have been recoqnised the Generally Recognized as Safe (GRAS) by FDA (Burdock and

Carabin, 2004). LAB starter cultures play a role in proteolysis and have enzyme potential to transform aromatic amino acids (ArAAs), branched-chain amino acids (BcAAs) and methionine to potent aroma compounds that have been identified as major aroma components of cheese flavours (Yvon *et al.*, 1999).

The bacterium used throughout this work was an *L .lactis* subsp. lactis biovar diacetylactisdairy strain LD61, which contains plasmids. Those plasmids contain lactose, protease and citrate utilization, those allow optimal growth in milk and meat (Raynaud *et al*., 2005; Monnet *et al*., 2008).

L. lactis has gained a strong position as a model organism for lactic acid bacteria species. However, this bacterium lives together with several microorganisms such as coagulate-negative staphylococci (CNS), yeast, lactobacilli and corynebacteria (Addis *et al.*, 2001; Blaiotta *et al.*, 2004). It is necessary to be able to study the behaviour of *L. lactis* in mixed cultures to determine whether its metabolism is modified by the presence of a microbial partner.

2.2 The genus *Staphylococcus*

The staphylococci are usually found in the skin of human and animals. The straphylococci have a G+C of 30-38%. A few physiological features, however, allow the relatively reliable differentiation of most strains belonging to this genus. The ability of staphylococci to produce acid anaerobically from glycerol in the presence of 0.4 µg of erythromycin per ml and their sensitive to lysostaphin from the basis of a discriminatory test to distinguish between these two groups.

.

Table 2.3 Characteristic differentiating species and subspecies of the genus *Lactococcus*.

Species	Source	Acid production from						
		Galactose	Lactose	Maltose	Melibiose	Melizitose	Raffinose	Ribose
L. lactis subsp. *lactis*	Raw milk and dairy products	+	+	+	-	-	-	+
L. lactis subsp. *cremoris*	Raw milk and dairy products	+	+	-	-	-	-	-
L. lactis subsp. *hodniae*	Leaf hopper	-	-	-	-	-	-	-
L. garviae	Bovine sample	+	+	V	V	-	-	+
L. plantarum	Frozen peas	-	-	+	-	-	-	-
L. raffinolactis	Raw milk	+	+	+	+	+	+	V
L. piscium	Disease rainbow trout yearling	+	+		+	+	+	+

Source: (Salminen and Wright, 1993)

* +, positive; -, negative; v, viable

Table 2.4 Physiological and other properties of dairy lactococci use for identification and differentiation.

Properties	*L. lactis* subsp. *lactis*	*L. lactis* subsp. *diacetylactis*	*L. lactis* subsp. *cremoris*
Growth at 10ºC	+	+	+
Growth at 40ºC	+	+	-
Growth at 45ºC	-	-	-
Growth in 4%NaCl	+	+	-
Growth in 6.5%NaCl	-	-	-
Growth at pH 9.2	+	+	-
Growth with methylene blue (0.1% milk)	+	+	-
Growth in presence of bile (40%)	+	+	+
NH_3 from arginie	+	+	-
CO_2 from citrate	-	+	-
Diacetyl and acetoin	-	+	-
Fermentation of maltose	+	+	Rarely
Hydrolysis of starch	-	-	-
Heat resistance (30 min at 60ºC)	V	V	V
Serological group	N	N	N
GC content of DNA (mol%)	33.8-36.9	33.6-34.8	35.0-36.2

Source: (Salminen and Wright, 1993)

* +, positive; -, negative; v, viable

Being facultative anaerobes, the staphylococci may use both of glycolytic (Embden-Meyerhof-Parnas, EMP and the hexose-monophosphate, HMP) pathways as main routes of glucose metabolism. The main end product of anaerobic glucose metabolism is (73-94%) L(+)-lactate (with traces of acetate, pyruvate and CO_2. Acetate and CO_2 are the main end product from glucose under aerobic conditions (Gardini *et al.*, 2002; Morot-Bizot *et al.*, 2006)

S. xylosus

S. xylosus is a common bacterial species from the skin micro flora of mammals frequently isolated from milk, meat, and other food products such as cheeses and sausages (Garcia-Varona *et al.*, 2000). *S. xylosus* is an anaerobic facultative bacterium, used as starter culture in combination with LAB in several types of sausages and cheeses. *S. xylosus* can survive at high salt concentration and in acidic environments, encountered conditions during fermented ripening and storing of meat and cheese products (Sørensen and Jakobsen, 1996; Simonovà *et al.*, 2006). This bacteria can form biofilm as represent on Figure 2.4.

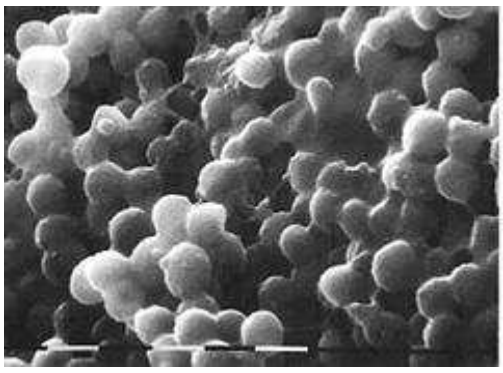

Figure 2.4 Biofilm of *Staphylococcus xylosus* (INRA, 2008).

S. xylosus also contributes to the ripening of traditional cheeses, particularly surface mold ripened soft cheeses, where it can induce an orange pigmentation as represent in Figure 2.5.

Figure 2.5 Munster cheese inoculated with *S. xylosus* on the surface (INRA, 2008).

S. xylosus plays a major role in the development of sensory properties of sausages by reduction of nitrates to nitrites and then to nitrous oxide, preventing rancidity through superoxide dismutase and catalase activities as shown in Figure 2.6 (Barriére *et al.*, 2001a; Barriére *et al.*, 2001b). It has been confirmed that ethyl esters and 3-alkanones were essential for salami odour and their production was associated with a high level of *S. xylosus* (Martin *et al.*, 2006)

Several aromatic substances such as peptides, amino acids, aldehydes, alcohols and free fatty acids produced by *S. xylosus* from its proteolytic and lipolytic activities have also considerable role in the development of the flavour of fermented meat (Motel *et al.*, 1996; Stahnke, 1999; Larrouture *et al.*, 2000; Mauriello *et al.*, 2001; Beck *et al.*, 2002; Beek *et al.*, 2004). The sensory important aldehydes, 3-methylbutanal, 2-methylbutanal and 2-methylpropanal related to leucine, isoleucine and valine catabolism were found to be only transiently present in the culture broth

during the exponential growth phase in batch cultures of *S. xylosus* and *S. carnosus* (de Vos Petersen et al. 2003). In fact, these aldehydes were rapidly oxidized into their corresponding carboxylic acids (Beck *et a*l., 2002). The further fate of the acids is currently unknown. Given the role of Branch chain amino acid (BCAA) catabolism towards formation of these flavor-important metabolites and their potential role in cellular physiology, the researchers found it essential with a further characterization of the metabolism of BCAAs in staphylococci.

Figure 2.6 Sausage inoculated with *S. xylosus* (INRA, 2008).

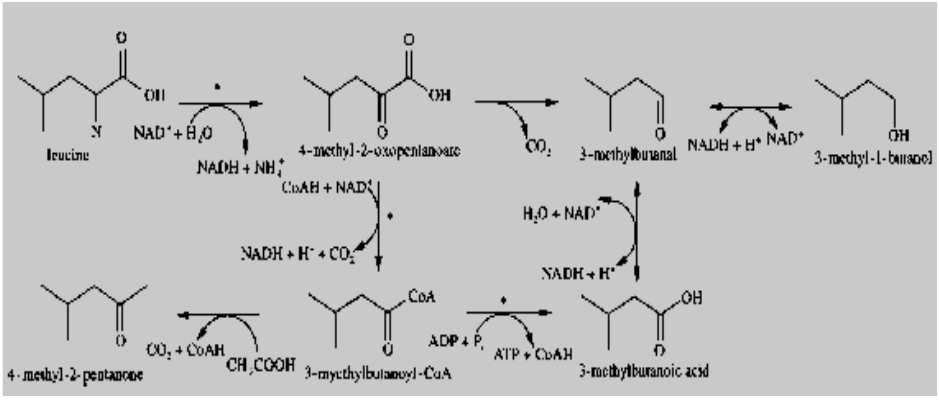

Figure 2.7 Catabolism of leucine (Beek *et al.*, 2004)

The production of 3-methylbutanoic acid is initiated at the onset of the stationary growth phase. Its immediate precursor, the sensorial most important

compound 3-methylbutanal, was recently demonstrated to be produced only transiently during the exponential growth phase. When the organism enters the stationary growth phase, the branched-chain aldehydes are converted to their corresponding carboxylic acids, probably *via* the formation and hydrolysis of the corresponding acyl-CoA complex. Non growing cells of *S. xylosus* rapidly oxidize the branched-chain aldehydes into the corresponding acids. A proposed pathway for the biosynthesis of odd-numbered isobranched-chain fatty acids and metabolite production by *S. xylosus* is shown in Figure 2.7. It has been shown that isobrached-chain fatty acid is used for cell membrane synthesis during exponential phase.

Some strains of *S. xylosus* are able to produce bacteriocin-like substances that have activities against a large spectrum of bacteria. In addition, an antimicrobial spectrum of bacteriocin-like substances produced by some strains of *S. xylosus* against *Listeria monocytogenes, Samonella arizonae, S. aureus, Pseudomonas aeruginosa*, *Escherichia coli*, *Enterococcus faecalis* and other bacteria have been reported (Essid *et al.*, 2007).

Even if *S. xylosus* has been defined as a non-pathogenic bacterium, some strains have been described in animal infection such as mastitis or as able to produce enterotoxins. (Planson *et al.*, 2006; Dordet-Frisoni *et al.*, 2007a; Dordet-Frisoni *et al.*, 2007b). This strongly highlights the need to increase knowledge in this field, which offers well understanding of the behaviour when this species act as starter for fermented foods.

2.3 Transcriptome analysis

Transcriptome is the set of mRNA molecules, produced in the cell, reflect the genes that are being expressed. The sequence of each gene has been developed

initially for different purposes to monitor the expression of all the genes in a given conditions. This approach permitted to the identification of regulations, but enables also the identification of patterns necessary for the control of the expression of sets of genes. Gene expression analysis using DNA microarray is important, since the physiology and metabolism of organism most often are the consequences of changes in the pattern of gene expression. Microarrays are orderly miniaturized arrays containing large sets of DNA sequences that have been attached to a solid substrate using automated equipment such that each spot corresponds to unique DNA (Schena *et al.*, 1995; Schena, 2002). These arrays are also sometimes referred as microchips, biochips, or gene chip. The whole genome sequence of *L. lactis* IL 1403 has been accomplished. DNA microarray has been developed in *L. lactis.* This technology has been employed for transcriptome analysis during environmental stresses of pure culture (Xie *et al.*, 2004).

Critical issues of microarray

This section will highlight some important issues related to microarray hybridization and detection from a practical point of view.

1) Probe DNA retention and quantitative analysis

In solution-based hybridization, the hybridization signal intensity depends on both target and probe DNA concentrations. In microarray-based gene expression studies, however, it is assumed that the concentration of all probe DNAs deposited on the microarrays are much higher than mRNA concentrations in the fluorescently labeled target samples. As a result, hybridization signal intensity is dependent only on the mRNA concentration in the target samples rather than on the arrayed probe DNA

concentration. Therefore, many factors that cause probe deposition variations will have negligible effects on hybridization signal intensity(Wu *et al.*, 2001).

For accurately gene expression, it is essential to ensure that the arrayed DNA probes are into the labeled target cDNAs. Generally, a DNA concentration of 100 to 200 ng/µl is used for spotting. This corresponds to 100 to 200 pg/spot for 1-nl deposition. The retention is about 20-30 % on silanized glass surface. However, probe DNA retention depends on slide surface, coating chemistry, post-processing, hybridization, and washing conditions. Therefore, to ensure accurate quantitative results for highly expressed genes, it is important to know how much spotted DNA can be actually retained after hybridization for each type of slide and for the hybridization conditions used.

