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Engineering of Biologic Functions

by

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Production and purification of biosurfactants and study of their influence on surface properties of stainless steel and Teflon

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Dedication

To my family

With love and gratitude

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Abbreviations

ATP adenosine 5'-triphosphate

BBG *Bacillus* Strain Collection of ProBioGEM

CFU colony forming unit

CMC critical micelle concentration

Da dalton

DNA deoxyribonucleic acid

dNTP deoxynucleotide triphosphate

EDTA ethylene diamine tetra-acetate

ESI electrospray ionization

GC gas chromatography

GRAS generally recognized as safe

HPLC high performance liquid chromatography

HEPES 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid

IPTG isopropyl β -D-thiogalactopyranoside

IT-MS ion trap mass spectrometry

m/z mass to charge ratio

MOPS 3-[N-morpholino]-propane sulfonic acid

MS mass spectrometry

MALDI-TOF MS matrix assisted laser desorption/ionization-time of flight mass spectrometry

MS/MS tandem mass spectrometry

NMR nuclear magnetic resonance

NRPS non-ribosomal peptide synthetase

OD optical density

ORF open reading frame

PCR polymerase chain reaction

PKS polyketide synthase

RBS ribosome binding site

Taq Pol thermoresistant polymerase of *Thermus aquaticus*

TQ-MS triple quadrupole mass spectrometry

RP-HPLC reversed phase-high performance liquid chromatography

SPE solid phase extraction

UV ultraviolet

v/v volume per volume

X-Gal 5-bromo-4-chloro-3-indolyl ß-galactopyranoside

XPS X-ray photoelectron spectroscopy

Abstract

In this study, a set of biosurfactant molecules was chosen in function of their structural diversity and their ability to be easily produced in industrial processes.

This set contains members of three families of lipopeptidic compounds produced by *Bacillus subtilis* strains including surfactin S1, iturin A and mycosubtilin (two members of the iturin family) and fengycin, as well as rhamnolipids produced by *Pseudomonas aeruginosa* PTCC 1637. After purification and/or characterization by several analytical methods, these compounds were examined for their ability to modify the surface hydrophobicity of the two substrata stainless steel and Teflon.

These modifications were evaluated by water contact angle measurements. The effects depend on the biomolecule, the concentration, and the substratum. Treatment of stainless steel with different concentrations between 1 and 100 mg l^{-1} of surfactin S1 and rhamnolipids showed an increase in the hydrophobicity. On the same substratum, fengycin increased hydrophobicity up to its critical micelle concentration (6.25 mg l^{-1}). With higher concentrations of fengycin, a decrease in hydrophobicity was observed. Surfactin, mycosubtilin and iturin A decreased hydrophobicity on Teflon. XPS analyses of surfaces treated by lipopeptides confirmed the presence of the different biomolecules. Relationships between structure, CMC, and modifications of surface properties are discussed.

Then, the attachment of *Bacillus cereus* 98/4 spores to conditioned surfaces with these biosurfactants was studied. There are promising correlations between hydrophobicity modifications of surfaces and the attachment of *B. cereus* 98/4 spores to these surfaces. Enhancement in hydrophobicity of surfaces increases the number of adhering spores to them and vice versa.

Finally, a strategy was developed to overproduce a less studied lipopeptide from *Bacillus licheniformis*, lichenysin that is slightly different structurally from surfactin but has demonstrated a potent property of biosurfactant. Bioinformatic analyses were also performed on the sequenced genome of *B. licheniformis* ATCC 14580 to confirm its ability to produce lichenysin and accordingly, a genetic engineering work was undertaken.

Résumé

Dans cette étude, un ensemble de molécules biosurfactantes a été choisi en fonction de leur diversité structurale et leur aptitude à être produites dans des procédés industriels.

Cet ensemble contient des membres des trois familles de composés lipopeptidiques produits par des souches de *Bacillus subtilis* comprenant la surfactine S1, l'iturine A et la mycosubtiline (deux membres de la famille des iturines) et la fengycine, ainsi que des rhamnolipides produits par *Pseudomonas aeruginosa* PTCC 1637.

Après purification et/ou caractérisation par plusieurs méthodes analytiques, ces composés ont été etudiés pour leur aptitude à modifier l'hydrophobicité de surface de deux substrats, l'acier inoxydable et le Téflon.

Ces modifications ont été évaluées par des mesures d'angle de contact de l'eau. Les effets dépendent de la biomolécule, de sa concentration et du substrat. Le traitement de l'acier inoxydable avec différentes concentrations, entre 1 et 100 mg l^{-1}, de surfactine S1 et de rhamnolipides a montré une augmentation de l'hydrophobicité. Sur le même substrat, la fengycine augmente l'hydrophobicité jusqu'à sa concentration micellaire critique (6,25 mg l^{-1}). Avec des concentrations plus élevées en fengycine, une réduction de l'hydrophobicité est observée. La surfactine, la mycosubtiline et l'iturine diminuent l'hydrophobicité sur le Téflon. Des analyses par XPS de surfaces traitées par les lipopeptides ont confirmé la présence des différentes biomolécules. Les relations entre structure, CMC et les propriétés de modifications de surface sont discutées.

L'adhésion de spores de *Bacillus cereus* 98/4, à des surfaces conditionnées par ces biosurfactants, a ensuite été étudiée. Il y a une bonne correlation entre les modifications de l'hydrophobicité et l'adhésion des spores de *B. cereus* 98/4 à ces surfaces. L'augmentation de l'hydrophobicité des surfaces augmente l'adhésion des spores et vice versa.

Finalement, une stratégie a été développée pour surproduire un lipopeptide moins étudié de *Bacillus licheniformis*, la lichenysine dont la structure differe légèrement de celle de la surfactine, et qui possède des meilleures propriétés biosurfactantes. Des analyses bioinformatiques ont été réalisées sur le génome séquencé de *B. licheniformis* ATCC 14580 pour confirmer sa capacité à produire la lichenysine et, en conséquence, un travail d'ingénierie génétique a été entrepris.

1. General introduction

1.1. Biosurfactants

1.1.1. Introduction

Biosurfactants are a structurally diverse group of surface-active molecules mainly synthesized by microorganisms (Cooper, 1980; Desai, 1997). Microbial biosurfactants include a wide variety of compounds, such as glycolipids, lipopeptides (LPs), polysaccharide-protein complexes, phospholipids, fatty acids, and neutral lipids. They are usually produced extracellularly or as part of cell membrane by bacteria, filamentous fungi and yeasts (Mata-Saddoval *et al*. 1999). Different kinds of bacteria have been employed by many researchers in producing biosurfactants using culture media. Most of such bacteria are isolated from contaminated sites usually containing petroleum hydrocarbon by products and/or industrial wastes (Rahman *et al*. 2006; Benincasa, 2007).

Interest in microbial surfactants has been steadily increasing in recent years, as they have numerous advantages compared to chemical surfactants including a lower toxicity, higher biodegradability (Zajic, 1997; Woo, 2004), higher foaming, better environmental compatibility (Georgiou, 1992; Banat, 1995), and effective properties at extreme temperature, pH levels, and salinity (Kretschner, 1982; Cho, 2005).

In the biosurfactant amphipathic structure, the hydrophobic moiety (tail) is either a long-chain fatty acid or a hydroxy fatty acid of varying length. The hydrophilic moiety (head) may be a carbohydrate, carboxylic acid, phosphate, amino acid, peptide, or an alcohol.
Table 1.1 shows a list of biosurfactants produced by different microorganisms.
Biosurfactants have applications in an extremely wide variety of industrial fields like food, cosmetic, pesticide, detergent, pharmaceutical industries, enhanced oil recovery, transportation of heavy crude oil, and bioremediation (Georgiou, 1992; Desai, 1997).
When considering the natural roles and potential applications of biosurfactants, it is important to emphasize that a wide variety of diverse microorganisms make these molecules and that biosurfactants have very different chemical structures and surface properties. It is therefore reasonable to assume that different groups of biosurfactants have different natural roles in the growth of the producing microorganisms.
This diversity makes it difficult to generalize about the natural role of biosurfactants (Rosenberg, 2006). One of their physiological roles is to permit microorganisms to grow on water-immiscible

Table 1.1. Major biosurfactant classes and microorganisms involved (Desai and Banat, 1997; Rosenberg 1999; Mulligan 2005; Raaijmarkers *et al*. 2006; Muthusamy 2008)

Surfactant class	**Microorganism**
Glycolipids	
Rhamnolipids	*Pseudomonas aeruginosa,a* *Pseudomonas* sp.
Trehalose lipids	*Rhodococcus erithropolis,* *Arthobacter* sp.
Sophorolipids	*Candida bombicola, Candida apicola,* *Candida lipolytica, Candida bogoriensis*
Mannosylerythritol lipids	*Candida antartica*
Lipopeptides	
Surfactin/iturin/fengycin	*Bacillus subtilis*
Viscosin/tolaasin/syringomycin	*Pseudomonas* spp.
Putisolvin/amphisin	*Pseudomonas* spp.
Lichenysin	*Bacillus licheniformis*
Serrawettin	*Serratia marcescens*
Phospholipids	
	Acinetobacter sp. *Corynebacterium lepus*
Fatty acids/neutral lipids	
Corynomicolic acids	*Corynebacterium insidibasseosum*
Polymeric surfactants	
Emulsan	*Acinetobacter calcoaceticus*
Alasan	*Acinetobacter radioresistens*
Liposan	*Candida lipolytica*
Lipomanan	*Candida tropicalis*
Particulate biosurfactants	
Vesicles	*A. calcoaceticus*
Whole microbial cells	*Cyanobacteria*

substrates by reducing the surface tension at the phase boundary, therefore making the substrate more readily available for uptake and metabolism. In addition to emulsification of the carbon source, they are also involved in the adhesion of microbial cells to the hydrocarbon and as a result allow growth on such a carbon source.
Surfactin could also play a physiological role by increasing the bioavailability of water-insoluble substrates and by regulating the attachment/detachment of microorganisms to and from surfaces (Rosenberg and Ron, 1999).

Ahimou *et al*. (2000) demonstrated that lipopeptide molecules adsorb on *B. subtilis* after their excretion in extracellular medium and induce changes of the cell surface hydrophobicity. The hydrophobicity alterations suggest an important role of lipopeptide molecules in *B. subtilis* in the the adhesion mechanisms onto various surfaces by hydrophobic interactions.
Biosurfactants have been shown to have an antagonistic effect towards other microbes in the environment. Furthermore, some of biosurfactants adher to cell membrane and enhance nutrient transport across cell membrane (Bodour *et al* .2003).

Factors controlling the production of biosurfactants through their effects on cellular growth or activity include the quality and quantity of carbon and nitrogen constituents in culture media and physico-chemical conditions such as pH, temperature, agitation, and oxygen avaibility (Desai and Banat 1997; Lang and Philp 1998).
Sheppard and Cooper (1997) have concluded that oxygen transfer is also one of the key parameters for the process optimization and scaling-up of surfactin production in *B. subtilis*.
Salt concentrations also affect biosurfactant production depending on its effect on cellular activity. However, some biosurfactants (lichenysin) were not affected by salt concentrations up to 10% (w/v) (Yakimov 2000), although slight reductions in the critical micelle concentrations (CMC) were detected (Abu-Ruwaida *et al*. 1991; Thimon et *al*.1992).
The type and/or the concentration of nitrogen present (whether NH_4^+, NO_3^-, urea or amino acid) can sometimes influence the biosurfactant produced (Robert *et al*. 1989; Haba *et al*. 2000). The nitrogen limitation appears to stimulate biosurfactant production and overproduction by some micro-organisms (Suzuki *et al*. 1974; Guerra-Santos *et al*. 1984).

Desai and Banat (1997) have reviewed a wide range of techniques to determine the presence of biosurfactants in culture media. The methods used up to now include colorimetric analyses for the detection of anionic surfactants (Shulga *et al*. 1992) and rhamnolipids (Hansen *et al*. 1993; Siegmund and Wagner 1991), hemolytic activity, emulsification index (EI) determination over a 24-h period (Cooper and Goldenberg 1987), drop-collapsing test (Jain *et al*. 1991), surface tension (ST) determination, TLC, HPLC, FTIR, NMR (H-1 and C-13) and MS methods. In addition, MALDI-TOF/MS is a rapid, sensitive and efficient method (the molecular masses can be determined with an accuracy of 0.01% to 0.02% (Vater *et al*. 2002) for structural characterization of biosurfactants by using whole microbial cells (thus avoiding cultivation and extraction) or crude culture filtrates. The latter is well suited for rapid primary screening of new microbial isolates for novel natural compounds in the context of biocontrol (Ongena and Jacques, 2008).

1.1.2. Physico-chemical properties

Biosurfactants are classified according to the ionic charge residing in the polar part of the molecule. Hence anionic, cationic, nonionic and zwitterionic biosurfactants exist. Amphoteric or zwitterionic ones have both positively and negatively charged moieties in the same molecule (Van Ginkel, 1989).

Biosurfactants can be also classified according to their Hydrophile–Lipophile Balance (HLB) that affects their physico-chemical properties (Tiehm, 1994). The HLB classification can be used to determine the suitability of using surfactants.

The HLB value indicates whether a surfactant will promote water-in-oil or oil-in-water emulsion by comparing it with surfactants with known HLB values and properties. The HLB scale can be constructed by assigning a value of 1 for oleic acid and a value of 20 for sodium oleate and using a range of mixtures of these two components in different proportions to obtain the intermediate values. Emulsifiers with HLB values less than 6 favor stabilization of water-in-oil emulsification, whereas emulsifiers with HLB values between 10 and 18 have the opposite effect and favor oil-in-water emulsification (Desai and Banat 1997). In general, a surfactant with a low HLB is lipophilic whereas a high HLB confers better water solubility (Sabatini *et al*. 1995). Table 1.2 shows the application of nonionic surfactants with different HLB values (Cross, 1987).