2) Target labeling quality

Target labeling is one of critical problems to realize transcriptome and various methods are available. Here, we will develop only the direct labeling method, which was used in this thesis. The first step for transcriptome is to retro-transcribe mRNA into cDNA. In the direct labeling method, the incorporation of fluorescent tags (Cy3- or Cy5-CTP) occurs during this retro-transcription. In direct labeling, fluorescent tags are directly incorporated into the nucleic acid target mixture before hybridization by enzymatic synthesis in the presence of either labeled nucleotide (e.g. Cy3- or Cy5-CTP) or PCR primers (Figure 2.8). The most common method is to label target mRNA or total RNA using reverse transcriptase. The incorporation of labeled nucleotides takes place at this step, by incorporation into the cDNA. In a reverse transcriptase reaction, random hexamer transform RNA to cDNA. During synthesis of cDNA with 3 unlabelled dNTP and one labeled fluorescent dye, the labeled dNTP

will be incorporated as well as the others. The resulting synthesized fragment will be labeled (Schena, 2002).

3) Photo bleaching and scanning parameters

Light is emitted from a fluorescent dye when it is illuminated by excitation light. Generally, the emitted fluorescent is directly proportional to the power of the excitation light. Therefore, to increase sensitivity, higher power of excitation light is preferred. However, if the excitation light is excessive, the incoming photons can damage the dyes and reduce the fluorescent signals during successive scans. More powerful light sources and or longer laser exposure time can lead to significant photo bleaching. Generally, should be less than 1% per scanning (Epstein and Butow, 2000).

Although Cy3 (0.15) has a longer quantum yield than Cy5 (0.28), Cy3 is more efficiently incorporated into cDNA during reverse transcription. Such dye characteristics can cause variation in the signal intensity obtained from these two dyes in the reverse labeling experiments. The signal should be balanced during scanning by using a higher PMT setting for the dye with a weaker signal to allow the detection of more spots with low signal intensity.

Different dyes have considerable differences in their photo stabilities. For example, Cy5 is more sensitive to photo bleaching than Cy3. The differences in photo stability among different dyes could potentially cause problems when multiple dyes are used in experiments because the ratios measured can lead to significant quantitative errors. To minimize photo bleaching, the Cy5 channel is always scanned first, followed by the Cy3 channel (Zhou *et al.*, 2004).

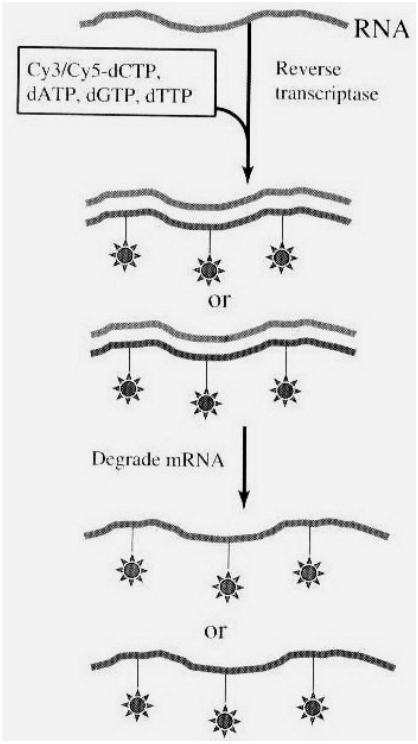

Figure 2.8 Direct incorporation of fluorescent dyed into target sample through reverse transcription (Zhou *et al.*, 2004)

2.4 Summary

As mentioned, microbial microflora in fermented foods are complex community and little is known about the physiology of microorganisms when they grow in mixed cultures. Literature is rich in papers devoted to the descriptive impact of one strain compared to another, mainly in terms of organoleptic qualities or food safety, but very few studies exist dealing with the mechanisms involved. However, the interpretation is always limited by the lack of knowledge regarding the general bacterial physiology in such matrices. Performing mixed culture interaction study was essential to explain the real community. Despite, the fact that *L. lactis* and *S. xylosus* can live in the same environment, little is known on their behaviour and metabolic characteristics in mixed culture.

This research project was part of the Genoferment ANR (Agence Nationale pour la Recherche, France) project including 12 different laboratories. The global aim of the project was to analyze the behaviour of 4 different microorganisms (*L. lactis, S. aureus, S. xylosus* and *Yarrowia lipolytica*), in mixed culture and in a food matrix model. All these microorganisms are present in cheese and/or sausages, during their manufacturing. All except *S. aureus* were used as starter cultures in these food manufacturing and *S. aureus* is a pathogen that can contaminate final products. The aim of this project was to study different combination of these micro-organisms and their interactions at the macroscopic or transcriptomic level.

The main of my research work was to analyze the behaviour of *L. lactis* and *S. xylosus* in a model growth medium. The first part was to perform fermentation of pure and mixed cultures of *L. lactis* and *S. xylosus* and determine their global physiology and potential differences when they share the same environment. Culture was grown in well defined conditions and evolution of metabolic parameters were analysed. The effect of oxygen concentration on their behaviour in pure and in mixed cultures were investigated. The second part of the project was evaluated whether it was possible to study the *L. lactis* transcriptome in mixed culture with *S. xylosus*. Indeed, cross-hybridizations of RNA from *S. xylosus* co-extracted with the *L. lactis* which could cause a major problem for microarray data interpretation. Measuring cross-hybridizations and estimating the feasibility of such an approach was a milestone before initiating such a transcriptome project.

CHAPTER III

MATERIALS AND METHODS

3.1 Cultures fermentation

3.3.1 Materials

3.1.1.1 Microorganism

L. lactis LD61 and *S. xylosus* C2a were used throughout the experiments. They were obtained from Ingénierie des Systèmes Biologiques et des Procédés, Toulouse, France. Cells were stored at – 80°C in CDM medium with 20% glycerol until used. Estimation of interaction was performed as follows.

3.1.1.2 Chemicals

All chemicals used for fermentation media and analytical work were analytical grade. They are listed as below (Table 6– 13).

3.1.2 Methods

3.1.2.1 Prepared inoculations

Cultures were prepared as follow.

a) Stock cultures (Cryo-tube)

All stock cultures were received by an aliquot of 1 ml in 20% glycerol, which were stored at -80 ºC as mention early.

b)10 ml tube cultures

The first seed inoculations were conducted in CDM 10 ml capped tubes. 100 µl 0f 1 ml aliquot cryo-tube. CDM was used with high concentration of phosphate

buffer [K_2HPO_4 15 g/l] and [KH_2PO_4 18 g/l]). These cultures were then inoculated aerobically at 30 ºC for 6 h with agitation speed at 200 rpm.

c) 100 ml Erlenmeyer flask cultures

The second seed inoculations were then taken from the first seed inoculations at the serial dilutions were performed at 10^{-1} to $10^{-3}$of the final volume. 100 ml CDM was used as same as the previous one in 500 ml Erlenmeyer flask capped with cotton lid. These cultures were then inoculated aerobically at 30 ºC with agitation speed at 75 rpm, overnight.

d) The last seed inoculations for all culture fermentation experiments

These seed inoculations were then taken from the overnight incubation at the concentration to reach for the bioreactor and Erlenmeyer flask experiments, the volume of inoculums was determined to obtain an initial OD_{580} of 0.01 as represented as the follow formulations,

$OD_i = OD_f/e^{\mu t}$

OD_i = the initial OD580 unit of the culture

OD_f = the final OD 580 of the culture

μ = specific growth rate of the interest culture (h^{-1})

t = time of incubation (h)

$V_iOD_i=V_fOD_f$

V_f = the final volume of the needed fermentation

OD_f = the final OD 580 unit of the culture

OD_i = the initial OD 580 unit of the culture

V_i = the initial volume of the fresh culture

3.1.2.2 Chemically defined media

CDM was used for the various fermentation experiments are given in Table 3.1 to 3.8 inclusive.

CDM preparation

All solutions were prepared with distilled water. All compounds were weighted individually and mixed by solution in the volumetric flasks.

Table 3.1 Solution 1: Sugars and salts

Compound	Concentration 1X (g/l)	Reference	Note
Glucose	10	Prolabo 24379.294	
Sodium acetate	1	Prolabo 27652.298	
Ammonium citrate	0.6	Sigma A8170	Without Fe
KH_2PO_4	3 or 9	Prolabo 26936.293 Panreac 121509.1211	
K_2HPO_4	2.5 or 7.5	Prolabo 26931.263	

This solution was prepared at concentration 10X (final volume required divided by 10).

Table 3.2 Solution 2: Salts 2

Compound	Concentration 1X (g/l)	Reference	Note
$MgCl_2$, $6H_2O$	0.2	Prolabo 25108.295	
$FeSO_4$, 7 H_2O	0.011	Prolabo 24244.298	
$CaCl_2$, $2H_2O$	0.050	Prolabo 22317.297	
$ZnSO_4$, $7H_2O$	0.005	Prolabo 29253.293	
$CoCl_2$, $6H_2O$	0.0025	Prolabo 22892.261	

This solution was prepared at concentration at 50 X.

Table 3.3 Solution 3: Amino Acids

Compound	Concentration 1X (g/l)	Reference	Note
Alanine	0.24	Sigma A7627	
Arginine	0.12	Sigma A3784 Fluka M010	
Asparagine	0.34	Sigma A0884	
Glutamine	0.51	Sigma G3126	
Glycine	0.17	Sigma G7126	
Histidine	0.11	Sigma H8000	
Isoleucine	0.20	Sigma I2752	
Leucine	0.47	Sigma L8000	

Table 3.3 Solution 3: Amino Acids (Cont.)

Compound	Concentration 1X (g/l)	Reference	Note
Lysine	0.35	Sigma L5626	
Methionine	0.12	Sigma M9625	
Proline	0.68	Sigma P0380	
Serine	0.34	Sigma S4500	
Threonine	0.23	Sigma T8625	
Tryptophane	0.05	Sigma T0254	
Valine	0.33	Sigma V0500	

This solution was prepared at concentration 20X.

Table 3.4 Solution 4: Phenylalanine

Compound	Concentration 1X (g/l)	Reference	Note
Phenylalanine	0.28	Fluka 78019	

This solution was prepared at 20X. The few drops of HCl 37% were added to dissolve.

Table 3.5 Solution 5: Tyrosine

Compound	Concentration 1X (g/l)	Reference	Note
Tyrosine	0.29	Sigma T3754	

This solution was prepared at concentration 20X. The few drops of NaOH 10N were added to dissolve.

Table 3.6 Solution 6: Bases

Compound	Concentration 1X (g/l)	Reference	Note
Adenine	0.010	Sigma A8626	
Guanine	0.010	Sigma G6779	
Uracile	0.010	Sigma U0750	
Xanthine	0.010	Sigma X0626	

This solution was prepared at concentration 50X. The few drops of f NaOH 10N were added to dissolve.

Table 3.7 Solution 7: Vitamins

Compound	Concentration 1X (g/l)	Reference	Note
Benzoic acid	0.010	Sigma A9580	4°C
Biotine	0.010	Sigma B4501	4°C
Cyano-cobalamine	0.001	Sigma V2876	4°C
Folic acid	0.001	Interchim 47620	
Inosine	0.005	Sigma I4125	
Nicotinic acid	0.001	Sigma N4126	
Oritic acid	0.005	Sigma O2750	4°C
Pantothinate	0.001	Sigma P2250	4°C
Pyridoxamine	0.005	Sigma P9380	-20°C
Pyridoxine	0.002	Sigma P5669	
Riboflavine	0.001	Sigma R4500	
Thiamine	0.001	Sigma T4625	
Thiotic acid	0.0025	Sigma T5625	
Thymidine	0.005	Sigma T9250	

This solution was prepared at concentration 20X.

Table 3.8 Solution 8: Cysteine

Compound	Concentration 1X (g/l)	Reference	Note
Cysteine	0.17	Sigma C7755, Fluka 30090	

This solution was prepared at concentration 20X.