Table 1.2. The uses of nonionic surfactants with different HLB values

Non-ionic surfactant HLB value	Uses
<3	Surface films
3-6	Water-in-oil emulsifiers
7-9	Wetting agents
8-15	Oil-in-water emulsifiers
13-15	Detergents
15-18	Solubilisers

Some biosurfactants and their surface activities are not affected by environmental conditions such as temperature and pH. McInerney *et al*. (1990) reported that lichenysin from *B. licheniformis* JF-2 was not affected by temperature (up to 50°C), pH (4.5–9.0) and by NaCl and Ca concentrations up to 50 and 25 g l^{-1} respectively. A lipopeptide from *B. subtilis* LB5a was stable after autoclaving (121°C for 20 min) and after 6 months at –18°C and its surface activity did not change from pH 5 to 11 and NaCl concentrations up to 20% (Nitschke *et al*. 1990).

Critical micelle concentratrion (CMC) is that concentration of surfactant favouring micelle formation. It is defined by the solubility of a surfactant within an aqueous phase and is commonly used to measure the efficiency of a surfactant. Biosurfactants have CMC about 10-40 times lower than that of chemical surfactants, i.e. less surfactant is necessary to get a maximum decrease in surface tension (Desai and Banat 1997).

Above the CMC, biosurfactant molecules aggregate to form supra molecular structures like micelles, bilayers, and vesicle.

Between 50 and 100 surfactant molecules usually (aggregation number) form micelles (Figure 1.1). Micelles arise when the lipophilic part of the surfactant molecule that is unable to form hydrogen bonding in an aqueous phase causes an increase in the free energy of the system. One way for the hydrocarbon tail to alleviate this free energy increase is to be isolated from water by adsorption onto surfaces, absorption into an organic matrix or the formation of micelle vesicles

where the hydrocarbon moiety of the surfactant become situated towards the centre with the hydrophilic part in contact with water (Haigh, 1996).

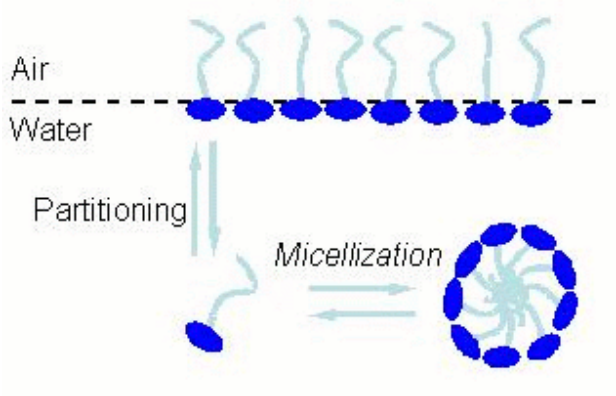


Figure 1.1. Formation of monolayer and micelles in aqueous solution.

Unlike chemically synthesized surfactants, which are usually classified according to the nature of their polar grouping, biosurfactants are generally categorized by their chemical composition and microbial origin. On the basis of their molecular mass, biosurfactants isolated from microorganisms are generally classified into two groups: 1) Low molecular mass biosurfactants, such as glycolipids, lipopeptides, corynomycolic acids, and phospholipids. Glycolipids and lipopeptide substances are involved in the lowering of surface and interfacial tensions in liquids. Low-molecular-weight biosurfactants that have low CMC increase the apparent solubility of hydrocarbons by incorporating them into the hydrophobic cavities of micelles (Miller and Zhang, 1994). Stable emulsions are not a usual trait of these surfactants. 2) High molecular mass molecules, such as emulsans, alasan, liposan, polysaccharides, and protein complexes. These biosurfactants are associated with production of stable emulsions but do not lower very much the surface tension. It is interesting to note that the production of stable emulsions enables bacteria to adhere to hydrophobic surfaces very strongly (Rosenberg and Rosenberg 1981; Neu *et al*. 1992) with implications on biodegradation capabilities.

1.1.3. Purification procedures

Biosurfactant recovery depends mainly on its ionic charge, solubility in water or organic solvents, and location (intracellular, extracellular or cell-bound). Most of biosurfactants are secreted into the medium, and they are isolated from either culture filtrate or supernatant obtained after removal of cells. Downstream processes for recovery of important biosurfactants include

ammonium sulfate precipitation, acid precipitation, solvent extraction, crystallization, adsorption, foam separation and precipitation, diafiltration and ultrafiltration (Desai and Banat, 1997). Table 1.3 summarize the recovery methods.

Table 1.3. Physicochemical property-based biosurfactant recovery methods and their relative advantages (Mukherjee *et al*. 2006)

Downstream recovery procedure	**Biosurfactant property responsible for separation**	**Instrument/apparatus/ setup required**	**Advantages**
Acid precipitation	Biosurfactants become insoluble at low pH values	No set-up required	Low cost, efficient in crude biosurfactants recovery
Organic solvent extraction	Biosurfactants are soluble in organic solvents due to the presence of hydrophobic end	No set-up required	Efficient in crude biosurfactant recovery and partial purification, reusable nature
Ammonium sulfate precipitation	Salting-out of the polymeric or protein rich biosurfactant	No set-up required	Effective in isolation of certain type of polymeric biosurfactants
Centrifugation	Insoluble biosurfactants get precipitated because of centrifugal force	Centrifuge required	Reusable, effective in crude biosurfactants recovery
Foam fractionation	Biosurfactants, due to surface activity, form and partition into foam	Specially designed bioreactors that facilitate foam recovery during fermentation	Useful in ontinuous recovery procedures, high purity of product
Membrane ultrafiltration	Biosurfactants form micelles above their critical micelle concentration (CMC), which are trapped by polymeric membranes	Ultrafiltration units with porous polymer membrane	Fast, one-step recovery, high level of purity
Adsorption on polystyrene resins	Biosurfactants are adsorbed on polymer resins and subsequently desorbed with organic solvents	Polystyrene resin packed in glass columns	Fast, one-step recovery, high level of purity, reusability
Adsorption on wood-activated carbon	Biosurfactants are adsorbed on activated carbon and can be desorbed using organic solvent	No setup required, can be added to culture broth, can also be packed in glass columns	biosurfactants, cheaper, reusability, recovery from continuous culture
Ion-exchange chromatography	Charged biosurfactants are attached to ion-exchange resins and can be eluted with proper buffer	Ion-exchange resins packed in columns	High purity, reusability, fast recovery
Solvent extraction (using Methyl tertiary-butyl ether)	Biosurfactants dissolve in organic solvents owing to the hydrophobic ends in the molecule	No set-up required	Less toxic than conventional solvents, reusable, cheap

1.1.3.1. Precipitation

Two different kinds of precipitation can be discriminated: acid (Deziel *et al*. 1999; Zang *et al*. 1992; Van Dyke *et al*. 1993) or ammonium sulphate precipitation. Through acidification of the medium to a final pH between 2 and 3, biosurfactants like surfactin exist in their protonated form and are therefore less soluble in an aqueous solution. The precipitate can be collected by centrifugation (after several hours at 4°C) and resuspended in an appropriate buffer (e.g. bicarbonate).

1.1.3.2. Solvent extraction

Biosurfactant extraction is often used for removing hydrophilic compounds prior to biosurfactant analysis. Different solvents and solvent mixtures like ethyl acetate, chloroform/methanol (2/1), butanol, hexan, acid acetic are applied (Mata-Sandoval *et al*. 1999). In general, the extraction yield can be improved by an acidification of the sample prior to extraction, as biosurfactant are then less soluble in water.

1.1.3.3. Adsorption

Most frequently, Amberlite XAD 2 or 16 polystyrene resin that absorb and release hydrophobic and amphiphilic substances owing to basically hydrophobic interactions are used (Gruber *et al*. 1991; Abalos *et al*. 2001; Haba *et al*. 2003; Dubey *et al*. 2005).
For primary enrichment, cell-free culture broth is directly applied to the adsorbent column and finally, biosurfactants are eluted with methanol and the solvent is evaporated subsequently.
Adsorption chromatography represents a good alternative to solvent extraction, the advantage being smaller solvent consumption.

1.1.3.4. Ion exchange

Another purification step of the biosurfactants mixture is anion exchange chromatography (Reiling *et al*. 1986). Since some of biosurfactants are charged negatively at higher pH values, they can be separated by a weak anion exchanger (e.g. (diethylamino) ethyl-Sepharose).

1.1.3.5. Membrane filtration

Membrane filtration is another alternative for biosurfactant enrichment and prepurification (Gruber *et al*. 1991). Generally, at concentrations above the critical micelle concentration, ultrafiltration with a membrane cut-off of 10 kDa leads to an almost complete retention of biosurfactants even at neutral pH (Gruber *et al*. 1991). A new continuous bioprocess based on membrane technology was recently developed by François Coutte (2009) to ensure the production and purification of surfactin.

1.1.3.6. Foam fractionation

Foam fractionation uses the peculiarity of biosurfactants of forming micelles and, thus, of foaming. When applied for the continuous removal of biosurfactants during fermentations, foam is allowed to press out of the bioreactor through a fractionation column. Afterwards, it collapses in a separate recipient by cooling, addition of acids or using shear forces (Gruber *et al*. 1991; Guez *et al*. 2008).

Continuous removal of biosurfactant during fermentation results in a several-fold net increase in biosurfactant yield. In addition, substantial reductions in the cost of product recovery and effluent treatment are achieved (Desai and Banat 1997).

1.1.3.7. Chromatographic separation of biosurfactant mixtures

After biosurfactant separation from bacterial cells and some water-soluble substances, chromatographic methods are applied to obtain pure biosurfactants. For smaller volumes, preparative TLC may be applied, whereas larger quantities are separated by column chromatography (e.g. silica gel or reversed-phase material).

- **Preparative TLC**

The process of preparative TLC is similar to that of analytical TLC. Generally, preparative silica gel plates are used with a solvent mixture of chloroform/methanol/water or acetic acid (65/15/2, v/v/v) or mixtures of equivalent polarities. As one chromatographic run sometimes is not sufficient to achieve pure biosurfactants, TLC is performed several times with solvents of

different polarities (Deziel *et al*. 1999) or after column chromatography for precision cleaning (Sim *et al*. 1997; Monteiro *et al*. 2007).

Samples are applied onto TLC plates after extraction from culture broth and concentration. The separated biosurfactants are eluted from silica by methanol or chloroform–methanol (Van Dyke *et al*. 1993; Sim *et al*. 1997; Monteiro *et al*. 2007). The advantages of TLC are its simple feasibility and the need for less equipment than for preparative column chromatography. On the other hand, only smaller quantities of samples can be treated and smaller amounts of organic solvents are consumed compared to column chromatography.

- **Normal phase**

Normal-phase column chromatography using silica is a standard purification method for biosurfactant separation if larger volumes have to be treated (Sim *et al*. 1997; Monteiro *et al*. 2007). The acidified biosurfactant mixture, in a solvent like chloroform, is fed into the column after various prepurification steps to separate bacterial cells and some water-soluble compounds. Flushing of silica with chloroform removes neutral lipids and some pigments (Sim *et al*. 1997). Biosurfactants can be eluted with chloroform–methanol solution.

- **Reversed phase**

In this method, biosurfactant mixtures are separated by reversed-phase chromatography according to the chain length of the hydroxy fatty acids (de Kostar *et al*. 1994). Prepurified biosurfactant mixtures are acidified and loaded onto an RP18 column. However, column material is more expensive than normal silica; therefore, it is used for semipreparative purposes only.

High performance liquid chromatography (HPLC) is not only appropriate for the complete separation of different biosurfactants but can also be coupled with various detection devices (UV, MS, evaporative light scattering detection (ELSD)) for identification and quantification of biosurfactants.

The high sensitivity of HPLC/MS allows for analysis down to trace concentrations. When MS is coupled with HPLC and a proper sample preparation, it is the most precise method for especially rhamnolipid identification and quantification.

Another advantage is the possibility to handle a high number of samples.

1.1.4. Structural analysis procedures

1.1.4.1. Mass spectrometry

Structure analysis of biosurfactant mixture (homologues) can be performed by tandem quadrupole mass spectrometers (developed in the late 1970s). Good biosurfactant ionisation is achieved by electrospray ionisation for direct infusion or HPLC/MS, as it represents a "soft" method with little fragmentation of primary molecules. Ionised molecules are selected by a mass analyser according to their mass-to-charge ratio (m/z) and are subsequently detected. Scanning of the whole mass spectrum between 100 and 750 Da (Q1 scan) in the negative MS mode allows for the selection of the target ions (different biosurfactants). Structural identification of the target ions can be accomplished by MS/MS experiments (product ion scans), which means that a target ion is fractionated in the collision cell and the fragment ions (e.g. hydroxy fatty acids, rhamnose fragments, amino acid residues) are detected (Heyd *et al*. 2008).

Lipopeptide supernatant, extract and/or colony can be analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS). A saturated solution of α-cyano-4-hydroxy-cinnamic acid is prepared in a 3:1 (v/v) solution of CH_3CN and H_2O containing 0.1% trifluoroacetic acid. The cell culture supernatant is diluted 10-fold with an α-cyano-4-hydroxy-cinnamic acid-saturated solution. Then, 0.5 µl of this solution is deposited on the target. Measurement is performed using a UV laser desorption-time of flight mass spectrometer (Bruker Ultraflex tof; Bruker Daltonics) equipped with a pulsed nitrogen laser (λ= 337 nm). The analyzer is used at an acceleration voltage of 20 kV. Samples are measured in the reflectron mode.

1.1.4.2. Fourier transform infra red (FTIR) spectroscopy

A classic method for structure analysis is IR spectroscopy. Irradiation of molecules with IR light induces an oscillation of chemical bonds at characteristic frequencies and, thus, energy is absorbed. The resulting transmission of radiation is measured and shows deformation bands which are characteristic of every molecule and allow for the chemical substances to be identified from spectrum files. By comparing the infra red spectra of 2 molecules, we are able to know whether they are the same. Another application of infra red spectrum is to give valuable information

about molecular structure. It also gives information about functional groups in given molecules (Heyd *et al*. 2008).