The final media preparation was performed as follow. Concentrated solutions were mixed in the order indicated (Table 3.1 to 3.8). The final volume was completed with distilled water. pH 6.6.was controlled before used.

3.1.2.3 Sterilization

a) Media sterilization

All culture media were sterilized by membrane filtration (0.2 µm; Sartorius)

b) Equipment sterilization

Pipettes, test tubes, Erlenmeyer flasks and bioreactors were sterilized in an autoclave at 121ºC for 20 min.

3.1.2.4 Analytical methods

a) pH Measurement

All pH measurements were performed by using a Metrohohm pH meter, which was calibrated prior to use by using 4.0 and 7.0 buffers.

b) Determination of sugar and fermentation product

Fermentation broths were collected hourly 30 min for substrate consumption and product formation analysis. Samples were centrifuged at 13,000 rpm for 3 min, supernatants kept and proteins of the supernatant precipitate with 0.3 M barium hydroxide and zinc sulphate prior to measurements. Glucose consumption and fermentation products (lactate, acetate, pyruvate etc.) were analysed by HPLC with Bio-Rad column ($HPX87XH^{+}$) at 48°C with 5 mM H_2SO_4 eluent, flow rate of 0.5 $ml.min^{-1}$ and refractometer detection (RID and UV 210 nm).

c) Total cell count

The cell counts were sampled every an hour. During fermentation bacterial growths were estimated by plate count on M17 (Difco ™) media for *L. lactis* and total flora in the mixed culture, and on TSA (AEF laboratory) containing 6.5% NaCl for *S. xylosus.* Cell count was preformed in 8x4 Folcon® well with the 500 µl of media volume, the colony forming was count in range 10 – 100 colonies. All bacterium were incubated at 30ºC for 24 h.

3.1.2.5 Fermentation culture conditions

1) Growth influences (glucose, lactate concentrations, and pH) tests of *S. xylosus* in Erlenmeyer flasks.

S. xylosus was inoculated in CDM and supplement with different glucose concentrations (1, 2.5, 5, 7.5 and 10 g/l), lactate concentrations (0, 25, 50 and 150 g/l) and pH (4, 4.5, 5, 5.5 and 6.6). Specific maximal growth rates were calculated to estimate the growth limitation by one of these parameters exists. The most suitable growth parameters combination was selected. Each experiment was performed in

duplicate.

2) Correlation between optical density and cell count with different media (M17 and TSA) of in Erlenmeyer flask.

S. xylosus cell viable counts and optical density at 580 nm was measured to establish a correlation between those two parameters. The *L. lactis* OD58 and CFU correlation was pre-determined in the laboratory (1 OD_{580} unit was 0.3 gL^{-1} dry- cell weight) and was used throughout their study. The two parameters were used to follow cell evolution during fermentations in order to obtain a more accurate evaluation of cell populations. Each experiment was performed in duplicate.

3) Interaction between *S. xylosus* and *L. lactis* in bioreactor

a) Uncontrolled oxygen conditions

The interaction study was carried out in 3 cultures (pure *L. lactis*, pure *S. xylosus* and mixed cultures). Pure cultures of each species, which were used as the reference to explain mode of interactions in mixed culture**.** Each experiment was performed in duplicate.

Bioreactor operation

The batch fermentations were carried out in bioreactor vessel was a 2 litre glass vessel (Sétric Génie Indudtrial, Toulouse, France) of 1.2 litres working volume. The Figure 3.1 gives bioreactor apparatus. The temperature was controlled by external coils system. A digital thermocouple was used to measure on temperature. Oxygen, pH, and thermocouple probes were calibrated before sterilization. The pH electrodes were calibrated by using pH 4.0 and pH7.0 buffer solution. The bioreactor, containing a liter of water, was remove from autoclave and connected to the bioreactor apparatus when the temperature was about 40-45 ºC. The

Figure 3.1 Bioreactor apparatus for uncontrolled oxygen interaction experiments

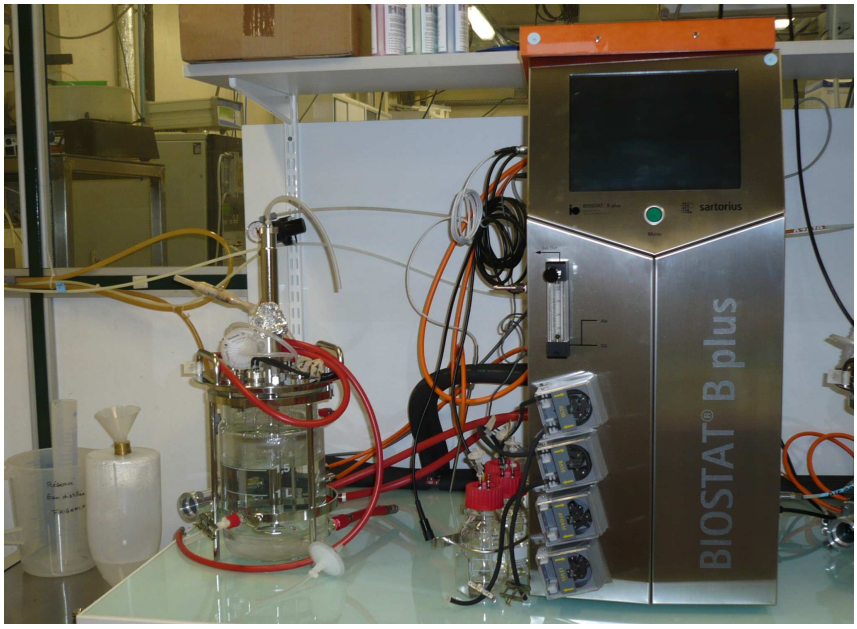

Figure 3.2 Bioreactor apparatus for 80 % oxygen interaction experiments

water was released from the sterilized bioreactor, the filtered CDM was feed into the bioreactor under sterilized condition. A Masterflex peristaltic pump was used for delivery of 5N KOH to the bioreactor vessel. The bioreactors were agitated at 250 rpm throughout experiments using a single 6 flat-bladed impeller (3.5 cm diameter). During the fermentation bioreactors were maintained at 30 °C and pH at 6.6. The dissolve oxygen concentrations were monitored until the end of fermentation.

b) 80 Oxygen concentrations

Bioreactor operation

The batch fermentations were carried out in bioreactor vessel was a 2 litre glass vessel (BIOSTAT®B plus, Sartorius) of 1.2 litres working volume. The Figure 3.2 gives bioreactor apparatus. The temperature was controlled by internal coils system. A digital thermocouple was used to measure on temperature. Oxygen, pH, and thermocouple probes were calibrated before sterilization. The pH electrodes were calibrated by using pH 4.0 and pH7.0 buffer solution. The bioreactor, containing a liter of water, was remove from autoclave and connected to the bioreactor apparatus when the temperature was about 40-45 ºC. The water was released from the sterilized bioreactor, the filtered CDM was feed into the bioreactor under sterilized condition. The bioreactor was maintained at 80% oxygen saturation. An internal peristaltic pump was used for delivery of 5N KOH to the bioreactor vessel. During the fermentation bioreactors were maintained at 30 °C and pH at 6.6.

Data interpretation

Substrate and fermentation products concentrations were determined. Specific production and/or consumption rates were calculated by correlation with the biomass present at the analysed point. The smooth curve method was used before

calculation of each fermentation values. Carbon balances of the cultured fermentations were performed. This was required to directly compare the production/consumption of each product between the different cultures.

3.2 The feasibility of transcriptome analysis study of *L. lactis* of mixed culture on specific *L. lactis* DNA microarray

3.2.1 The Feasibility of transcriptome analysis by using total RNA

3.2.1.1 Materials

1. Biochip of *L. lactis*.

L. lactis. IL-1403-specific PCR product (mean length: 535 bp) were provided by Eurogentec and spotted in duplicate on glass slide by the biochips platform (Toulouse Génopôle, France). Two thousand three of the 2,310 Open Reading Frames (ORFs) identified on genome were effectively available on the array.

2. RNA extraction Kit was provided by Qiagent
3. Bioanalyser 2100 model was provided by Agilent technology
4. Mikro-dimembretor unit was provided by Braun, Melsungen
5. Cy-3 and 5 were provided by Invitrogen
6. Reverse transcription and labelling kit (Qiagent)
7. Random primer (Invitrogent)
8. *L. lactis* specific primer (Eurogentec)

3.2.1.2 Methods

a) Total RNA extraction

Frozen cell pellets corresponding to 6 mg of dry weight were dropped on to a precooled 5-ml Teflon vessel. A 7 mm bead made of the tungsten carbide was added. The flask was then shaken at 2,600 rpm for 2 min in a Mikro-dimembretor unit (Braun, Melsungen). Powder was re-suspended in 4 ml of RLT buffer (Qiagent) supplied with beta-mercaptoethanol. RNA extraction was performed with Qiagent's RNaesy extraction kit according to supplier recommendations. RNA quality and quantification were analyzed using a bio-analyzer 2100 model (Agilent Technologies).

b) Reverse transcription and labeling

RNA of the both species were individually reverse transcribed to cDNA and labeled with a cyanine dye (Cy5 for *L. lactis* and Cy3 for *S.xylosus*) by using a LabelStar array kit (Qiagent) according to supplier recommendations. RNA quality was controlled before label by using Agilent 2100 Bio-analyzer. Random and specific oligonucleotide was used as primers (Invitrogen ® and Eurogentech ®, respectively)

c) Genomic DNA extraction

For the interaction of both two species genomic DNA of *S. xylosus* was added to reduce cross-hybridization. The DNA extraction was performed as described. DNA of *S. xylosus* was extracted after incubate overnight with M17 at 37 °C, 200 rpm. Four ml of cells were pelleted and resuspended in 250µl C1 solution (saccharose 20%, Tris 50 mM pH 7.5, EDTA 5 mM, lysozyme 1 mg/ml, RNAse 10 U/ml). Cell lysis was performed by addition of 200 µl C2 solution (Tris 10 mM pH

8, EDTA 5 mM, SDS 1%). After addition of proteinase K (0.4 mg/ml),incubation overnight at 55°C, and an Phenol/chlorophorme/isoamylic alcohol extraction, genomic DNA was precipitated with isopropanol (v/v), washed with ethanol 70% and re-suspended in TE buffer (Tris 10 mM, EDTA 10 mM). The final DNA pellet was treated with RNase and digested with Sau3AI.

d) Hybridization and detection

All DNA microarrays were performed in a Ventana™ automatic machine system. The DNA microarrays were pre-hybridized 30 min at 50 °C in a pre-hybridization solution (1% bovine serum albumin, 2x SSC (0.30 M NaCl plus 0.03 M sodium citrate) and 0.2 % (SDS). The microarray was automatically washed and hybridized for 5 h at 50°C in 200 µl of ChipHype buffer containing Cy-3 labeled cDNA and gDNA of *S. xylosus* with 0, 200 and 400µg. After hybridization, the microarray was washed twice in Ribowash (Discovery; Ventana Medical System, Inc.), twice with 2x SSC for 30 sec and with 0.1 SSC for 30 sec. The microarray was dried by centrifugation for 1 min. Fluorescent signals were captured with a laser scanner (GenePix 4000A; Axon Instrument, CA) and analyzed with GenePix version 6.01software.

e) Cross-hybridization test

The feasibility of transcriptome analysis study of *L. lactis* in mixed culture was analyzed by cross-hybridization tests. The test consists in labeling independently the same amounts of RNA from *L. lactis* and RNA from *S. xylosus* and to hybridize those the cDNA mix with different amounts of *S. xylosus* genomic DNA. Concentrations employed were summarized in Table 3.9. The condition without genomic DNA was used as maximal cross-hybridization control. The effect of

genomic DNA on cross-hybridization was determined by comparison of the signals with the control. The amounts of added genomic DNA were determined according to previous studies in the laboratory.