1.1.4.3. Nuclear Magnetic Resonance (NMR) spectroscopy

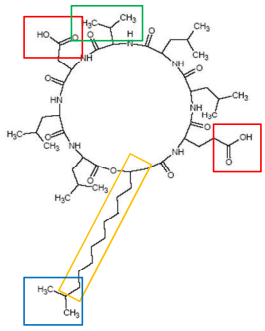
In the last few years, NMR spectroscopic analysis was performed in principle to confirm the structure of biosurfactants produced by recently isolated bacteria or mutant strains compared to the structure of biosurfactants mentioned in the literature (Itoh *et al*. 1971; Syldatk *et al*. 1984).
NMR spectroscopy is based on transitions in atoms with a magnetic moment when applying an external magnetic field. Structure information is obtained from three parameters: chemical shifts of the absorption frequency, coupling (mutual influence of adjacent nuclei), and integral height. Different techniques, such as COSY (correlation spectroscopy), HMQC (heteronuclear multiple quantum coherence) can be applied for NMR (Wei *et al*. 2005, Monteiro *et al*. 2007).
Measurements of deuterium-exchanged samples are generally carried out in chloroform-deuterated methanol (2/1, v/v) using tetramethylsilane as the internal standard.
NMR spectroscopy allows for an even more accurate structure and purity analysis than IR spectroscopy.

1.1.5. Biosurfactants produced by *Bacillus* spp.

1.1.5.1. Introduction

Bacteria of the genus *Bacillus* produce a number of cyclic compounds, which are biologically active. Various strains of *B. subtilis* produce more than twenty different molecules with antibiotic activity including many lipopeptides that also show biosurfactant properties. The cyclic structure of the peptide part protects the lipopeptides (Figure 1.2) from enzymatic cleavage and maintains its general stability, so they are commonly resistant to peptidases and proteases (Nagorska *et al*. 2007). They are synthesized by large multienzymatic proteins called non-ribosomal peptide synthetases (NRPSs). These biosynthetic systems lead to a remarkable heterogeneity among the LP products generated by *Bacillus* with regard to the type and sequence of amino acid residues, the nature of the peptide cyclisation and length and branching of the fatty acid chain. Variations in length and branching of the fatty acid chains and amino acid substitutions allow the lipopeptides identified so far to be divided into three groups: the surfactin or lichenysin (Peypoux

Surfactin family



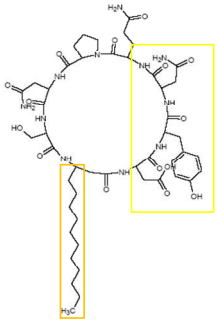
Variants

		Lenght and branching of the acyl chain
Esperin**	**L-Glu-L-Leu**-D-Leu-**L-Val**-L-Asp-D-Leu-**L-Leu-COOH**	
Lichenysin***	**L-XL_1-L-XL_2**-D-Leu-**L-XL_4**-L-Asp-D-Leu-**L-XL_7**	*i*-C_{13}, *ai*-C_{13}, *n*-C_{14}, *i*-C_{15}, *ai*-C_{15}
Pumilacidin	**L-Glu-L-Leu**-D-Leu-**L-Leu**-L-Asp-D-Leu-**L-XP_7**	
Surfactin	**L-Glu-L-XS_2**-D-Leu-**L-XS_4**-L-Asp-D-Leu-**L-XS_7**	*i*-C_{14}, *n*-C_{14}, *i*-C_{15}, *ai*-C_{15}

** the βcarboxyl of Asp_3 is engaged in the lactone
*** or halobacillin
XL_1 = Gln or Glu ; XL_2 = Leu or Ile ; XL_4 and XL_7 = Val or Ile ;
XP_7 = Val or Ile ;
XS_2 = Val, Leu or Ile ; XS_4 = Ala, Val, Leu or Ile ; XS_7 = Val, Leu or Ile

n, linear
i, iso
ai, anteiso

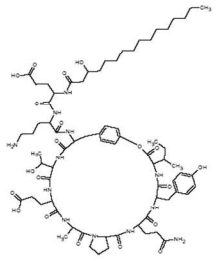
Iturin family



Bacillomycin D	**L-Asn**-D-Tyr-D-Asn-**L-Pro-L-Glu-D-Ser-L-Thr**	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}
Bacillomycin F	**L-Asn**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Asn-L-Thr**	*i*-C_{16}, *i*-C_{17}, *ai*-C_{17}
Bacillomycin L	**L-Asp**-D-Tyr-D-Asn-**L-Ser-L-Gln-D-Ser-L-Thr**	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}
Bacillomycin LC*	**L-Asn**-D-Tyr-D-Asn-**L-Ser-L-Glu-D-Ser-L-Thr**	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}, *i*-C_{16}
Iturin A	**L-Asn**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Asn-L-Ser**	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}
Iturin A_L	**L-Asn**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Asn-L-Ser**	*n*-C_{16}, *i*-C_{16}
Iturin C	**L-Asp**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Asn-L-Ser**	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}
Mycosubtilin	**L-Asn**-D-Tyr-D-Asn-**L-Gln-L-Pro-D-Ser-L-Asn**	*n*-C_{16}, *i*-C_{16}, *ai*-C_{17}

* or bacillopeptin

Fengycin family



Fengycin A	L-Glu-D-Orn-**D-Tyr**-D-aThr-L-Glu-**D-Ala**-L-Pro-L-Gln-**L-Tyr**-L-Ile	*ai*-C_{15}, *i*-C_{16}, *n*-C_{16}
Fengycin B	L-Glu-D-Orn-**D-Tyr**-D-aThr-L-Glu-**D-Val**-L-Pro-L-Gln-**L-Tyr**-L-Ile	*ai*-C_{15}, *i*-C_{16}, *n*-C_{16}, C_{17}
Plipastatin A	L-Glu-D-Orn-**L-Tyr**-D-aThr-L-Glu-**D-Ala**-L-Pro-L-Gln-**D-Tyr**-L-Ile	*n*-C_{16}, *ai*-C_{17}
Plipastatin B	L-Glu-D-Orn-**L-Tyr**-D-aThr-L-Glu-**D-Val**-L-Pro-L-Gln-**D-Tyr**-L-Ile	*n*-C_{16}, *ai*-C_{17}

Figure 1.2 Structures of representative members and diversity within the three lipopeptide families synthesized by *Bacillus* species. Boxed structural groups are those that were shown to be particularly involved in interaction with membranes and/or are supposed to be important for biological activity in addition to the cyclic nature of the molecule. To the best of our knowledge, no clear data are available to date for fengycins in this context. Boxed blue, type of branching (linear, *iso*, *anteiso*); boxed orange, acyl chain length; boxed red, ionisable or polar groups; boxed green, hydrophobicity of residue in position 4; boxed yellow, L-Asx(1)–D-Tyr(2)–D-Asn(3) sequence (Ongena and Jacques 2008).

et al. 1999; Mulligan, 2005), iturin (Tsuge *et al*. 2005) and fengycin families (Ongena *et al*. 2005b). They are composed of seven α-amino acids (iturins, surfactin and lichenysin) or ten α-amino acids (fengycins).

In artificial media, cells in the transition from exponential phase to stationary phase mostly produce surfactins, while fengycin synthesis is delayed to early stationary phase and iturins only accumulate later (Jacques *et al*. 1999; Koumoutsi *et al*. 2004).

These compounds have many pharmacological activities: antibacterial (Thimon *et al*. 1992; Toure *et al*. 2004; Stein 2005), antifungal (Thimon *et al*. 1992), antiviral (Kracht *et al*. 1999), and antimycoplasma properties (Vollenbroich *et al*. 1997), inhibition of the fibrin clot formation and hemolysis (Arima *et al*. 1968; Cameotra *et al*. 2004); formation of ion channels in lipid bilayer membranes (Sheppard *et al*. 1997); antitumour activity against Ehrlich's ascites carcinoma cells (Cameotra *et al*. 2004); and inhibition of the cyclic adenosine-3,5-monophosphate phosphodiesterase (Hosono *et al*. 1983). A comparison between the activities of the different variants of the three families of lipopeptides produced by *B. subtilis* shows that the amphiphilic character is not the sole trait explaining these biological activities. Within each family, some structural homologues are seemingly more active than others (Fickers *et al*. 2009). Specific functions present in the peptidic moiety are also important (Ongena and Jacques 2008). Lipopeptides are capable of penetrating into membrane cells, with the lipophilic hydrocarbon chain interacting with the plasma membrane lipid moiety, while the polar amino acids in the peptide part interacts with the polar phosphatidyl moieties. Whether lipopeptides are able to damage the integrity of the plasma membrane or create ion-conducting pores depends on the nature of the lipopeptides and on the phospholipids of the membranes.

These amphiphilic molecules are responsible for the biocontrol of plant pathogens by *B. subtilis*. Indeed, recent advances show that they can act not only as 'antagonists' or 'killers' by inhibiting phytopathogen growth but also as 'spreaders' by facilitating root colonisation and as 'immuno-stimulators' by reinforcing the host resistance potential (Ongena and Jacques 2008).

1.1.5.2. Surfactins family

1.1.5.2.1. Surfactin

Surfactin was first isolated in 1968 from *B. subtilis* IAM 1213 (Arima *et al*. 1968). Surfactin or closely related variants such as lichenysin have been isolated from *Bacillus coagulans*, *Bacillus pumilus* and *B. licheniformis*. It was demonstrated to have exceptional surfactant properties and is still considered as one of the most powerful biosurfactant (Maget-Dana and Ptak, 1992; Heerklotz and Seelig, 2001).

- **Structure**

Surfactin contains a heptapeptide with the chiral sequence LLDLLDL, interlinked with a β-hydroxy fatty acid to form a cyclic lactone ring structure (Figure 1.2 and 1.3). The length of the carbon chain of β-hydroxy fatty acids ranges from C13 to C16 (Li *et al*. 2009). The structure and chain length of the lipid moieties are critically dependent on the culture conditions of the producing bacterium (Besson *et al*. 1992; Oka *et al*. 1993; Akpa *et al*. 2001). For example, the supplementation of the branched amino acids, Val or Ile, led to typical variations, in relation with the amino acid structure (Besson *et al*. 1992; Grangemard *et al*. 1997).

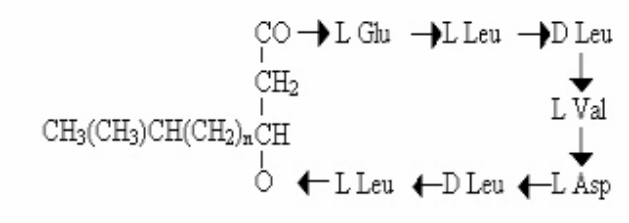


Figure 1.3 Surfactin S1 structure.

Surfactin's three-dimensional structure has been experimentally determined by high resolution ^{1}H NMR combined with molecular modeling techniques (Bonmatin *et al*. 1994). In its backbone folding, surfactin adopts a "horse saddle" topology, also called a β-sheet structure, which along with strong surfactant properties is probably responsible for its biological properties.

Membrane penetration by surfactin is facilitated in the presence of cations (Maget-Dana *et al*. 1995). The two acidic residues Glu-1 and Asp-5 form a well-suited "claw", which can easily

stabilize a surfactin-Ca^{2+} 1:1 complex via an intramolecular bridge (Maget-Dana *et al*. 1992). This effect of Ca2+ ions on the surfactin conformation promotes the deeper insertion of the lipopeptide into the membrane.

- **Physico-chemical properties**

It is a powerful biosurfactant with exceptional emulsifying and foaming properties that reduces ST of water from 72 mN m^{-1} to values in the range of 25-30 mN m^{-1}(Bonmatin *et al*. 2003).
Surfactin (mixture) has a critical micelle concentration (CMC) of 10 mg l^{-1} (Ishigami *et al*. 1995). In literature, different CMC values have been reported, which depend on measurement conditions.
Dufour *et al*. (2005) showed that the loss of the cyclic structure does not suppress the surface-active properties in surfactin, but only reduces them. The number of carbon atoms in fatty acid chain has an important role in tensioactive properties of it, so that CMC value decreased as carbon chain length rises. Li *et al*. (2009) observed that the CMC value of surfactin-C16 is smaller than that of the surfactin-C13 and C14.

The residue type also influences these properties. In a study, when Leu (7) was substituted by Ile (7) or Val (4) with Ile (4), the CMC decreased. Since surface properties increase steadily when increasing the hydrophobicity (hereby, the apolar domain favour micellization) of residues at positions 4 and 7, the hydrophobic character of the apolar domain governs intermolecular hydrophobic interactions (Grangemard *et al*. 1997).

The presence of salts in aquous phase influences these parameters; for example, as the concentration of Na^{+} was increased CMC, ST and micropolarity of the surfactin micelles decreased (Thimon *et al*. 1992; Li *et al*. 2009). Li *et al*. 2009 concluded that addition of sodium ions facilitates micellization and enhances the surface activity and solubilizing properties of the surfactin-C16.
Surfactin forms rod-like micelles with an aggregation number of ~170 (Heerklotz 2001).
The influence of Na^{+} concentrations on the aggregation number (N) was also investigated by Li *et al*. (2009). The values of N decreased as the concentration of Na^{+} was increased. These values were smaller than those obtained by static light scattering measurement by Ishigami (121 in

concentration 0.02 M l^{-1} Na^+ and 75 in 0.3 M l^{-1} Na^+). It is possible that the presence of Na^+ leads to a stronger and tighter micelle structure and the surfactin forms smaller micelles. However, the aggregation numbers obtained by the steady-state fluorescence method (Li *et al*. 2009) need to be justified under special conditions, so further work is needed to quantify the aggregation number.
Temperature has an important impact on mobility of hydrophobic fatty acid chains of surfactin molecules, while pH exerts influence mainly on ionization of the peptide loop (Song *et al*. 2007).