Table 3.9 Test conditions of cross-hybridization

Conditions	Labelled cDNA of *L. lactis* µg	Labelled cDNA of *S. xylosus* µg	Unlabelled gDNA of *S. xylosus* µg
1	5	5	0
2	5	5	50
3	5	5	400

f) Data analysis

Data was further processed by statistical analysis methods using Bioplot software developed by Biochips platform of Toulouse, France. For each wavelength, spot intensity background was estimated with the fluorescent measure on a set of empty spots. A cut off threshold (T) was calculated with the empty spots, using formula $T = K_{empty} + 3.\ \sigma$, where K_{empty} was the mean of log intensity of all empty spots, and σ was corresponding standard deviation. Intensity up to this threshold value was considered as significant. This was corresponded to signals of significantly expressed genes for *L. lactis* and cross-hybridization signals for *S. xylosus*. The ratio of number of spots with signal up to the cut-off in the wave length of *S. xylosus* and *L. lactis* was determined the cross-hybridization percentage on the whole array.

Comparison of those percentages obtained in each condition was shown if addition of genomic DNA has a positive effect.

3.2.2 The feasibility of transcriptome analysis by directly using genomic DNA

3.2.2.1 Materials

1) 100 mM dNTPs were provided by Invitrogen

2) 3'-5' Klenow enzyme was provided by BioLabs

3) MicroSpin ™ G-25 column was provided by GE Healthcare

3.2.2.2 Methods

a) Genomic DNA extraction

For the interaction of both two species genomic DNA of *S. xylosus* was added to reduce cross-hybridization. The DNA extraction was performed as described. DNA of *S. xylosus* was extracted after incubate overnight with M17 at 37 °C, 200 rpm. Four ml of cells were pelleted and resuspended in 250µl C1 solution (saccharose 20%, Tris 50 mM pH 7.5, EDTA 5 mM, lysozyme 1 mg/ml, RNAse 10 U/ml). Cell lysis was performed by addition of 200 µl C2 solution (Tris 10 mM pH 8, EDTA 5 mM, SDS 1%). After addition of proteinase K (0.4 mg/ml), incubation overnight at 55°C, and an Phenol/chlorophorme/isoamylic alcohol extraction, genomic DNA was precipitated with isopropanol (v/v), washed with ethanol 70% and re-suspended in TE buffer (Tris 10 mM, EDTA 10 mM).

b) Genomic DNA fragmentation

Genomic DNA fragmentation was performed a describe follow. 80 µg/ml genomic DNA was diluted by sterilized water. An aliquot of 1 ml was conducted in

1.5 ml micro-centrifuge tube. 20 units speed sonicator was used throughout this method. DNA solution was stored into the ice during the time of operation. ¼ of sonicated probe was dipped into DNA solution for a minute and then let it stand for a minute. This method was performed with total 5 times to get 1-1.2 kb genomic DNA length. The size of fragmented genomic DNA was confirmed by 1.5 % agarose gel electrophoresis.

d) Genomic DNA labeling

Genomic DNA labeling was conducted with several protocols as described in appendix III section. EAD4 was referred to the team of metabolic engineering of prokaryote, Toulouse, France. Roche ™ protocol was performed as according to supplier recommendations.

e) Hybridization and detection

All DNA microarrays were performed in a Ventana™ automatic machine system. The DNA microarrays were pre-hybridized 30 min at 50 °C in a pre-hybridization solution (1% bovine serum albumin, 2x SSC (0.30 M NaCl plus 0.03 M sodium citrate) and 0.2 % (SDS). The microarray was automatically washed and hybridized for 5 h at 50°C in 200 µl of ChipHype buffer containing Cy-5 labeled genomic DNA and Cy-3 gDNA genomic DNA of *L. lactis* and *S. xylosus*, respectively. After hybridization, the microarray was washed twice in Robo-wash (Discovery; Ventana Medical System, Inc.), twice with 2x SSC for 30 sec and with 0.1 SSC for 30 sec. The microarray was dried by centrifugation for 1 min. Fluorescent signals were captured with a laser scanner (GenePix 4000A; Axon Instrument, CA) and analyzed with GenePix version 6.01software.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Fermentation of two species

4.1.1 Factors influence on growth conditions (Glucose, lactate concentrations, and pH) of *S. xylosus*

There are no publications to indicate the growth conditions of *S. xylosus.* To estimate the optimum growth of *S. xylosus* in the same defined conditions with *L. lactis* therefore, the growth influent parameters were estimated the feasibility of *S. xylosus* under the same condition with *L. lactis.* Each growth influent parameters were described as follow.

4.1.1.1 Glucose influence

The several glucose concentrations were used to estimate the growth influence of *S. xylosus.* The lower and higher concentrations were received from the preliminary experiments, the growth limitation of *S. xylosus* when glucose is more than 10 g/l. The specific growth rates of *S. xylosus* of different glucose concentrations were not significantly difference as shown in Figure 4.1. The selected different glucose concentrations were not strongly effect to the growth of *S. xylosus.* Therefore, the 10 g/l glucose was used for further experiment with the conventional CDM as being used for *L. lactis* cultivation.

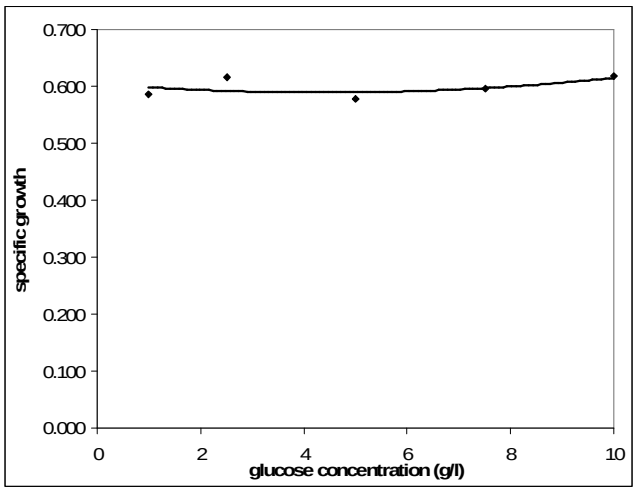

Figure 4.1 Glucose influence of *S. xylosus* to specific growth rate (h^{-1}).

4.1.1.2 Lactate influence

The several lactate concentrations were used to estimate the growth influence of *S. xylosus.* The lower and higher concentrations were estimated from the possibility of glucose consumption to produce lactate as indicate by the theoretical equation of carbon balance. The specific growth rates of *S. xylosus* of different lactate concentrations were not significantly difference as shown in Figure 4.2. The results indicate that 150 mM lactate might be the growth limitation of *S. xylosus.* However, this did not matter for the cultivation under 10 g/l glucose. Therefore, the lactate inhibition for *S. xylosus* at this glucose concentration will not occur.

4.1.1.3 pH influence

The several pH conditions (4, 4.5, 5, 5.5 and 6.6) were used to estimate the growth influence of *S. xylosus.* The specific growth rate of *S. xylosus* was lower at pH 4.0. In contrast, the higher specific growth rate was occurred at pH 6.6, (Figure

4.3), which has been used for *L. lactis* cultivation to avoid the pH inhibition. Therefore, the pH 6.6 will be used for the further experiments.

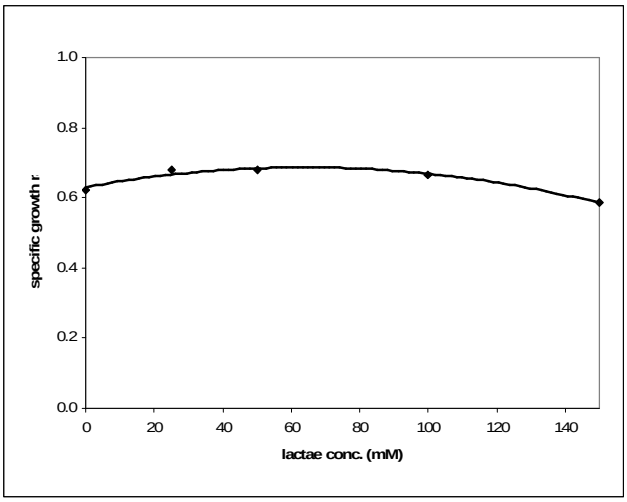

Figure 4.2 Lactate influence of *S. xylosus* to specific growth rate (h^{-1}).

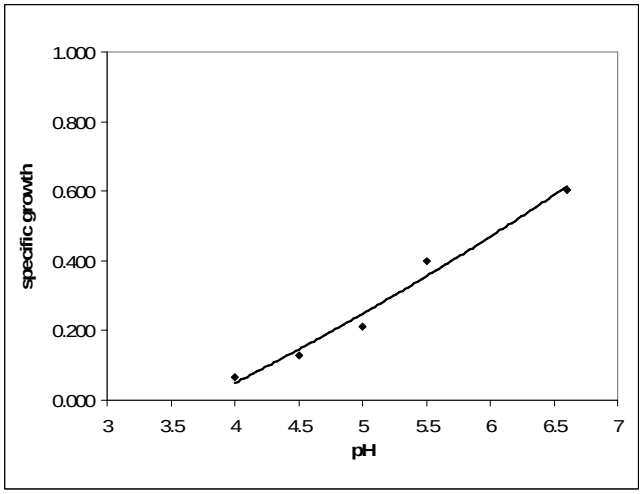

Figure 4.3 pH influence of *S. xylosus* on specific growth rate (h^{-1})

4.1.1.4 Correlation between optical density and cell count in different media (M17 and TSA) of *S. xyosus* in Erlenmeyer flask and bioreactor

The correlation between optical density and cell count in different media (M17 and TSA) was estimated. The amount of cell count from two different media were very similar as shown in Figure 4.4. The correlation between optical density and cell count were increased during the first of cultivation to 10 h. Therefore, TSA will be used for further experiments with *S. xylosus* cell count.

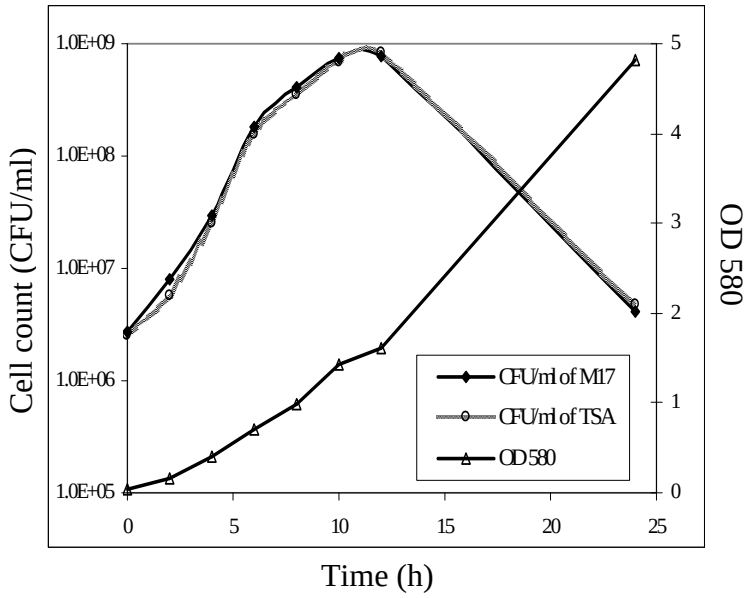

Figure 4.4 Correlation between optical 580 nm density and cell count with different mediums (M17 and TSA) of in Erlenmeyer flask and bioreactor

4.1.2 Interaction between *S. xylosus* and *L. lactis* in bioreactors

To estimate the differences in metabolic behavior of *L. lactis* and *S. xylosus* when they were cultivated in mixed cultures, the growth rate, glucose consumption rate and metabolites production of pure cultures in the two tested conditions were

determined. Then, this will be used as references for the analysis of the two species mixed culture.

4.1.2.1 Pure culture of *L. lactis*

Two conditions of fermentation were carried out with modified oxygen concentration in the growth conditions: uncontrolled oxygen supply and oxygen concentration controlled at 80%. In the uncontrolled oxygen condition, oxygen concentration reached 0% after 4h for *L. lactis* (data not shown). Whatever the conditions, the growth of *L. lactis* started immediately after inoculation and continued until the complete exhaustion of glucose. No lag phase was observed. Biomasses of *L. lactis* between the two conditions were similar, reaching about 2 g/L (Figure 4.5). Biomass reduction after the entry to stationary phase was of about 1 g/L, from 8 to 10 h in uncontrolled oxygen condition and only about 0.4 g/L in controlled oxygen condition. The maximum specific growth rate, biomass yield and glucose consumption rate were not significantly different between the two conditions (Table 4.1).

Table 4.1. Biomass yield on glucose, maximum specific growth rate and rate of glucose consumption obtained during pure culture of *L. lactis* in different conditions.

Conditions	**Biomass yield (g.m mol glucose^{-1})**	**μ_{max} (h^{-1})**	**$q_{glucose}$ max (mmol. g^{-1}. h^{-1})**
Uncontrolled oxygen	0.04	1.20	38.70
80 % controlled oxygen	0.04	1.17	37.27

For *L. lactis*, despite a similar maximum glucose consumption rate in both conditions, glucose consumption at 80% oxygen was slower than without oxygen control (Figure 4.6). In fact, glucose was completely exhausted after 7 h with uncontrolled oxygen compared to 7.5 h with 80% oxygen condition. Lactate was produced similarly in both conditions and the production of lactate ceased when glucose exhausted at the beginning of the stationary phase (Figure 4.6).

The products of pure *L. lactis* in both conditions were different. Lactate, acetate, formate and ethanol were produced in uncontrolled oxygen condition (Figure 4.6A) although in the presence of 80% oxygen, *L. lactis* produced lactate, acetate and acetoin (Figure 4.6B). In uncontrolled oxygen condition pyruvate was converted into acetyl coenzyme-A (acetyl CoA), the latter being transformed into mixture of acetate and ethanol. After oxygen was completely exhausted (after 5h data not show), formate was also produced, indicating that pyruvate formate lyase was active under anaerobic condition, which is consistent with the enzyme being extremely oxygen-sensitive. For 80% oxygen condition, a mixture of acetate and acetoin was produced,

indicating that the classical aerobic metabolism of LAB characterized by the induction of NADH oxidase and the control of the dehydrogenases by the reducing power occurred.

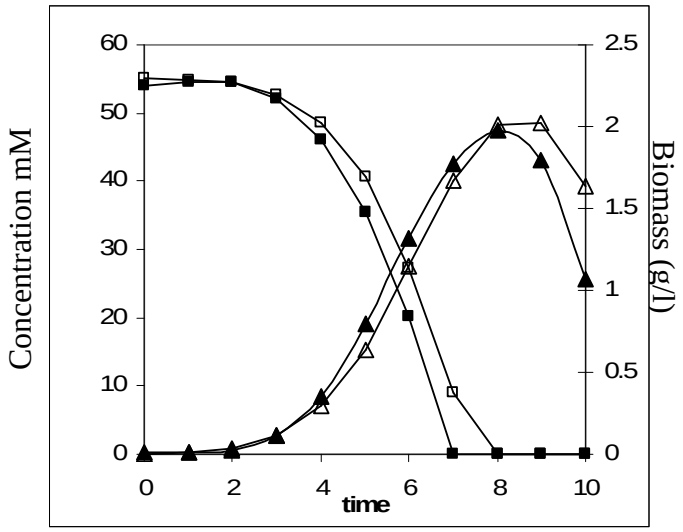

Figure 4.5 Cell growth of pure *L. lactis* on CDM **uncontrolled oxygen condition** (**dense**) and **80% oxygen control condition** (**empty**) : biomass (▲) and glucose (■)

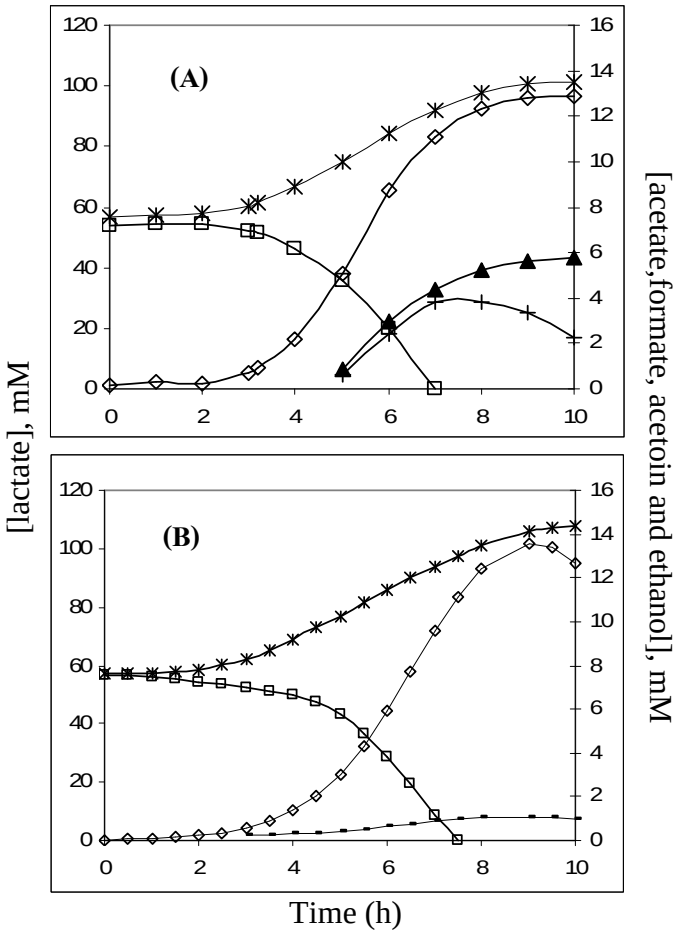

Figure 4.6 Fermentation products of pure *L. lactis* in uncontrolled oxygen (A) and 80% oxygen condition (B) : glucose (□), lactate (◊), acetate(x), formate (▲), acetoin (-) and ethanol (+)

4.1.2.2 Pure culture of *S. xylosus*

In pure culture of *S. xylosus*, biomass in 80% oxygen condition was greatly increased when compared with uncontrolled oxygen condition (Figure 4.7). As shown in Figure 4.7, in uncontrolled oxygen condition, pO_2 decreased in the bioreactor from 85% to 0% in 4 h.

Glucose consumption in uncontrolled oxygen condition was lower than in the controlled oxygen condition. The main products were acetate and lactate. In

uncontrolled oxygen condition, lactate started to be produced after 5 h and virtually no acetate was produced. Under 80% oxygen condition, both lactate and acetate were produced after 6 h. Lactate concentration under uncontrolled and 80% oxygenconditions at 10 hours was about 2.3 and 5.4 mM, respectively. Between 7 to 9 hours, in 80% oxygen condition, acetate production by *S. xylosus* was highly increased (from 10 to 20 mM), but acetate concentration in the medium decreased between 9 to 10 h (Fig. 4.8).

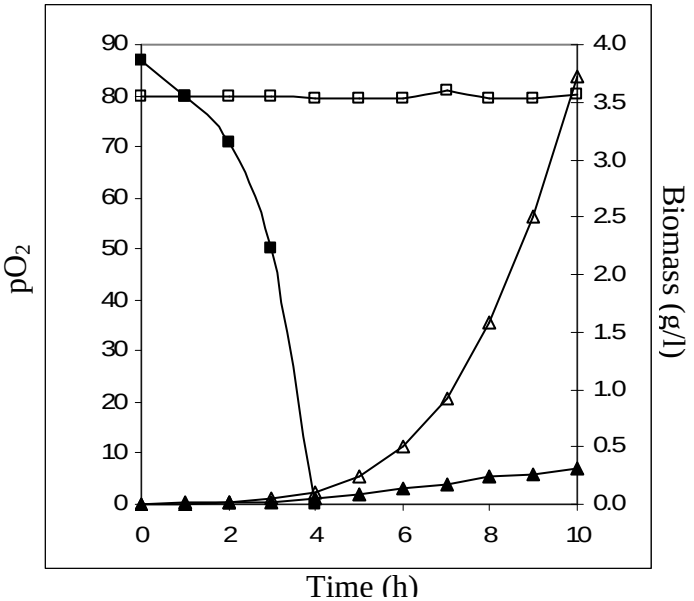

Figure 4.7 Evolution of pO_2 and biomass in pure *S. xylosus* culture in **uncontrolled oxygen** (**dense**) and **80% oxygen** condition (**empty**): pO_2 (■) and biomass (▲)

The catabolic rate, the growth rate and the biomass yield were strongly improved in 80% oxygen condition compared with uncontrolled oxygen condition (Table 4.2). On the contrary, the catabolic carbon balance values calculated at 10 hours were about 26% in uncontrolled oxygen condition and about 16% in 80%

oxygen condition. This can be explained by the fact that glucose consumption by *S. xylosus* was mostly used for cell growth under excess oxygen condition. Indeed, *S. xylosus* could have gain more energy in aerobiosis than in oxygen limitation, either by producing acetate from acetyl CoA leading to ATP synthesis, or by aerobic metabolism involving TCA cycle. In uncontrolled oxygen condition, *S. xylosus* revealed an anaerobic fermentation, which earned low source of energy. Therefore, biomass in uncontrolled oxygen condition was lower than that in 80 % oxygen condition.

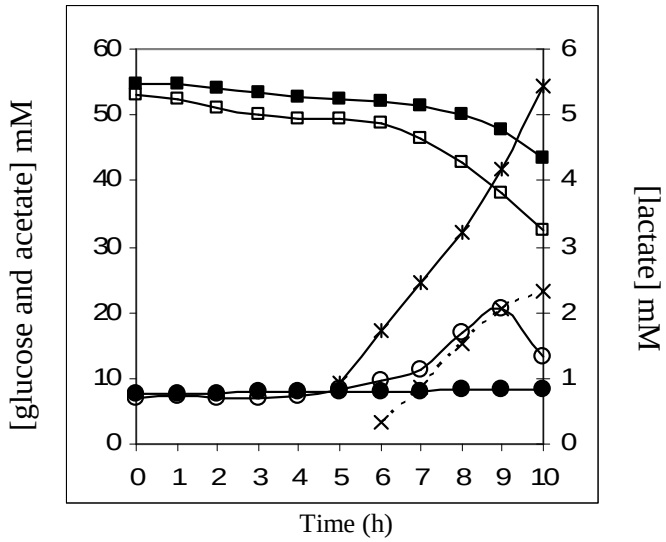

Figure 4.8 Evolution of glucose and fermentation product concentrations (mM) of pure *S. xylosus* in uncontrolled oxygen (**dense**) and 80% oxygen condition (**empty**): glucose (□), lactate (○) and acetate (x)

Table 4.2 Biomass yield of glucose, maximum specific growth rate and rate of glucose consumption obtained during pure culture of *S. xylosus* in two different conditions.

Conditions	Biomass yield (g. mmol^{-1} of glucose)	μ_{max} (h^{-1})	$q_{glucose}$ max (mmol. g^{-1}. h^{-1})	Carbon balance
Uncontrolled Oxygen	0.03	0.62	10.27	26
80 % Oxygen	0.18	0.76	30.88	16

4.2.3 Mixed culture

Mixed cultures were conducted similarly to the pure cultures but with the two species, with initial concentration of inoculum determined to reach the same amount as individually pure culture. The substrate consumption and product formation of mixed culture fermentations were compared with the pure cultures of both species to explain the behaviours of each present species.

4.2.3.1 Uncontrolled oxygen condition

In uncontrolled oxygen condition, final biomass concentration of mixed culture was higher than that of pure culture of *L. lactis* and *S. xylosus*, results from the global biomass measurement contained within the two species. The exponential growth phase was very similar between pure *L. lactis* and mixed culture with maximal specific growth rates of about 1.2 h^{-1} (Table 4.3), while the growth ceased at about 7-8 h and the biomass concentration started to decrease (Figure 4.9).