- **Biological activity**

Because of its amphiphilic nature, surfactin can also readily associate and tightly anchor into lipid layers. It can thus interfere with biological membrane integrity in a dose-dependent manner. At moderate concentrations, the lipopeptide forms domains segregated within the phospholipids that may contribute to the formation of ion-conducting pores in membranes and at high concentrations, the detergent effect prevails (Bonmatin *et al*. 2003). This lipid bilayer destabilisation process is facilitated by the tri-dimensional form of the surfactin molecule featuring charged side chains protruding into the aqueous phase and apolar moieties reaching into the hydrophobic core of the membrane (Deleu *et al*. 2003; Heerklotz *et al*. 2004). It shows the importance of hydrophobic interactions in penetration of surfactin into the membranes. Surfactin-induced pores show some selectivity for potassium over other cations (Maget-Dana 1985; Sheppard 1991).
Surfactin in concentrations of 30-64 μM is cytotoxic to several human and animal cell lines (Vollenbroich *et al*. 1997) and provoke hemolysis (Dufour *et al*. 2005).
It has been reported that surfactin lyses protozoan membranes (Gould *et al*. 1971) and inhibits starfish oocyte maturation at a concentration of 3 μM (Toraya *et al*. 1995). It also shows insecticidal activity against the fruit fly *Drosophila melanogaster* (Assie *et al*. 2002). Inactivation of enveloped viruses such as vesicular stomatitis virus (VSV), simian foamy virus (SFV), and suid herpesvirus 1 (SHV-1) by surfactin depends on its hydrophobicity: the C14 and C15 isoforms were more antiviral than C13 (Kracht *et al*. 1999). Surfactin is well qualified to maintain virus and mycoplasma safety in biotechnological products.
The presence of cholesterol in the phospholipid layer attenuates the destabilizing effect of surfactins (Carrillo *et al*. 2003), which suggests that the susceptibility of biological membranes may vary in a specific manner depending on the sterol content of the target organisms. This could explain why

surfactins display hemolytic, antiviral, antimycoplasma and antibacterial activities but intriguingly, no marked fungitoxicity (Ongena and Jaques 2008).

When it is associated with the antifungal iturin A, another lipopeptide co-produced by *B. subtilis*, surfactin has strong synergistic effects (Grangemard *et al*. 1997).

Surfactin-producing *B. subtilis* strains have high swarming motility and biofilm formation, whereas surfactin non-producing strains did not swarm or form biofilm (Connelly *et al*. 2004). Surfactin promotes bacterial cell motion by lowering the surface tension (Kinsinger *et al*. 2003; Mukherjee and Das, 2005). It does not induce biofilm formation as a surfactant but rather as a signaling molecule. Surfactin might act as an 'autoinducer' or 'quorum-sensing' signal made by *B. subtilis* under certain conditions that might regulate the expression of genes involved in biofilm formation (Lopez *et al*. 2008). Harsh *et al*. (2004) observed that *B. subtilis* 6051 forms a stable, extensive biofilm and secretes surfactin, which acts to protect plants against attack by pathogenic bacteria.

Moreover, surfactin have been found to inhibit the adhesion of pathogenic organisms to solid surfaces or the infection sites. *B. licheniformis* strain 603 produced a lipopeptide that prevents adhesion of cells to a glass surface at the concentration of 1.6 $\mu g\ ml^{-1}$. It exhibited a considerable growth-inhibiting activity against *Corynebacterium variabilis* and a much lower activity against *Acinetobacter sp.* (Batrakov *et al*. 2003). Surfactin also decreased the amount of biofilm formed by *Salmonella typhimurium*, *Salmonella enterica*, *Eschericha coli* and *Proteus mirabilis* in polyvinyl chloride wells, as well as vinyl urethral catheters (Seydlova and Svobodova 2008).

- **Effective agents on growth and production**

Different culture media are used for growth and production which contain carbon and nitrogen. Carbon source usually is a carbohydrate such as glucose. Sucrose and fructose have also been mentioned as efficient carbon sources while the presence of glycerol greatly decreased surfactin production (Peypoux *et al*. 1999). Amino acids, ammonium sulphate and yeast extract can be used as nitrogen source. The presence of salts is of importance too.

The common media used include Cooper medium (Cooper *et al*. 1981) and Landy medium (Landy *et al*. 1948). The earlier studies carried out in nutrient broth gave a very low yield of 0.1 $g\ l^{-1}$ (Arima *et al*. 1968). In subsequent studies, a minimal mineral salts medium, containing NH_4NO_3 (0.05 M) as the inorganic nitrogen source and glucose (4%) as the carbon source, was defined by Cooper (Cooper's medium). The replacement of Cooper's nitrogen source and the introduction of O_2

limitation, which redirects the energy flux into product synthesis, have led to a productivity of 7 g l^{-1}, about 10-fold higher than Cooper's basal yield (Kim *et al*. 1997). Some studies were performed for optimization of culture media too (Jacques *et al*. 1999).

The most important parameters for growth and production are temperature and agitation. The most common used temperatures are 30°C and 37°C.

1.1.5.2.2. Lichenysin

B. licheniformis is a Gram-positive endospore-forming organism that can be isolated from soils and plant material all over the world (Sneath *et al*. 1986). This species is closely related to the well studied model organism *B. subtilis* (Table 1.4).

Recent taxonomic studies also indicate that *B. licheniformis* is closely related to *B. subtilis* (*B. licheniformis* shares 872 genes with *B. subtilis*) (Table 1.4) and *Bacillus amyloliquefaciens* on the basis of comparisons of 16S rDNA and 16S-23S internal transcribed spacer (ITS) nuleotidic sequences (Xu, 2003).

Lichenysins are surface-active lipopeptides with antibiotic properties produced non-ribosomally by different strains of *B. licheniformis.* Lichenysin A, produced by *B. licheniformis* strains ATCC 10716, BAS50 and BNP29, is a cyclic lipoheptapeptide characterized as one of the highest biosurfactant activities reported (Yakimov *et al*. 1995), while it is produced in much lower amounts than surfactin (Yakimov *et al*. 1996).

Table 1.4. Comparison of genome of *B. licheniformis* and *B. subtilis*

	***B. licheniformis* chromosome**	***B. subtilis* chromosome**
Size (bp)	4,222,748	4,214,810
Number of genes	4,286	4,112
% coding	87.9	87.0
% G+C	46.2	43.5
rRNA operons	7	10
tRNA genes	72	86

- **Structure**

Lichenysin A, as it was proposed before by NMR analysis and FAB-MS/MS analysis, has seven amino acid residues in its peptide moiety with the following sequence: Gln-Leu-Leu-Val-Asp-Leu-Ile. It is a mixture of isomeric and homologous compounds differing by the lipid parts representing by linear and branched L-hydroxy fatty acids ranging in size from C12 to C17 (Yakimov *et al.* 1999). It has the chiral sequence LLDLLDL. Yakimov (1995) initially proposed Gln (1) and Asn (5) for lichenysin structure, but in his next experiments in 2000, he confirmed Gln and Asp for lichenysin.

In the positive FAB ionization MS mode, the most abundant molecular ions [M+H] were detected at m/z 1035, 1021 and 1007. The negative FAB ionization MS of native lichenysin A showed deprotonated molecular ions at m/z 1061, 1047, 1033, 1019, 1005 and 991 with m/z 1033, 1019 and 1005 being the major ions. The main L-hydroxy fatty acid residues in the lipophilic part of lichenysin A molecules was shown to be C13, C14 and C15 acids (Yakimov *et al.* 1999).

Lichenysin A differs structurally from surfactin in three aspects, namely qualitatively, in two constituent amino acids (it has glutamine instead of glutamate as the first residue, and isoleucine instead of leucine as the last), and quantitatively, in the composition of its lipid substituents. It is likely that the Glu/Gln difference is the most relevant to the difference in activities of these two lipopeptides. This local variation causes significant changes in the properties of the molecules. In other words, the less polar peptide moiety and the presence of a longer β-hydroxy fatty acid in the lichenysin A molecules appear to have an important influence on the surface activity of this lipopeptide. This may be due to the delicate hydrophile-lipophile balance.

- **Physico-chemical properties**

Grangemard *et al.* (2001) showed that lichenysin has a higher surfactant power than surfactin, CMC being strongly reduced from 220 to 22 μM (in the presence of 5 mM Tris) and a much higher hemolytic activity because 100% hemolysis was observed with only 15 μM instead of 200 μM. Lichenysin is also a better chelating agent than surfactin because its association constants with Ca^{2+} and Mg^{2+} are increased by a factor of 4 and 16, respectively. This effect is assigned to an increase in the accessibility of the carboxyl group to cations owing to a change in the side chain topology induced by the glutamate/glutamine exchange. Data support the formation of a lichenysin-Ca^{2+} complex in a molar ratio of 2:1 instead of 1:1 with surfactin, suggesting an

intermolecular salt bridge between two lichenysin molecules. When Ca^{2+} ions are present in the solution, micellization occurs via a dimer assembly, with a possible long-range effect on the spatial arrangement of the micelles or other supermolecular structures (Grangemard *et al*. 2001). It is a heat stable lipopeptide (100˚C, 20 min) and is also stable against pronase (200 µg ml^{-1}, 3 h), HCl (pH 2, 30 min) and NaOH (pH 12, 30 min).

Purified lichenysin A decreases the ST of water from 72 to 28 mN m^{-1}. It also shows a lowering of ST at high NaCl concentrations up to 30% (w/v). The very low CMC 12 mg l^{-1} shows that lichenysin A is effective at dilute concentrations. Fractions of lichenysin A with branched β-OH acids in the lipid tail demonstrated lower ST activity than the fractions of lichenysin A having straight β-OH acids (Yakimov *et al*. 1996).

- **Biological activity**

The antibacterial activity of lichenysin A has been demonstrated against some bacteria. The lipopeptide lichenysin A inhibited the growth of most of the bacteria tested on nutrient agar plates, but this inhibition was less than that observed with surfactin (Yakimov *et al*. 1995).

To clarify the role of the polar groups on the antimicrobial activity, lichenysin A was treated with alkali to open the lactone linkage and derivatized with diazomethane. The open form of lichenysin A had approximately the same antimicrobial activity as the closed form, but when the free polar groups were esterified, the activity disappeared (Yakimov *et al*. 1995).

- **Effective agents on growth and production**

In contrast to the lipopeptide surfactin, lichenysins seem to be synthesized during growth under aerobic and anaerobic conditions (Konz *et al*. 1999). It is well known that the production of most lipopeptides is dependent on the composition of the culture medium (Yakimov *et al*. 1995) and the nutrient conditions can affect the composition of the product peptide (Konz *et al*. 1999).

Addition of branched-chain α-amino acids to the medium caused similar changes to both cellular fatty acid and to β-OH fatty acid composition in the lipophilic part of lichenysin A. Production of lichenysin A was enhanced about 2 and 4-fold by addition of L-glutamic acid and L- asparagine, respectively. It is suggested that these amino acids may be involved in the control of lipopeptide formation.

The strain BAS50, a lichenysin A producer, grew in medium with a range of 0 to 13% (w/v) NaCl, at temperatures of 25 to 55˚C and pHs of 5.4 to 8.5. During the different growth phases, the ST of the anaerobic cultures was similar to that of aerobic cultures, but its minimum value was 35 mN m^{-1}. Optimal growth and surfactant production under anaerobic conditions occurred in Cooper's medium with 5% NaCl at 40 to 45˚C.

Glucose and sucrose but not fructose and maltose supported the best surfactant production. Increasing the glucose or sucrose concentration above 2% (w/v) does not affect surfactant production. Solutions of molasses with concentrations of up to 4% (v/v) in Cooper's medium supplemented with 0.1% $NaNo_3$, 0.05% yeast extract supported the growth of BAS50 and surfactant production at temperatures of up to 50°C.

1.1.5.3. Iturin family

Iturins are produced by *B. subtilis* and other closely related *Bacilli*, e.g. *B. amyloliquefaciens* (Souto *et al*. 2004). The iturin group comprises iturins A–E, bacillomycins D, F, and L, and mycosubtilin (Stein, 2005). Iturin A, the best known member, was isolated from *B. subtilis*, strain taken from the soil in Ituri (Zaire) during the year 1957 (Delcambe and Devignat 1957). They contain a cyclic heptapeptide acylated with β-amino fatty acids and the constant chiral sequence LDDLLDL (Figures 1.2 and 1.4). They are neutral or monoanionic lipopeptides and contain a mixture of isomers ranging from 14 to 17 carbon atoms in the *n, iso* and *anteiso* configuration (Isogai *et al*. 1982). In different family members, the amino acid residues in the heptapeptides vary slightly (Figure 1.2).

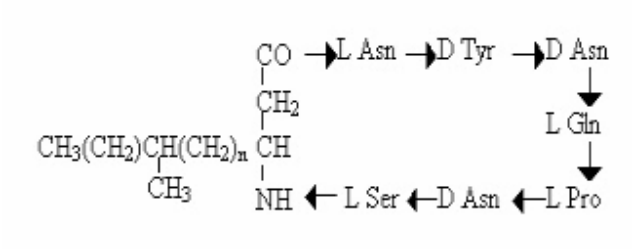


Figure 1.4. Iturin A structure.

Iturin A has CMC of 40 mg ml^{-1} (Maget-Dana *et al*. 1994) and reduces surface tension of water to 54 mN m^{-1}. Unlike surfactin, the presence of salts does not influence ST of iturin but decreases CMC. It has the ability to form foam and to stabilize it (Razafindralambo *et al*. 1998). The mixture

of surfactin and iturin in ratio of 2/3 exerts synergic effects (Razafindralambo *et al*. 1997b). It has been shown that, in addition to the monomer or micellar organizations, at higher concentrations than CMC, another type of aggregate different from the micelle is preferred, probably a lamellar vesicle, whose proportion increases as the concentration of iturin is raised. For iturin A micelles, an aggregation number of 7 has been proposed (Grau *et al*. 2001).