Glucose in mixed culture was completely consumed after 7 h as in pure *L. lactis* culture. The specific glucose consumption rate ($q_{glucose}$) in mixed culture was distinctly higher than those observed in pure *S. xylosus* culture. In contrast, it was

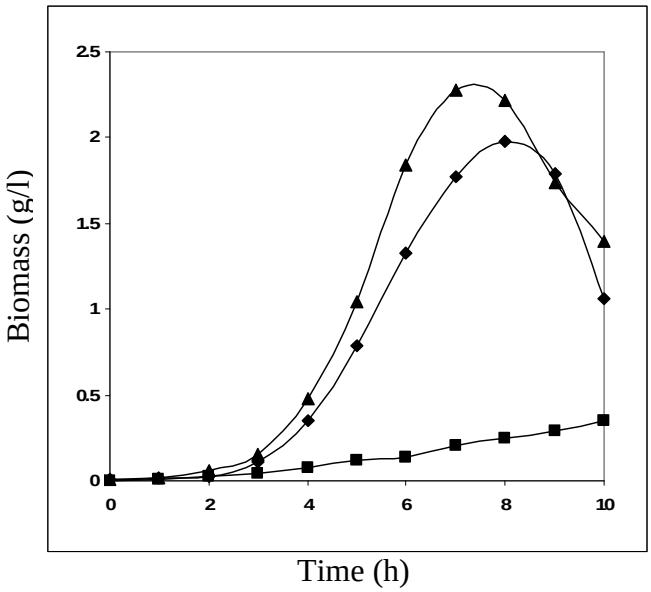

Figure 4.9 Growth curve comparison of pure *L. lacis* (♦), pure *S. xylosus* (■) and mixed culture (▲) in **uncontrolled oxygen condition**

very similar to this pure *L. lactis* culture (Table 4.3), which shown that in mixed culture there was no additive effect of the two specific glucose consumption rates of *L. lactis* and *S. xylosus* as observed in pure cultures.

Production of lactate, acetate, ethanol and formate in mixed culture occurred as in *L. lactis* pure culture (Figure 4.10). However, the concentration of lactate in mixed culture was lower than that in pure *L. lactis* culture. This can be explained by the fact that glucose was shared by the two different species and was more consumed than the pure cultures for cell growth.

The specific production rates of all the products, *i. e.* lactate ($\mathbf{\nu}_{lactate}$), acetate ($\mathbf{\nu}_{acetate}$), formate ($\mathbf{\nu}_{formate}$), and ethanol ($\mathbf{\nu}_{ethanol}$), in the mixed culture were not significantly different than these in pure *L. lactis* culture. Indeed, the main involvement in the mixed culture behavior was the *L. lactis* metabolism, since *S. xylosus* exhibited a weak product formation and had a lower growth rate than *L.*

lactis. Hence, the catabolic profile observed in the mixed culture was very similar to the one obtained in pure *L. lactis* culture.

Catabolic carbon balance calculated between glucose consumption and products formation in mixed culture was about 92 which value was higher than these of pure *S. xylosus* culture but lower than those pure *L. lactis* culture (26 and 93, respectively). As for specific production rates explained above, the value of carbon balance obtained in mixed culture was very close to the characteristic one of pure *L. lactis* culture. This can be explained by the weak involvement of *S. xylosus* in the global catabolism of the mixed culture.

In these uncontrolled oxygen conditions, after glucose exhaustion in mixed culture, the products concentrations remained stable, indicating that lactate and acetate in the medium were not consumed by *S. xylosus*.

Table 4.3 Calculated fermentation values from pure and mixed cultures of both species in uncontrolled oxygen condition.

Parameter	Fermentation values		
	L. lactis LD61 Pure culture	*S. xylosus* C2A Pure culture	Mixed culture
μ_{max} (h^{-1})	1.20	0.62	1.17
$q_{glucose}$ (mmol. g^{-1}. h^{-1})	38.7	10.27	36.82
$\nu_{lactate}$ (mmol. g^{-1}. h^{-1})	38.26	12.80	37.91
$\nu_{acetate}$ (mmol. g^{-1}. h^{-1})	3.20	2.57	2.15
$\nu_{formate}$ (mmol. g^{-1}. h^{-1})	2.60	-	2.09
$\nu_{ethanol}$ (mmol. g^{-1}. h^{-1})	2.99	-	2.99
$Y_{cell, glucose}$ ($g.mmol^{-1}$)	0.04	0.03	0.05
$Y_{lactate, glucose}$ ($cmol.cmol^{-1}$)	0.89	0.24	0.84
$Y_{acetate, glucose}$ ($cmol.cmol^{-1}$)	0.04	0.02	0.04
$Y_{ethanol, glucose}$ ($cmol.cmol^{-1}$)	0.02	-	0.03
$Y_{formate, glucose}$ ($cmol.cmol^{-1}$)	0.02	-	0.03
Carbon balance	93	26	92

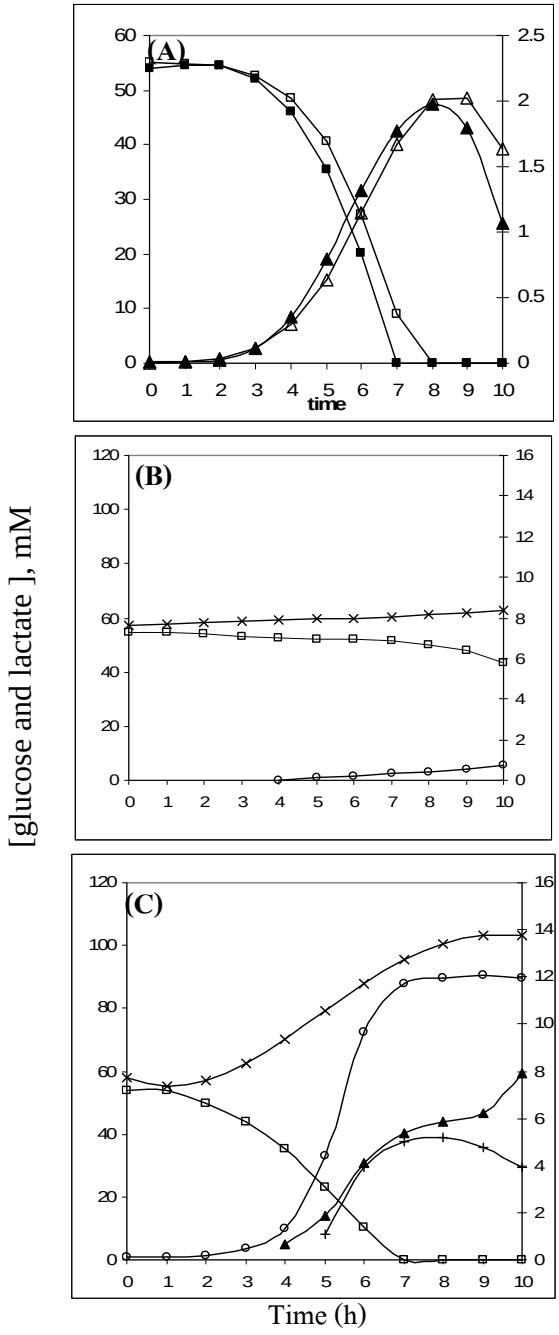

Figure 4.10 Evolution of substrate and fermentation products (mM) on CDM under uncontrolled oxygen condition during the first 10 h, of the pure culture of *L. lactis* (A), the pure culture of *S. xylosus* (B) and the mixed culture (C), glucose (□), lactate (○), acetate(x), formate (▲),and ethanol (+)

4.1.3.2 80% oxygen condition

The final biomass concentration in mixed culture reached about 3.5 g/l, which was by far higher than in pure *L. lactis* culture and in the same order of magnitude than in pure *S. xylosus* culture (Figure 4.11). Mixed culture containing the two species, the global biomass concentration was higher than that in pure cultures. However, the global profile of the growth curve in mixed culture, with stationary and death phases, was similar to the one observed in pure *L. lactis* culture.

The maximum specific growth rates in pure *L. lactis* culture and in mixed culture were slightly similar, as in uncontrolled oxygen condition, at about 1.02 to 1.2 h^{-1} (Table 4.4).

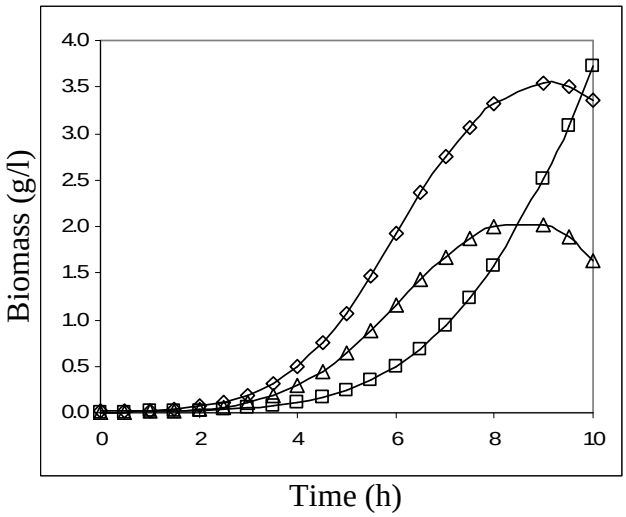

Figure 4.11 Growth curve comparison of pure *L. lactis* (▲), pure *S. xylosus* (■) and mixed culture (♦) in **80% oxygen**

The specific glucose consumption rates in mixed culture and pure *L. lactis* culture were similar (Table 4.4). The behavior of products formation in mixed culture was similar to these observed in pure *L. lactis* culture, with lactate, acetate and

acetoin produced. Lactate production in mixed culture ceased when glucose was exhausted at a value lower than that in pure *L. lactis* culture (Figure 4.12). This demonstrated that glucose consumption by both species in co-culture was used for cell growth rather than lactate production. However, lactate yield from glucose between mixed culture and pure *L. lactis* culture were slightly different because most of the lactate was produced by *L. lactis*.

Lactate concentration in mixed culture decreased after glucose was completely consumed (Figure 4.12C), demonstrating that *S. xylosus* consume lactate for growth after the main carbon source (glucose) was exhausted. This hypothesis was confirmed by the profile of *S. xylosus* biomass which still increased during that phase, like the cell viable count of *S. xylosus* in mixed culture (Fig 4.12). The specific lactate consumption rate by *S. xylosus* at 10 h was about 9.95 $mmol.g^{-1}.h^{-1}$. Lactate was used for *S. xylosus* growth, albeit at a slower rate, and acetate production. Indeed, lactate was converted to pyruvate and then into acetate in aerobic condition. Acetate production yield ($Y_{acetate,\ lactate}$) from lactate after glucose exhausted was about 0.56 $cmol.cmol^{-1}$ as shown in table 4.4 (calculated from reduced lactate and increased acetate concentration at 10 h).

Table 4.4 Fermentation values calculated from pure and mixed culture of both species in 80% oxygen condition

Parameter	Fermentation value		
	L. lactis Pure culture	*S. xylosus* Pure culture	Mixed culture
μ_{max} (h^{-1})	1.20	0.76	1.02
$q_{glucose}$ (mmol. g^{-1}. h^{-1})	37.27	30.88	38.64
$q_{lactate}$ (mmol. g^{-1}. h^{-1})	-	-	9.95
$\boldsymbol{\nu}_{lactate}$ (mmol. g^{-1}. h^{-1})	28.89	1.11	22.36
$\boldsymbol{\nu}_{acetate}$ (mmol. g^{-1}. h^{-1})	0.69	0.99	1.84
$Y_{cell, glucose}$ (g.$mmol^{-1}$)	0.04	0.18	0.05
$Y_{lactate, glucose}$ (cmol.$cmol^{-1}$)	0.90	0.06	0.72
$Y_{acetate, glucose}$ (cmol.$cmol^{-1}$)	0.04	0.10	0.13
$Y_{acetoin, glucose}$ (cmol.$cmol^{-1}$)	0.01	-	0.02
$Y_{acetate, lactate}$ (cmol.$cmol^{-1}$)	-	-	0.56
Carbon balance	95	16	87

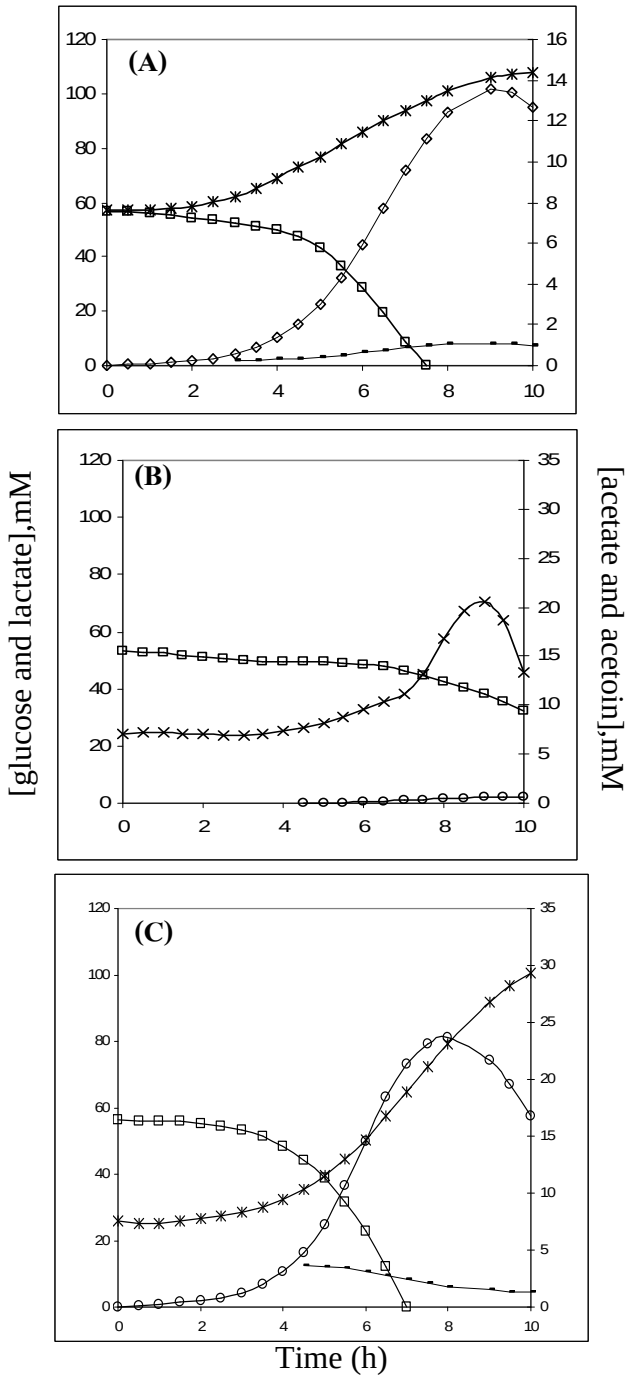

Figure 4.12 Evolution of substrate and fermentation products (mM) on CDM with 80% oxygen condition during the first 10 h, of the pure culture of *L. lactis* (A), the pure culture of *S. xylosus* (B) and the mixed culture (C) glucose (□), lactate (○), acetate (x) and acetoin (-)

4.1.4 Viable cell count

In uncontrolled oxygen condition, the amount of viable cells of pure *L. lactis* culture was strongly decreased (~2 log) during the stationary phase (Figure 4.13). In contrast, the number of pure *S. xylosus* culture was slightly decreased because glucose remained in the medium until 24 h (35 mM), while it was exhausted pure *L. lactis* culture. Similarly, the number of *S. xylosus* in mixed culture decreased since no substrates (glucose or lactate) were used during this phase.

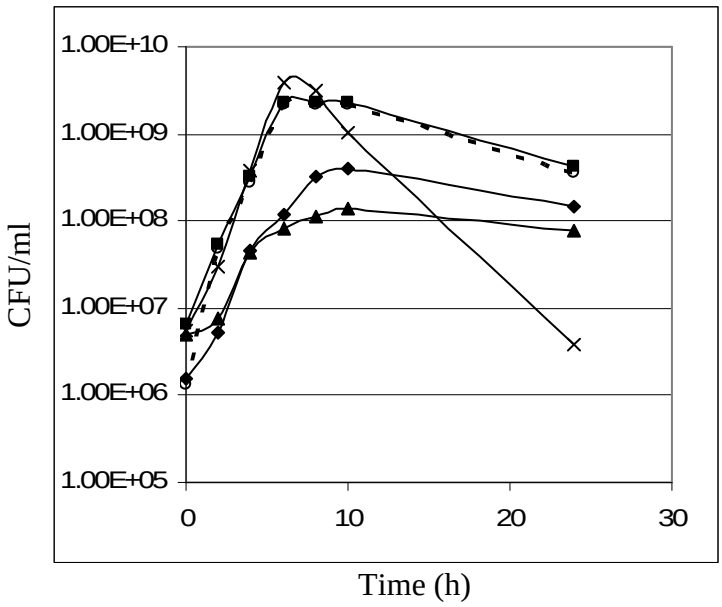

Figure 4.13 Viable cell count of two species in pure and mixed culture in uncontrolled oxygen condition: Total cell in mixed culture (■), pure *L. lactis* (X), mixed *S. xylosus* (▲),mixed *L. lactis* (--○--) and pure *S. xylosus* (♦)

In 80% oxygen condition, the viable cell count of pure *S. xylosus* culture decreased more than 3 log after 10 h until the end of fermentation (24 h) compared to mixed *S. xylosus* culture (Figure 4.14). This can be explained by glucose exhaustion between 10 and 23 h of fermentation (data not show) in pure culture. At the opposite, in mixed culture, *S. xylosus* continued to grow at the expense of lactate produced by *L. lactis*. Hence, the amounts of total cell and of mixed *S. xylosus* culture were very related after 10 h. On the contrary, the amount of total cell was very similar to the pure *L. lactis* during the first 8 h of fermentation.

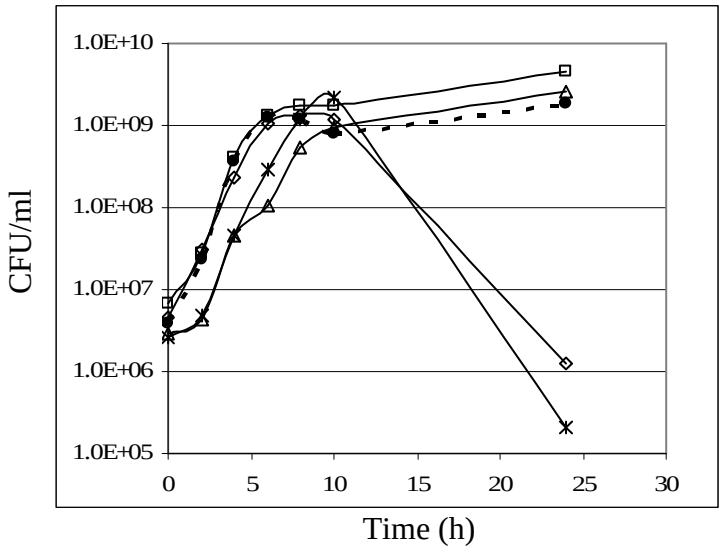

Figure 4.14 Viable cell count of two species in pure and mixed cultures in 80% oxygen condition: Total cell in mixed culture (□),pure *L. lactis* (X), mixed *S. xylosus* (Δ),mixed *L. lactis* (--●--) and pure *S. xylosus* (◊).

Finally, *S. xylosus* was effected under 80% oxygen conditions. The presence of lactate consumption after glucose was exhausted, result from cell still alive. In contrast, under uncontrolled oxygen the presence or absence of substrate were not

significantly effected on cell decreased. Physiology of *L. lactis* was not different under the two tested conditions. The viable cells were significantly decreased with glucose exhaustion.

4.2 The feasibility of transcriptome analysis of *L. lactis* in mixed culture study

4.2.1 The Feasibility of transcriptome analysis of *L. lactis* in mixed culture study by using genomic DNA

4.2.1.1 Genomic DNA labeling efficiency

DNA is more stable and convenience to treat than RNA. Consequently, the feasibility of trancriptome analysis by directly using genomic DNA was conducted. The suitable genomic DNA labeling was established with several protocols. The Genomic DNA of two species was separately extracted, labeled with several protocols as mentioned earlier. Klenow 3′-5′ exo-nuclease activity (provided by BioLab™) was used throughout these experiments. Cy-5 will be used as a reference for selecting the suitable protocol. The Nanodrop results of each protocol are represented on Table 4.5. The best Cy-5 dye incorporation has obtained from IFR (Institute of Food Research**,** Norwich Research Park, Colney, Norwich, UK**)** protocol when compared to the others. The dNTP concentrations of IFR protocol were lower than the others. However, of the incubation time of IFR protocol takes more time comparing to the others. Hence, the suitable protocols for genomic DNA labeling, which will be used throughout the using genomic DNA for the feasibility study of transcriptome analysis of *L. lactis* in mixed culture.

Table 4.5 Comparison between several genomic DNA labeling protocols

Origin of protocol	EAD4	IFR	Roche™
Amount of fragmented gDNA (µg)	2	2	2
Final primer concentration (ng/µl)	Random primer :10 Specific primer: 10	Random primer: 10	Mixed hexonucleotide; unknown concentration
Final concentration of dNTP (mM)	0.3	0.12	0.25
• dATP, dTTP, dCTP	0.1	0.06	0.25
• dCTP			
Final concentration of dCTP-Cy5	5	3	5
Klenow enzyme (unit)	50	50	2
Time of incubation (h)	2	Overnight	1
Temperature of incubation (ºC)	37	37	37
Final volume of reaction solution (µl)	50	50	50
Purification method	MicroSpin ™ G-25	MicroSpin ™ G-25	MicroSpin ™ G-25
Nanodrop results			
• Cy-5 (pmol/µl)	3.7	4.3	0.1
• gDNA marker (ng/µl)	58.3	84.5	15.1

4.2.1.2 Labeled genomic DNA hybridization test

The efficiency of labeled genomic DNA of each species was conducted on the same DNA array of *L. lacis* IL1403. The first, genomic DNA of each pure species were separately extracted and labeled by Cy-5 and Cy3, respectively. The second, the labeled genomic DNA of each species were hybridized and detected under UV light the signals of fluorescent dye. The genomic DNA hybridization was shown on the right handside of Figure 4.15. They were nothing on DNA array after hybridization at 50 ºC for 5 h. with Ventana™ machine. This result could be applied to compare the result of the other hybridization under the same conditions and same species

(Figure 4.15, left). The green, red, and orange spots of Figure 4.15 (right) were the homologous sequences to the spots of each PCR product in DNA array. In contrast, labeled genomic DNA was not hybridized into the array due to the temperature and time for hybridization may not be suitable for labeled genomic DNA, however, attempt was done to keep these conditions as to compare our study with the others in our laboratory. Therefore, the feasibility study of transcriptome analysis by using genomic DNA was not possible under these conditions.