Iturin A retained 100% biological activity after heating for 30 min at 100 ºC and 60% after autoclaving for 20 min at 120 ºC as measured with the fungal inhibition test (Yu *et al*. 2002). Biological effects of the iturin family are due to their capability of forming ion-conducting pores. These molecules disrupt the yeast plasma membrane by forming small vesicles and by aggregating membrane-spanning particles. It has been shown that the different biological activities of iturins, especially their antifungal property, depend on both the lipid tail and peptide ring, with a key role played by the D-Tyr residue in the peptide backbone which needs to have a free hydroxyl group for an optimal interaction with the target cells and forming pores (Bonmatin *et al* .2003). This residue was found to be essential for functional activity while the impact of the other residues was less important (Besson *et al*. 1979). Iturin-induced pores show a slight selectivity for anions over cations.

As far as the lipid moiety is concerned, the iturinic acids with 16 and 17 carbon atoms are assumed to be the best fitting for hydrophobic interactions with the ergostrol and phospholipid chains, since the lipopeptides containing a majority of these acids are the most active (Bonmatin *et al*. 2003; Toure *et al*. 2004; Fickers *et al*. 2009).

Their fungitoxicity increases with the number of carbon atoms in the fatty acid moiety, i.e., the C17 homologues are 20-fold more active than the C14 forms (Leclere *et al*. 2005). Intensity of production of homologue compounds depends on the strain and the culture medium (Akpa *et al*. 2001).

Iturin A and bacillomycin L provoked hemolysis and released K^+ from erythrocytes (Aranda *et al*. 2005). Iturin A induced morphological changes in human erythrocytes (Thimon *et al*. 1994). Mycosubtilin altered the permeability of the plasma membrane, releasing nucleotides, proteins, and lipids from yeast cells (Besson and Michel 1989) and lysing erythrocytes (Besson *et al*. 1989). Mycosubtilin formed pores in dimyristoylphosphatidylcholine (DMPC) membranes by interacting with the phospholipids, forming a complex with cholesterol; thereby stabilizing the ion pore (Maget-

Dana and Ptak 1990). The lipopeptides of the iturin family are more active in membranes containing cholesterol, such as mammalian cells, than in the ergosterol-containing fungal cells. However, the underlying mechanism is based on osmotic perturbation due to the formation of ion-conducting pores and not membrane disruption or solubilization as caused by surfactins (Aranda *et al*. 2005). In this respect, it should also be noted to its concentration and self-association property (Bonmatin *et al* . 2003), although the self-associated process has not been understood at the molecular level.

Members of this family exhibit a rather limited antibacterial activity (Maget-Dana and Peypoux, 1994) restricted to *Micrococcus luteus* (Besson *et al*. 1978) and no antiviral activities (Yu *et al*. 2002; Hiradate *et al*. 2002). They also display a strong *in vitro* antifungal action against a large variety of yeast and fungi. Overproduction of mycosubtilin by the recombinant *B. subtilis* strain BBG100, had significant antagonistic properties against phytopathogenic fungi, *Botrytis cinerea*, *Fusarium oxysporum* and *Pythium aphanidermatum*, and yeasts, *Pichia pastoris* and *Saccharomyces cerevisiae* (Leclere *et al*. 2005). This strain is a derivative of *B. subtilis* ATCC 6633 and has a 15-fold higher mycosubtilin production rate than the parental strain. Besides the antifungal activities, mycosubtilin is also involved in *Bacillus* spreading. Leclere *et al*. (2006) demonstrated that overproduction of mycosubtilin is directly related to an enhanced invasive behaviour. Addition of the purified lipopeptide to the medium caused the enhancement of swarming motility of *B. subtilis* 168, which is known as a non-spreading strain (Julkowska *et al*. 2004). The role of mycosubtilin in this process is based on an increase of the wettability and a decrease of the surface tension of the medium. Numerous studies have shown the potential of the iturin family as alternative antifungal agents. Isomers of iturin A purified from culture broth were responsible for inhibition of *Rhizotecnia solani* growth *in vitro* (Yu *et al.* 2002). Moreover, Souto *et al.* (2004) indicated that those excreted secondary metabolites efficiently inhibited mycelia growth of *Fusarium oxysporum, Rhizoctonia solani, Fusarium solani* and *Sclerotinia sclerotiorum.*

1.1.5.4. Fengycin family

The third family of LPs comprises fengycins A and B, which are also called plipastatins (Figure 1.5). These molecules are lipodecapeptides with an internal lactone ring in the peptidic moiety and with a β-hydroxy fatty acid chain (C_{14} to C_{18}) that can be saturated or unsaturated. The structure of fengycin A contains D-Ala6 instead of the D-Val6 of fengycin B. Fengycins have

stereoisomeric composition different from those of plipastatins. Fengycins contain D-Tyr3 instead of the L-Tyr3 of plipastatins and L-Tyr9 instead of the D-Tyr9 of plipastatins (Volpon *et al*. 2000). Fengycin-producing strains were identified in *B. cereus* and *B. thuringiensis* in addition to *B. subtilis* and *B. amyloliquefaciens*.

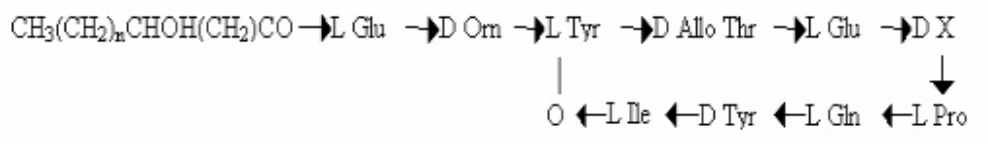


Figure 1.5. Plipastatin structure. X=Ala or Val in plipastatin A and B, respectively.

Hathout *et al*. (2000) reported from *B. thuringiensis kurstaki* HD-1 an antifungal compound structurally resembling plipastatins and fengycins. Fengycins and plipastatins inhibit phospholipase A2, an enzyme affecting inflammation, acute hypertensions, and blood platelet aggregation (Volpon *et al*. 2000). *B. thuringiensis* strain CMB26 produced an analogue of fengycin with a double bond in the fatty acid. It was fungicidal, bactericidal, and insecticidal, and was more effective against fungi than iturin or surfactin (Kim *et al*. 2004).

In low molar ratios from 0.1 to 0.5 of fengycin/dipalmitoyl-phosphatidylcholine (DPPC) membrane, fengycin forms pores and at a ratio of > 0.66, it acts as a detergent that solubilizes membrane (Deleu *et al*. 2005). Fengycins are less hemolytic than iturins and surfactins (40-fold less than it) but retain a strong fungitoxic activity more specifically against filamentous fungi (Vanittanakom *et al*. 1986; Hofemeister *et al*. 2004; Koumoutsi *et al*. 2004). Mechanistically, the action of fengycins is less well known compared to other lipopeptides but they also readily interact with lipid layers and somewhat retain the potential to alter cell membrane structure (packing) and permeability in a dose-dependent way (Deleu *et al*. 2005). Deleu *et al*. 2008 recently reported that the mechanism of fengycin action is probably based on a two-state transition controlled by the lipopeptide concentration. One state is the monomeric, not deeply anchored and nonperturbing lipopeptide, and the other state is a buried, aggregated form, which is responsible for membrane leakage and bioactivity. The molecular mechanism underlying this membrane perturbation is not yet fully understood.

Lipopeptides act in a synergistic manner as suggested by several studies on surfactin and iturin (Maget-Dana *et al*. 1992), surfactin and fengycin (Ongena *et al*. 2007) and iturin and fengycin (Koumoutsi *et al*. 2004; Romero *et al*. 2007).

1.1.5.5. Biosynthesis of lipopeptides in *Bacillus*

Lipopeptides from *B. subtilis* are synthesized by non-ribosomal peptide synthetases (NRPS) or hybrid polyketide synthases/non-ribosomal peptide synthetases (PKS/NRPS) (Figure 1.6). These modular proteins are responsible for the biosynthesis of several hundred bioactive compounds (Figure 1.7). They are megaenzymes organized in interactive functional units called modules that catalyze the different reactions leading to polyketide or peptide transformation. Each module is subdivided in several catalytic domains responsible for each biochemical reaction. A typical NRPS module usually comprises about 1000 amino acid residues and is responsible for one reaction cycle of selective substrate recognition and activation as an adenylate (A-domain), tethering of a covalent intermediate as an enzyme-bound thioester (PCP-domain), and peptide bond formation (C-domain) (Fiking, 2004). The basic set of domains within a module can be extended by substrate-modifying domains, including domains for substrate epimerization (E-domain), hydroxylation, methylation, and heterocyclic ring formation, which are either inserted at specific locations into the module or act in *trans* as independent catalytic units. A thioesterase domain (Te-domain) is usually present in the last module to ensure the cleavage of thioester bond between the nascent peptide and the last PCP-domain. In several cases, this thioesterase is responsible for the cyclisation of the peptide. Three large open reading frames coding for surfactin synthetases are designated *srfA-A, srfA-B* and *srfA-C* (Peypoux *et al*. 1995) (Figures 1.6 and 1.8). They present a linear array of seven modules (one module per residue), three modules are present in the products of both *srfA-A* and *srfA-B* and the last one in *srfA-C*. The fatty acid chain is added to the amino acid activated in the first module. A first thioesterase fused with the carboxy-terminal end of the last activation PCP domain is responsible for the release of the synthesized product from the enzymatic template. A second thioesterase/acyltransferase (Te/At-domain) encoded by a fourth gene, *srfA-D* stimulates the initiation of the biosynthesis (Steller *et al*. 2004).

Lichenysin A synthetase has modular structure like surfactin. The *lchAA* gene product (LchAA) contains three modules, with a C-terminal epimerization domain attached to the third; *lchAB* encodes LchAB, and has similar structure to LchAA; *lchAC* encodes LchAC, one module with an

additional carboxy-terminal putative thioesterase domain (Grangemard *et al*. 2001). The third and sixth modules of LchAA and LchAB, respectively, contain at their C-terminal ends motifs found in all synthetases producing D-amino acid containing peptides and known as epimerization domains.

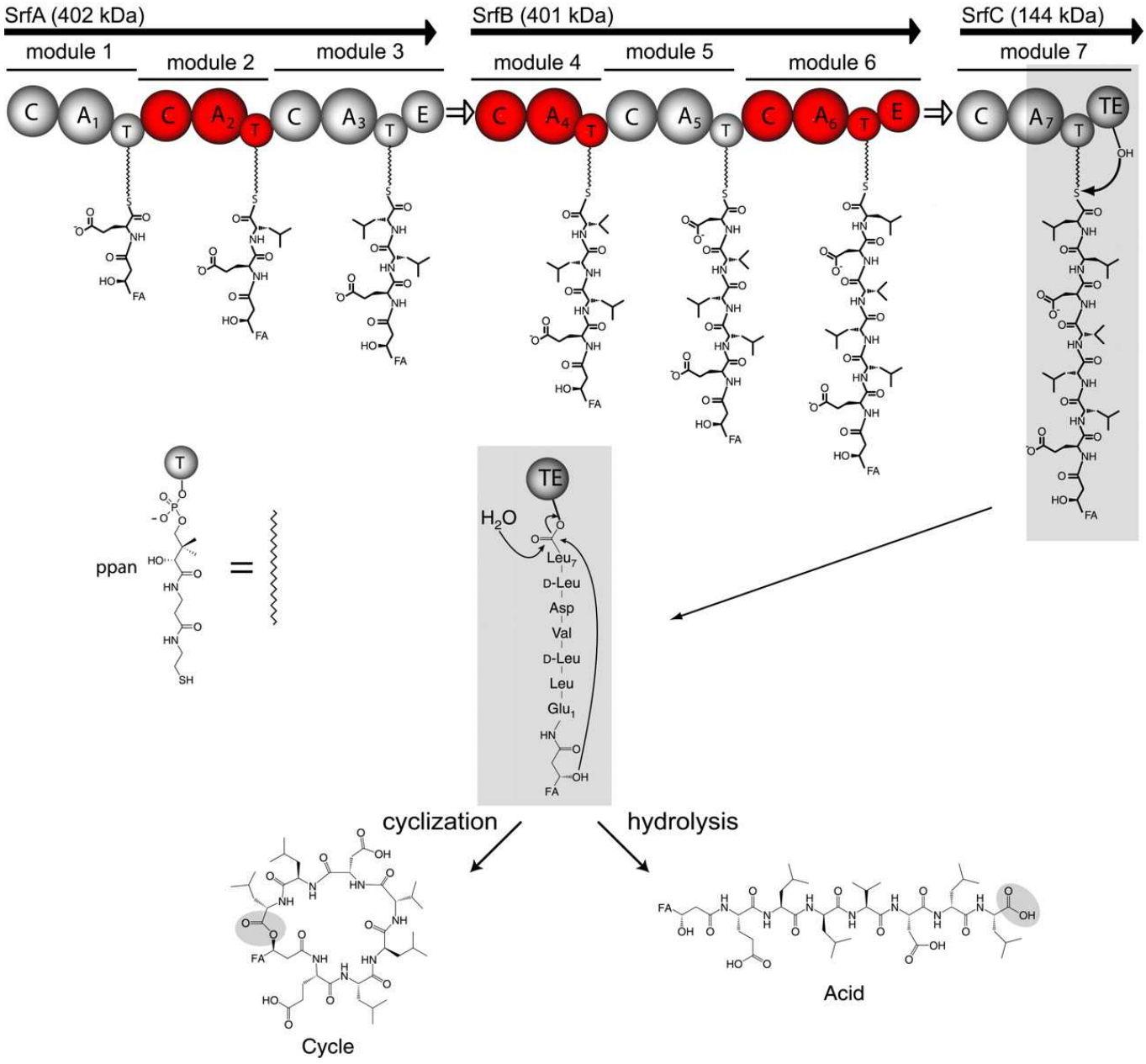


Figure 1.6. The synthesis of surfactin by NRPS. A: adenylation, T: thiolation, C: condensation, Ppan: phosphopantethein (Sieber and Marahiel, 2003).