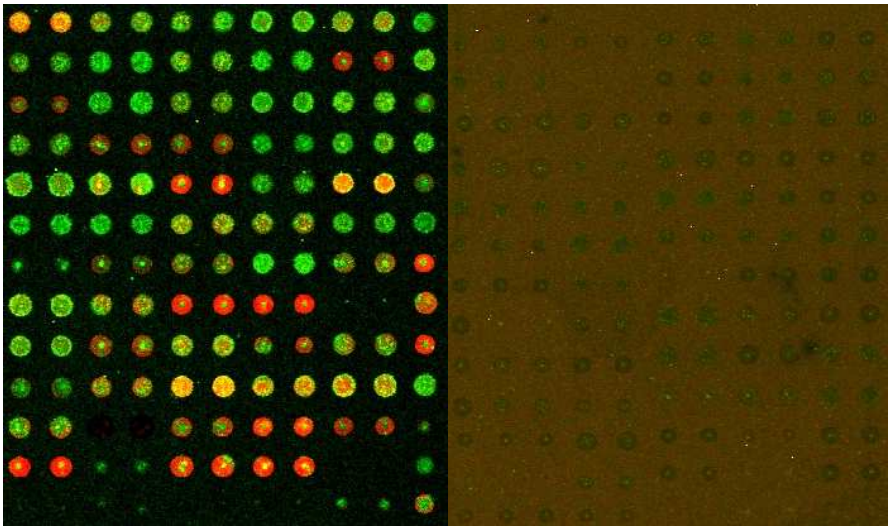

Figure 4.15 Hybridized *L. lactis* chip photos under exposure of UV light of (Right) Labeled cDNA, which was labeled and reverse-transcribed from total RNA of the two species (Left) Labeled genomic DNA which was labeled from genomic DNA of the two species

4.2.2 The feasibility of transcriptome analysis by directly using total RNA

4.2.2.1 Total RNA lebeling efficiency

L. lactis and *S. xylosus* are two Gram-positive bacteria with low %GC content and belonging to the Firmicute phylum. A DNA array based on the *L. lactis* IL1403 has been developed in the laboratory. This array contains 1948 of the 2321 open reading frames present in the genome. This array is made of PCR products of about 500 bp length. As the mixed culture contained both *L. lactis* and *S. xylosus* species, RNA from the two bacteria will be extracted together and retro-transcribed to cDNA before hybridization. It is then possible that cDNA from *S. xylosus* may cross-hybridize with the PCR probes spotted on the array. This results in misleading interpretation of the array data. It is important to quantify the feasibility of using this array to study the *L. lactis* transcriptomic response when grown in mixed culture with *S. xylosus*.

The comparative DNA hybridization has been estimated in glass slide array by using the intensity signal of fluorescent dye as key to monitor transcription of genes of interest. Therefore, the efficiency of labeled cDNA with fluorescent dye is one of the main parameters to control for accomplish transcriptome analysis.

Total RNA of both *L. lactis* and *S. xylosus* were labeled by several methods to find the best efficiency to label cDNA which was produced from RNA by reverse transcriptase enzyme cocomitant with dye labeling. The different fluorescent dyes were used for individual species. Total RNA of *S. xylosus* was labeled with Cy-3 and *L. lactis* with Cy-5. The best labeling efficiency, as determined by the pico molar

incorporation and based on dye ratio of Cy3-dCTP and Cy5-dCTP in newly synthesized DNA, was quantified by UV spectrophotometer.

The suitable protocol for total RNA labeling method was used for this experiment as described above in materials and methods. As labeling efficiency depended on RNA quality, it was first confirmed the quality of RNA by Agilent chip technology before labeling.

Table 4.6 cDNA labeling efficiency of *S. xylosus* with Cy3 and Cy5

Dye	FOI	Labeled cDNA concentration (pmol/µl)
Cy3	26.14	2.5
Cy5	0.58	0.4

The cDNA labeling of *S. xylosus* did not show good efficiency with Cy5 (0.58) compared to Cy3 labelling. FOI (Frequency of incorporation) value was calculated and was used to explain the frequency of dye incorporation, as shown in Table 4.6. A reason might be that Cy5 is larger molecule than Cy3, and is more difficult to incorporate into cDNA than Cy3.

4.2.2.2 Cross-hybridization test

The interactions of *L. lactis.* and *S. xylosus* by transcriptome analysis were performed. This experiment started by the estimation of cross-hybridization and tests to reduce it by the addition of genomic DNA from the partner species. This cross-hybridization limitation method has been previously employed in the lab with the yeast *S. cerevisiae* (Maligoy *et al*., 2008).

The labeled cDNA were suitable for hybridization, and the cross-hybridizations were tested in three different conditions (Figure 4.16). For the first condition, individually labeled cDNA from *L. lactis* and *S. xylosus* mixed and hybridized to check the cross hybridization. The result of signal intensities of each spot on *L. lactis* specific DNA microarray confirmed that *S. xylosus* was cross-hybridized with the PCR probes of *L. lactis* (Figure 4.16)

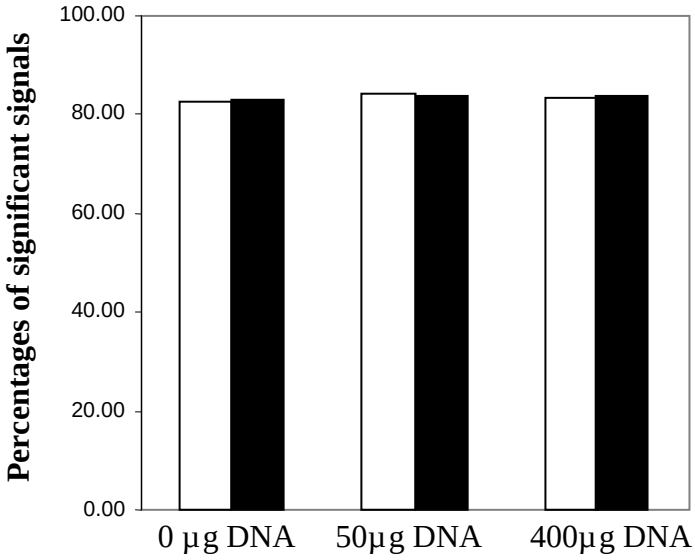

Figure 4.16 Three different gDNA concentrations of *S. xylosus* (0, 200 and 400 µg, respectively) were hybridized with *L. lactis* (empty) cDNA labeled with Cy5 and *S. xylosus* (dense) with Cy3.

It was previously demonstrated in our laboratory that the barricade of transcriptome analysis by microarray test in mixed species population is cross-hybridization, which usually happens by the partner species cDNA on the spots of microarray slide. Thus the microarray must be sufficiently species-specific to avoid cross-hybridizations. Transcriptome analysis of *L. lactis* in co-culture with *Saccharomyces cerevisiae* has been accomplished by adding unlabeled genomic DNA (gDNA) of partner yeast on specific *L .lactis* DNA microarray (Maligoy *et al.*, 2008).

That addition of genomic DNA of the partner microorganism can reduce the cross-hybridization signal by sequester cDNA from the partner. This strategy was applied to this approach. For this, genomic DNA was extracted from *S. xylosus*. DNA

was digested by Sau3AI restriction enzyme before used. Two different concentrations of genomic DNA, 50 and 400 µg were used to estimate the potential of this addition to reduce cross-hybridizations. As shown in Figure. 4.16, middle and right, none of the addition of genomic DNA from *S. xylosus* was able to reduce cross-hybridizations of *S. xylosus* cDNA with the array. This can be explained that by the fact that the array is made of PCR fragment of about 500 bp and that of *S. xylosus* is phylogenetically closely related to *L. lactis*. In this case, the sequences are not different enough to obtain specific hybridization, and for the genomic DNA from *S. xylosus* to capture specifically cDNA from *S. xylosus*.

Therefore, interaction between both species can not be analyzed on this DNA microarray. Oligonucleotide microarray might be an alternative technology. The small length of oligonucleotides compares to PCR fragments enable to increase specificity of the hybridization. Furthermore, the genome sequence of the microbial partner can be taken into account during the array design by the use of completely specific sequences. Such a technology will be necessary for an efficient transcriptomic analysis of mixed cultures between *L. lactis* and *S. xylosus*.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

The main objective of this study was to characterize the possible interactions between two bacteria of interest in food industry, *L. lactis* and *S. xylosus*, often sharing the same environment. Cultures were performed in bioreactors in chemically defined medium under both uncontrolled and controlled oxygen conditions. Biomass of *S. xylosus* was strongly increased during glucose consumption in aerobic condition compared with oxygen limitation. At the opposite, *L. lactis* growth was not influenced by the oxygen condition. Lactate, formate, ethanol and acetate were produced by *L. lactis* when the culture was grown in uncontrolled oxygen. The production of acetate and acetoin appeared under 80% oxygen condition in pure *L. lactis* culture, while no ethanol and less lactate were produced. *S. xylosus* exhibited a higher biomass yield than *L. lactis*, particularly under aerobic conditions. However, they produced lower product concentrations, lower lactate in oxygen limitation and lower lactate and acetate in oxygen excess.

The interactions between *L. lactis* and *S. xylosus* were obviously observed in excess oxygen condition because *S. xylosus* could efficiently use glucose to increase biomass concentration more than in uncontrolled oxygen condition, and once the glucose was exhausted, lactate was used as source of energy. The exhaustion of glucose had a dramatic effect on cell survival of both species.

The second objective was to perform transcriptome analysis of *L. lactis* in

mixed culture, carried out on DNA microarray of *L. lactis*. The reduction of cross-hybridizations by adding genomic DNA of *S. xylosus* was shown not to be efficient enough. Trancriptional responses of *L. lactis* with *S. xylosus* in mixed culture on *L. lactis* DNA microarray could not be accomplished. These two bacteria are too closely related for their cDNA to be distinguished by the DNA array available in the laboratory. The perspective is to design and use specific oligonucleotide microarrays.

Most of fermented foods are performed in uncontrolled oxygen condition which *S. xylosus* is unable to consume lactate. At the opposite, aeration of the fermented product after lactic acid fermentation could be a way to decrease acidification of the product and to improve the taste if *S. xylosus* consumes lactate. The lactate consumption by *S. xylosus* could be analyzed in more details, particularly to better characterize the mechanism and its regulation. Moreover, it will be interesting to explore the biodiversity of *S. xylosus* with regard to this parameter which could be used to improve the selection of starter cultures for fermented foods. These approaches will enable to better understand the microbial ecosystems associated with fermented foods.

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APPENDIX I

DIRECT LABELLING GENOMIC DNA

The protocol modified from Institute of Food Research (IFR), Norwich Research Park, Colney, Norwich, UK

1. Reagent preparation

1) TE buffer pH 8

a) 50 µl of 1M Tris pH 8

b) 10 µl of 500 mM EDTA

c) 4940 µl of autoclaved water

2)1/10 dNTP mix in TE buffer

a) 6 µl of 100 mM dATP

b) 6 µl of 100 mM dGTP

c) 6 µl of 100 mM dTTP

d) 3 µl of 100 mM dCTP

e) 479 µl of TE buffer

} Invitrogen 100mM dNTPs

3) 1/6 random primer in water

4) 3'-5' klenow enzyme

5) 10x NE Buffer 2

} BioLabs M0212M

2. Method

1) 2µg of fragment DNA, which is dried by speed vac. Resuspend the pellet with 21 µl water.

2) Add the solutions as the follow

1µl of 1/6 random primer

5 µl of 10x NE Bufer14 µl of water

Incubate at 95 °C for 5 min and then put on ice 5 min.

3) On ice, add the solution as follow after that vortex to get well-mix

5µl of 1/10 dNTP mix

3 µl of Cy-3 or Cy-5 dCTP

1 µl of klenow enzyme

Mark: The total volume of mixed solution is 50 µl.

4) Spin briefly and incubate the mix solution at 37°C overnight and protect it from light.

5) Put it on ice and use Microspin G 25 column for the labelled DNA purification.

The pure labelled DNA has to keep away from light and store at -20°

BIOGRAPHY

Miss Panadda Nongbeung was born on June 12, 1983 in Loei. She obtained her Bachelor of Science degree in Food Technology from Department of Food Technology, Faculty of Agricultural, Ubon Ratchathani University, Ubon Ratchathani, in 2005. After graduation, she decided to study master degree in the field of bioprocess engineering. During study, she received SUT financial support. After she finished coursework in Thailand, she worked as a research assistant in the team of metabolic engineering of prokaryote, Laboratory for Biosystem and Chemical Engineering, (LISBP), Toulouse, France under Fronco-Thai exchange graduated student program under living allowance of Ambassade de France en Thaïlande and DUO-Thailand Fellowship Program, the financial support of an ANR (French Research National Agency) grant under the Genoferment program, France. During this time she had chance to practice skills in the field of molecular biology techniques, microarray technology and bio-fermentation technology. Her research topic was Interaction of *Lactococcus lactis* and *Staphylococcus xylosus* in mixed culture. The results from part of this study have been presented as oral presentation at The 20th Annual Meeting and International Conference of the Thai Society for Biotechnology (TSB) on October 14-17th, 2008, Maha Sarakham, Thailand.