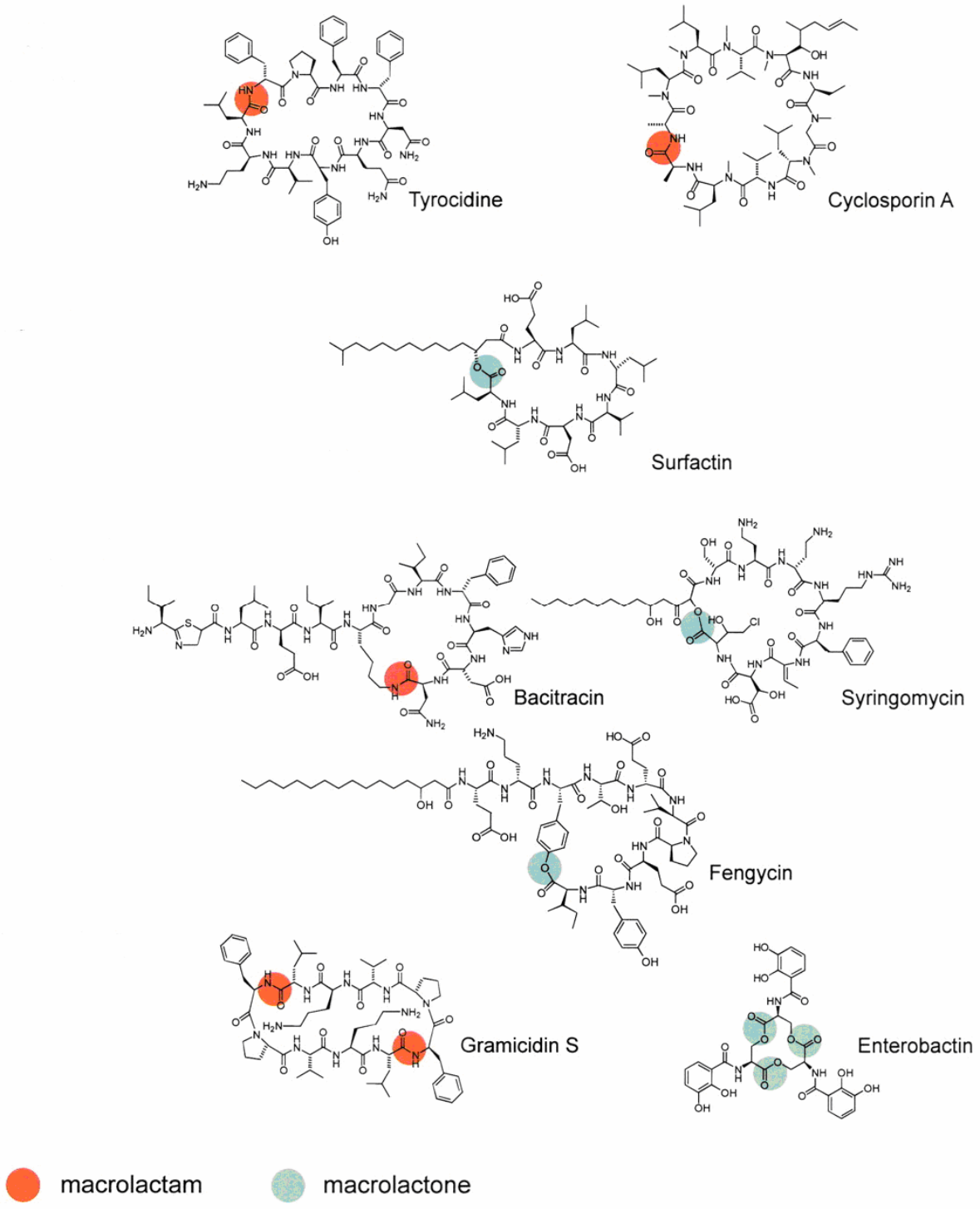


Figure 1.7. Peptides structure synthesized by NRPS direction (Sieber and Marahiel, 2003).

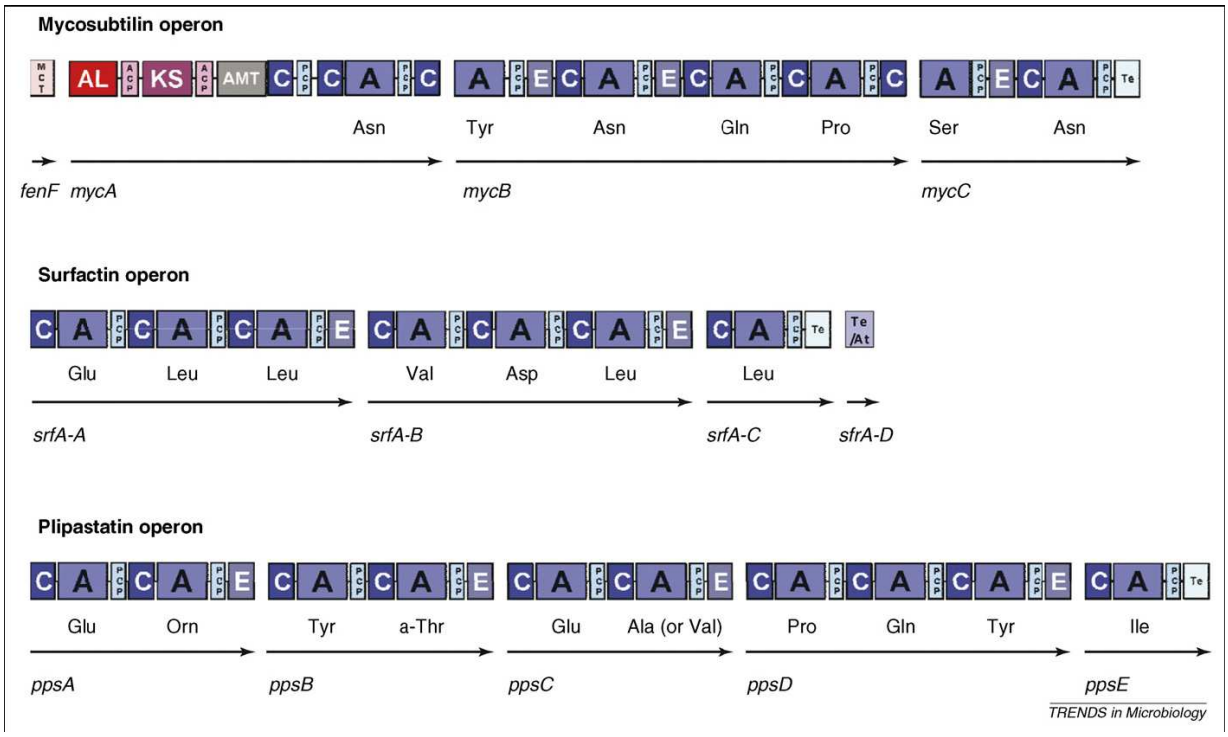


Figure 1.8. Operons of mycosubtilin, surfactin and plipastatin synthetases. Schematic representation of operons (ORFs, domains of NRPSs or PKSs and amino acid incorporated by the different modules) encoding catalytic machinery responsible for the biosynthesis of representative members of each family of lipopeptides produced by *B. subtilis*: mycosubtilin for the iturin family, plipastatin for the fengycin family and surfactin (Ongena *et al*. 2007).

Similarly, fengycin or plipastatin are synthesized by NRPSs encoded by an operon with five open reading frames *fenA-E* (or *ppsA-E*) (Steller *et al*. 1999). The first three enzymes contain two modules, the fourth contains three modules and the last enzyme consists of one module. Contrary to surfactin and fengycin, iturin derivatives are synthesized by a PKS–NRPS hybrid complex (Tsuge *et al*. 1999; Moyne *et al*. 2004). The operon consists of four ORFs called *fenF*, *mycA*, *mycB* and *mycC* or *ituD*, *ituA*, *ituB* and *ituC* for mycosubtilin or iturin respectively. The last three genes encodes the NRPSs which are responsible for the incorporation of the first residue for *mycA* (or *ituA*), the following four residues for *mycB* (or *ituB*) and the two last residues for *mycC* (or *ituC*). The difference between structures of iturin A and mycosubtilin in which the last amino acids are inverted can be explained by an intragenic domain change in *mycC* and *ituC*. *FenF* (*ituD*) encodes a malonyl-CoA transacylase (MCT-domain) and the *mycA* also contains genes related to polyketide synthases. These genes are responsible for the last steps of the biosynthesis of the fatty acid chain (last elongation and β-amination) before its transfer to the first amino acid of the peptidic moiety (acyl-

CoA ligase (AL-domain), acyl carrier protein (ACP-domain), β-keto acyl synthetase (KS-domain), amino transferase (AMT domain)) (Aron *et al*. 2005).

NRPSs are easily accessible to genetic manipulations, providing powerful tools for generation of novel antibiotics with new properties (Sieber and Marahiel, 2003).

1.1.6. Biosurfactants produced by *Pseudomonas aeruginosa*: rhamnolipids

1.1.6.1. Introduction

Bacteria of the *Pseudomonas* genus are known to produce glycolipid-type surfactants (rhamnolipids). They are among the most extensively studied biosurfactants. They are secondary metabolites and composed of one or two units of rhamnose linked to one or two fatty acid chains with lengths of 8, 10, 12 and 14 carbons, as well as of 12- or 14-carbons with a single double bond (Wang *et al*. 2007). The different types vary in the number of sugar groups per molecule and the length of the lipid chain (Figure 1.9).

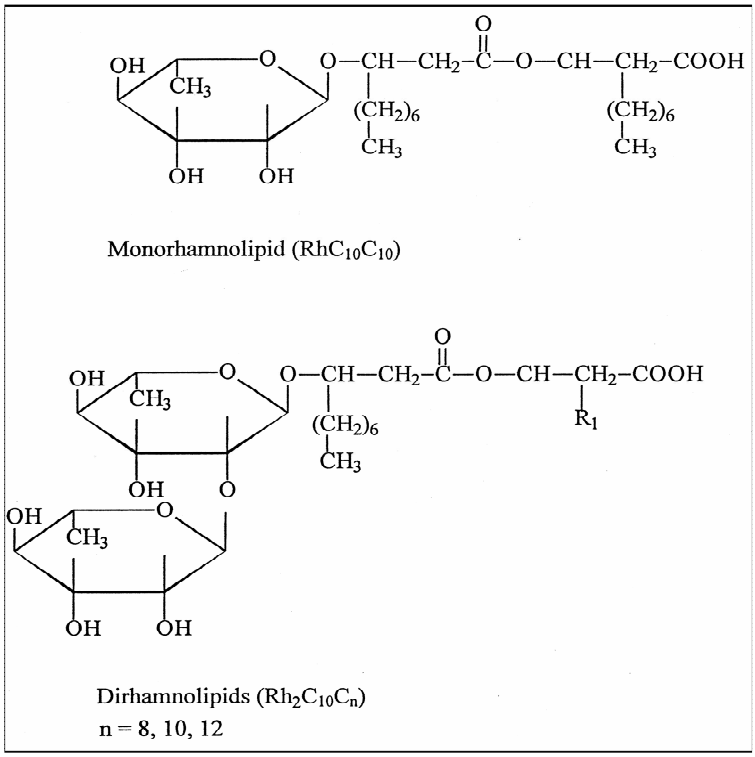


Figure 1.9. Structure of rhamnolipids produced by *P. aeruginosa*.

The type of rhamnolipid produced depends on the bacterial strain, the carbon source used, and the process strategy (Robert *et al*. 1989; Mulligan and Gibbs, 1993). Rhamnolipid accumulation in the supernatant starts at the end of the logarithmic phase, since rhamnolipids are secondary metabolites. The exact physiological role of rhamnolipids is still undetermined.

The various combinations of these groups generate a large number of possible rhamnolipid congeners. Up to 28 different structural homologues are currently known (Deziel *et al*. 1999; Mata-Sandoval *et al*. 1999). The two main rhamnolipids produced by *P. aeruginosa* in liquid cultures are L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (Rh-C_{10}-C_{10}) and L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (Rh-Rh-C_{10}-C_{10}) which have quite different physico-chemical properties (Benincasa *et al*. 2004). In recent years, some publications have reported that Rh-C_{10}-C_{10} and Rh-Rh-C_{10}-C_{10} are in fact produced as part of a complex mixture of rhamnolipids.

1.1.6.2. Physico-chemical properties

These anionic compounds are soluble in water and water/alcohol solutions between pH 6.5 - 7.5. They are powerful natural emulsifiers capable of reducing the surface tension of water from roughly 76 mN m^{-1} to 25 to 30 mN m^{-1}. They reduce the interfacial tension of water/oil systems from 43 to values below 1 mN m^{-1} too.

Rhamnolipids have an excellent emulsifying power with a variety of hydrocarbons and vegetable oils (Abalos *et al*. 2001). This biosurfactant activity of rhamnolipids makes them excellent candidates for assisting in the breakdown and removal of oil spills. They also possess high emulsifying activity (Van Dyke *et al*. 1993; Mata-Sandoval *et al*. 1999).

The hydrophilic alpha-L-rhamnose sugar combined to the hydrophobic tail gives the molecules soap-like properties. While an extra rhamnose ring confers more hydrophilicity to rhamnolipids (monorhamnolipids vs. dirhamnolipids), additional carbons in the fatty acid chains can increase their hydrophobicity. These properties can affect the stability of rhamnolipids in the aqueous phase (as monomers or micellar conglomerates), their capability to solubilize hydrophobic organic compounds, and the bioavailability of such compounds (Mata-Sandoval *et al*. 1999). More hydrophilic rhamnolipids like Rh-C_{10} or Rh_Rh-C_{10} yielded CMC as high as 200 mg l^{-1} whereas lower values of 5-60 mg l^{-1} have been reported for mixtures containing mainly monorhamnolipid

Rh-C_{10}-C_{10} (Syldatk *et al*. 1987; Dyke *et al*. 1993; Thangamashi *et al*. 1993). The dirhamnolipid Rh_2-C_{10}-C_{10} shows intermediate CMC values of 40-65 mg l^{-1} (Syldatk *et al*. 1987; Zang *et al*. 1992; Thangamani *et al*. 1994).

1.1.6.3. Identification methods

The methods used for the isolation and chemical analysis of rhamnolipids in the few studies all involved an initial chromatographic separation of the mixtures into various fractions by thin-layer chromatography (TLC), often followed by high-performance liquid chromatography (HPLC). Although these methods give excellent information on the structure of the different isolated rhamnolipids, they are of little help in the study of the complete profile of the mixtures, as some congeners may be lost throughout the various purification steps (Deziel *et al*. 1999).

HPLC coupled with mass spectrometry currently presents the most precise method for rhamnolipids identification and quantification (Deziel *et al*. 2000; Heyd *et al*. 2008).

1.1.6.4. Effective agents on growth and production

Production of rhamnolipids occurs during the stationary phase of growth. Some experiments demonstrated that it is controlled by quorum sensing (Ochsner and Reiser, 1995; Pearson, 1997).

One of the advantages of rhamnolipids over other biosurfactants is their ease of isolation from the culture (they are exo-biosurfactants), and the fact that they can be produced in high yield using relatively cheap carbon sources such as hydrocarbons (C11 and C12 alkanes: Robert *et al*. 1989), vegetable oils, or even wastes from food industry (Mata-Sandoval *et al*. 1999) or agriculture (Wang *et al*. 2007). Concerning the carbon source, Syldatk et *al*. (1985) demonstrated that although different carbon sources in the medium affected the composition of biosurfactant production in *Pseudomonas* spp., substrates with different chain lengths exhibited no effect on the chain lengths of fatty acid moieties in rhamnolipids.

It was noted that in addition to carbon sources, pH and age of the culture affects the yield of rhamnolipid production.

Dependence of *Pseudomonas* spp. biosurfactant production on nutritional and environmental factors has been extensively studied by Guerra-Santos *et al*. (1986), Syldatk and Wagner (1987), and Robert *et al*. (1989). Syldatk *et al*. (1985) found that the addition of a nitrogen source causes inhibition of

rhamnolipid synthesis in resting cells of *Pseudomonas* sp. strain DSM-2874. The limitation of multivalent cations also causes overproduction of them. Guerra-Santos *et al.* (1986) demonstrated that by limiting the concentrations of salts of magnesium, calcium, potassium, sodium and trace elements, a higher yield of rhamnolipid can be achieved in *P. aeruginosa* DSM 2695. Iron limitation stimulated biosurfactant production in *P. fluorescens* (Pearson *et al*. 1990a and 1990b) and *P. aeruginosa* (Guerra-Santos *et al*. 1984 and 1986).

1.1.6.5. Antimicrobial activity

Rhamnolipids also demonstrate antibacterial and antifungal activities, suggesting possible roles in the medical and agricultural fields. For example, members of the *Pseudomonas chlororaphis* species were used as biocontrol strains and sprayed directly onto plant seeds to protect the seeds against fungal pathogens (Tombolini *et al*. 1999). This antimicrobial activity was shown against *B. subtilis*, *Staphylococcus aureus, Proteus vulgaris and Enterococcus faecalis*, and against some phytopathogenic fungal species such as *Penicillium* spp., *Alternaria* spp., *Gliocadium virens* and *Chaetonium globosum* (Stranghellini *et al*. 1997; Benincasa *et al*. 2004).

1.1.6.6. Biosynthesis

The synthesis of rhamnolipids proceeds by sequential glycosyl transfer reactions, each catalyzed by a specific rhamnosyltransferase (Burger *et al*. 1963) with TDP-rhamnose acting as a rhamnosyl donor and 3-(3-hydroxyalkanoyloxy) alkanoate acting as acceptor (Maier and Soberon-Chavez, 2000). Rhamnosyltransferase 1 is encoded by the *rhl*A and *rhl*B genes, which are organized in an operon and responsible for biosynthesis of mono-rhamnolipid. The active enzyme complex is located in the cytoplasmic membrane, with the RhlA protein being localized in the periplasm and the catalytically active RhlB component crossing the membrane (Ochsner *et al*. 1994). Rhamnosyltransferase 2 is encoded by the *rhl*C gene that is located in another operon with an upstream unknown gene (PA1131) in *P. aeruginosa* PAO1, and not organized with RhlAB (Rahim *et al*. 2001). RhlAB is the key enzyme complex in rhamnolipid biosynthesis, but this biosynthesis is modulated by the complicated transcriptional regulatory network in *P. aeruginosa* (Soberon-Chavez and Aguirre-Ramirez, 2005).

1.1.6.7. Applications

Lindhardt *et al*. (1989) have suggested using rhamnolipids as a source of L-rhamnose for scientific and industrial purposes.

Rhamnolipids can further be used to improve the properties of butter cream, decoration cream and/or non-dairy cream filling for Danish pastries, croissants and other fresh or frozen fine confectionery products (Benincasa *et al*. 2004) and have also applications in cosmetics, pharmaceuticals and detergent industry (Heyd *et al*. 2008), oil transportation and recovery.

Rhamnolipids produced by *P*. *aeruginosa* strains are among the most effective surfactants when applied for the removal of hydrophobic compounds from contaminated soils (bioremediation).

They can enhance growth of microorganisms on alkanes. Trevors *et al*. (1991) showed *P*. *aeruginosa* UG2 biosurfactants enhanced biodegradation of some hydrocarbons in soil.

1.1.6.8. Colorimetric methods for concentration determination

Colour reactions are generally performed by binding a dye to the rhamnolipid (e.g. cetyltrimethylammonium bromide (CTAB) agar test) or by reaction of the rhamnose moiety with a coloured chemical compound (e.g. anthrone method and orcinol test), which can be quantified afterwards by photometry. These assays are still applied most frequently in rhamnolipid analysis. One of the main disadvantages of the indirect and colorimetric methods described below is the ignorance of sample composition and, hence, the occurrence of various rhamnolipid species.

- **CTAB agar test**

This semi-quantitative agar plate cultivation test is based on the formation of an insoluble ion pair of anionic surfactants with the cationic surfactant CTAB and the basic dye methylene blue (Siegmund *et al*. 1991). As the constitution of the agar medium, containing 0.2 g l^{-1} CTAB and 0.005 g l^{-1} methylene blue, can be altered, this quick and simple test is well suited for medium optimisation and screening new anionic biosurfactant production strains or mutants (Perfumo *et al*. 2006). Rhamnolipids are detected as dark-blue halos around the colonies, with the spot diameter being dependent on rhamnolipid concentration. Nevertheless, care has to be taken in quantification, as the spot diameter is influenced by variable cell growth of the bacteria, cultivation time, migration of the rhamnolipids, and filling level of the agar plates.

- **Anthrone method**

This colorimetric test is based on the reaction of rhamnose in the presence of a strong acid with anthrone (9,10-dihydro-9-oxoanthracene), forming a dye by heating, which can be measured at 625 nm by a photometer against a calibration curve with rhamnose or rhamnolipid (Figure 1.10).

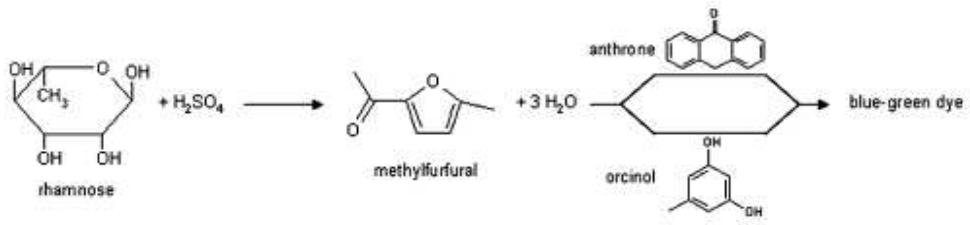


Figure 1.10. Reaction scheme of the anthrone method and orcinol assay

As it is a quick and simple assay that does not require any expensive instrumentation, it is often used just for the detection of rhamnolipids or for quantification of the total rhamnolipid content down to 20 mg l^{-1}. For this purpose, the composition of the rhamnolipid mixture has to be known. However, it generally changes during rhamnolipid production or in adsorption experiments (Noordman *et al*. 2000), which may render the method inacurate. Additionally, interferences of several solvents, inorganic salts (e.g. NaCl), carbonyl or oxidising compounds, and proteins with the reaction have been reported (Hodge *et al*. 1962). An advantage consists in the possibility of treating larger quantities of samples on the micro titre plate scale.

- **Orcinol assay**

In analogy to the anthrone method, this method modified by Chandrasekaran and BeMiller (1980) is based on using a dye for the quantification of the rhamnolipid content in a sample measuring the absorption at 421 nm (Figure 1.10). In this case, the rhamnose molecule of rhamnolipids reacts with sulphuric acid and orcinol (1,3-dihydroxy-5-methylbenzene) at high temperature (30 min at 80°C) to give a blue-green colour.

1.2. Techniques for study of surface properties

1.2.1. Contact angle measurement

Over the years, several methods to measure the hydrophobicity, including contact angle methods, microbial adhesion to hydrocarbon (MATH) and hydrophobic interaction chromatography have been used. Bunster *et al*. (1989) studied the surface activity of bacteria by measuring the contact angles obtained after adding the bacteria to a water drop. Contact angle can be measured by producing a drop of pure liquid on a solid. The contact angle is located at the interface between the droplet and the solid surface. In the absence of surfactant, water molecules on a hydrophobic surface adhere strongly to each other and so the water droplet retains a round appearance with a contact angle of more than 90°, while in the presence of biosurfactants, the adherence forces are reduced causing the droplet to spread out flat creating a contact angle of less than 90° (Figure 1.11).



Figure1.11. Contact angle (θ) measured by goniometer. Middle image shows a water droplet on Teflon and the right image shows the same after conditioning of surface with surfactin.

Goniometer (Figure 1.12) or contact angle goniometer is an instrument that is used to precisely measure static and dynamic contact angles of liquids on solids. The modern contact angle goniometer was invented by Dr William Albert Zisman at the Naval Research Laboratory in Washington DC and built by ramé-hart Co. in New Jersey. The current generation ramé-hart goniometer replaces the microscope with a digital camera and imaging software to collect and measure contact angle. Additionally, the new generation of instruments can calculate surface energy, surface tension as well as perform advancing and receding measurements and other more advanced tasks. Hydrophilicity occurs when a water drop forms with a small contact angle and wetting is nearly complete; surface energy is very high. If the water contact angle approaches 0°,

the material is said to be superhydrophilic. Strictly speaking a hydrophilic surface is one that attracts water. Hydrophobicity occurs when a water drop forms with a large contact angle. In this condition wetting is considered poor and surface energy is low.



Figure 1. 12. A digital goniometer (Digidrop, GBX Scientific Instruments, France, www.gbxinstru.com)

1.2.2. X-ray photoelectron spectroscopy (XPS)

1.2.2.1. Introduction

The first experimental XPS spectrometer was developed by Siegbahn and his team at the University of Uppsala (Sweden). He was the first to measure core levels of chemical shifts in 1957 and to use the electron spectroscopy for chemical shifts analysis (ESCA). In 1967, Kai Siegbahn published a comprehensive study on XPS bringing instant recognition of the utility of XPS. In cooperation with Siegbahn, Hewlett-Packard in the USA produced the first commercial monochromatic XPS instrument in 1969. Siegbahn received the Nobel Prize in 1981 to acknowledge his extensive efforts to develop XPS into a useful analytical tool.

X-ray photoelectron spectroscopy (XPS) is a quantitative spectroscopic technique that measures the elemental composition, empirical formula, chemical state of the elements that exist within a material (Rouxhet *et al*. 1991). It has indeed become a technique of major importance in the field of material science, owing to the importance of interfacial phenomena such as adsorption and adhesion. This includes the domain of biomaterials, the performances of which rely strongly upon the interactions between the surface and cells or biological fluids. It can be applied to systems with biological nature (microbial cells and food products) (Van der Mei *et al*. 2000;

Ahimou *et al*. 2007), but it requires particular precautions as the samples are exposed to high vacuum during the analysis. XPS spectra are obtained by irradiating a material with a beam (Figure 1.13) of aluminum or magnesium X-rays while simultaneously measuring the kinetic energy (KE) and number of electrons that escape from the top 1 to 10 nm of the material being analyzed.

XPS is a surface chemical analysis technique that can be used to analyze the surface chemistry of a material in its "as received" state, or after some treatment such as: conditioning, fracturing, cutting or scraping in air or ultra high vaccum (UHV) to expose the bulk chemistry, ion beam etching to clean off some of the surface contamination, exposure to heat to study the changes due to heating, exposure to reactive gases or solutions, exposure to ion beam implant and exposure to ultraviolet light.

A correlation has been found between the surface chemical composition and the physico-chemical properties of microorganisms (Rouxhet *et al*. 1994; Van der Mei *et al*. 2000). In a study, the increase of polysaccharide concentration at the surface of germinating fungal spores, revealed by XPS, was related to a change of surface morphology and an increase of adhesiveness (Dufrene *et al*. 1999). In other study, the surface chemical composition of strains of *B. subtilis* was determined by X-ray photoelectron spectroscopy, indicating that XPS data are relevant to the natural state of the cell surface. This includes relationships between surface chemical composition and adhesion or hydrophobicity of microorganisms (Ahimou *et al*. 2007).

Leone *et al*. (2006) analyzed chemical composition of the bacterial surface by XPS permiting to elucidate the presence of surface sites containing carboxylate, phosphate and amine functional groups.

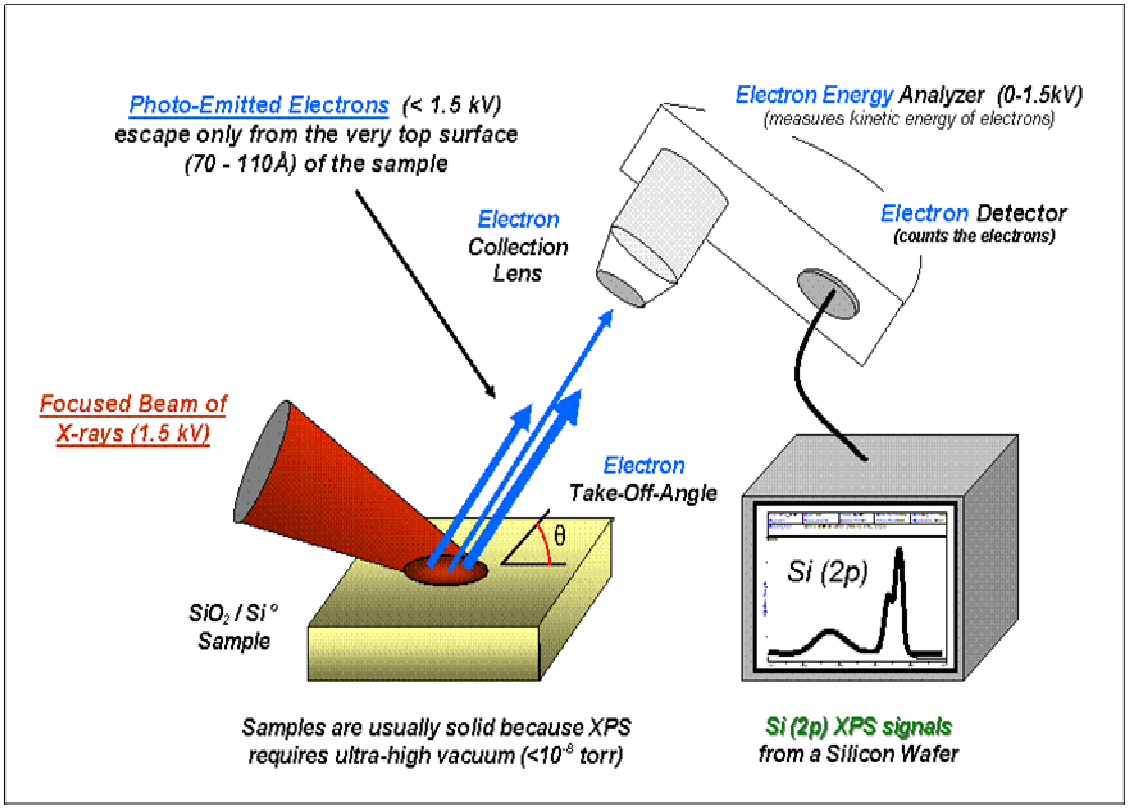


Figure 1.13. A schematic of XPS equipment.

XPS is also known as ESCA, an abbreviation for Electron Spectroscopy for Chemical Analysis. XPS detects all elements (Figures 1.14) with an atomic number (*Z*) of 3 (lithium) and above. This limitation means that it cannot detect hydrogen (*Z*=1) or helium (*Z*=2). Detection limits for most of the elements are in the parts per thousand ranges. Detections limits of parts per million (ppm) are possible, but require special conditions: concentration at top surface or very long collection time (overnight).

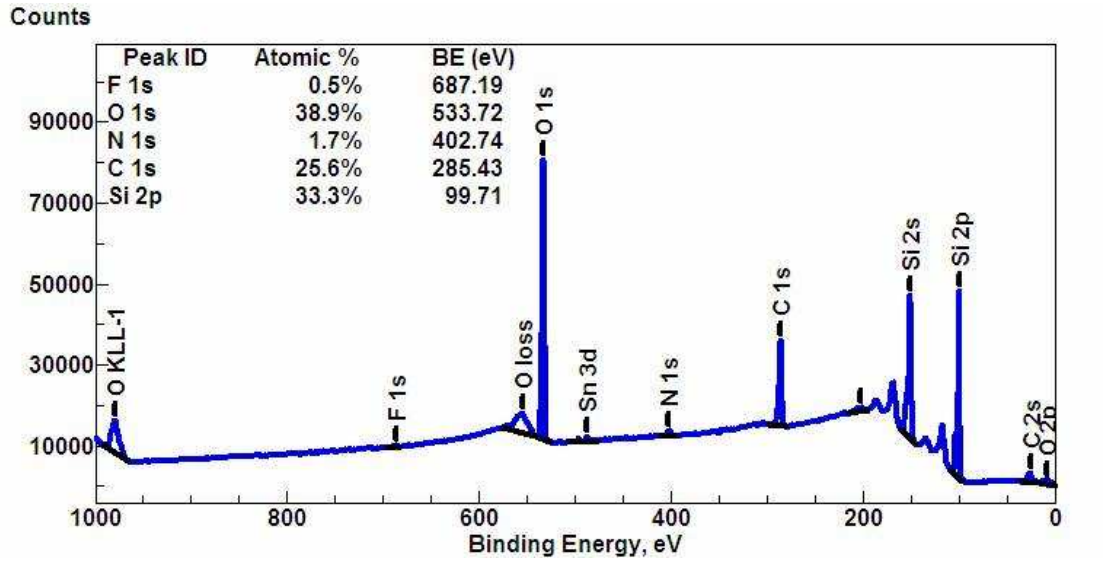


Figure 1.14. An Example of a "Wide Scan Survey Spectrum" using XPS. It is used to determine what elements are and are not present.

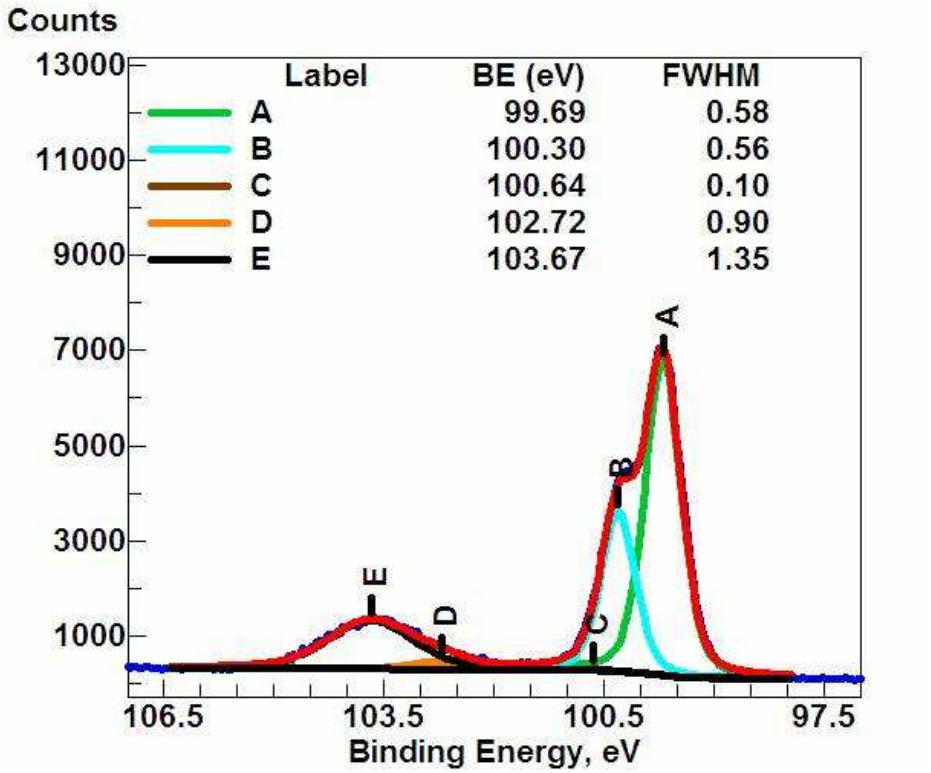


Figure 1.15. An example of "High Energy Resolution XPS Spectrum".

High Energy Resolution XPS Spectrum is also called High Resolution Spectrum. This is used to decide what chemical states exist for the element being analyzed. In Figure 1.15 the Si (2p) signal reveals pure Silicon at 99.69 eV, a Si2O3 species at 102.72 eV and a small SiO2 peak at 103.67 eV. The amount of Si2O at 100.64 eV is very small.

1.2.2.2. Applications

XPS is routinely used to determine:

- What elements are present within ~10 nm of the sample surface, and in which quantity
- What contamination, if any, exists in the surface of the sample
- Empirical formula of a material that is free of excessive surface contamination
- The chemical state identification of one or more of the elements in the sample
- The binding energy (BE) of one or more electronic states
- The thickness of one or more thin layers (1–8 nm) of different materials within the top 10 nm of the surface
- The density of electronic states

XPS is used in a lot of industrial sectors including:
Adhesion, agriculture, battery, beverage, biotech, canning, catalyst, ceramic, chemical, computer, cosmetic, electronics, environmental, food, fuel cells, geology, glass, laser, lighting, lubrication, magnetic memory, mineralogy, mining, nuclear, packaging, paper and wood, plating, polymer and plastic, printing, recording, steel, textiles and thin-film coating.

Materials routinely analyzed by XPS includes inorganic compounds, metal alloys, semiconductors, polymers, pure elements, catalysts, glasses, ceramics, paints, papers, inks, woods, plant parts, make-up, teeth, bones, human implants, biomaterials, viscous oils, glues, ion modified materials.
Organic chemicals are not routinely analyzed by XPS because they are readily degraded by either the energy of the X-rays or the heat from non-monochromatic X-ray sources.

1.3. Biofilm formation in food industry and detrimental effects

Biofilm forms when bacteria adhere to surfaces in aqueous environments and begin to excrete a slimy, glue-like substance that can anchor them to all kinds of material such as metals, plastics, soil particles, medical implant materials, and tissue. A biofilm can be formed by a single bacterial species, but more often biofilms consist of many species of bacteria, as well as fungi, algae, protozoa, debris and corrosion products. Essentially, biofilms may form on any surface exposed to bacteria and some amount of water. Once anchored to a surface, biofilm microorganisms carry out a variety of detrimental or beneficial reactions (by human standards), depending on the surrounding environmental conditions.

In food environments, material surfaces are most often covered by a conditioning film (Mettler and Carpentier 1999; Storgards *et al*. 1999b), formed by the adsorption of various organic materials (proteins, fat, minerals, etc.) due to successive runs of food processing and cleaning procedures. Along with organic material, detergents (Cloete and Jacob, 2001), surfactants (Nitschke and Costa, 2007) and disinfectants (Sinde and Carballo, 2000) may also condition surfaces and further affect their hygienic properties**.** Development of adsorbed layers, often termed "conditioning" of a surface, is considered to be the first stage in biofilm formation and has been widely demonstrated. Since the conditioning film strongly affects the physico-chemical properties of the substratum (Marshall 1996; Storgards *et al*. 1999a) and thus influence bacterial attachment, an understanding of these initial interactions is crucial in identifying control measures.

One of the decisive arguments when choosing materials for processing line equipment, along with their mechanical and anticorrosive properties, has become hygienic status (low soiling level and/or high cleanability). Of these materials, stainless steel, which is widely used for constructing food process equipment, has previously been demonstrated to be highly hygienic (Holah and Thorpe, 1990). It can be produced in various grades and finishes, affecting bacterial adhesion because of their various topographies and physico-chemical properties (Bellon-Fontaine *et al*. 1990; Jullien *et al*. 2008).

Microbial biofilms which form on all types of surfaces in the food industry adversely affect the quality and safety of final products. They usually form by various species of microorganisms, which protect each other against the effects of biocidal (antibacterial) agents and are resistant to

these agents. Several reports have shown that sessile bacterial cells are more resistant to environmental changes and cleaning or disinfection treatments.

The fact that a number of microorganisms are food-borne pathogens, e.g. *Staphylococcus aureus* or *Listeria monocytogenes*, makes a serious problem directly affecting human health.

It has been shown in literature that the presence of a conditioning film may enhance or inhibit bacterial adhesion (Jullien *et al*. 2008). According to Parker *et al*. (2001), coating stainless steel (SS) surfaces with skimmed milk proteins decreased the attachment of both vegetative cells and spores of thermophilic bacteria. Conversly, Flint *et al*. (2001) observed that the attachment of *Bacillus stearothermophilus* cells to SS was significantly increased by the presence of milk on the surfaces. In a study (Peng *et al*. 2001), adhesion of *B. cereus* vegetative cells to stainless steel was positively correlated with the cell surface hydrophobicity (R=0.979).

Bacillus species have been found to be involved in biofilm formation in different dairy processes (Flint *et al*. 1997). Unfortunately, both spores and bacteria embedded in biofilms are of concern to the food industry because of their strong adherence and high resistance to cleaning procedures (Wirtanen and Mattila-Sandholm 1995). Moreover, adhering bacteria may detach and a further cross-contamination of products during processing may occur.

Bacillus cereus is a common contaminant in raw milk. Spores of *B. cereus* are very hydrophobic and readily adhere to various inert substrata such as those found during food processing such as stainless steel, glass and rubber, and short cleaning-in-place programmes do not always eliminate all the spores. Once this first step of adhesion has been completed, colonization may occur when environmental conditions become favourable to spore germination.

This species was demonstrated by Kramer and Gilbert (1989) to contaminate 43.8% of cream and dessert dishes and some UHT-processed milk. *Bacillus* contamination levels may vary greatly, with up to 10^5 cfu ml^{-1} in raw milk and up to 10^2 cfu ml^{-1} in pasteurized milk (Crielly *et al*. 1994). *B. cereus* is often implicated in food-borne gastroenteritis but may also give rise to common milk spoilage. Spores adhering to surfaces are more difficult to eliminate by disinfectants than spores in solution. Many *B. cereus* spores germinate rapidly in milk upon heat activation and, if allowed to propagate on surfaces, may form biofilms that are extremely difficult to eliminate. Spore-forming bacteria cause special problems for the food industry. It is not always possible to apply enough heat during food processing to kill spores, thus we have to take

advantage of knowledge of the spore-formers to control them. *B. cereus* is more difficult to control specifically in the dairy industry, where it is now causing the main problems. There are several reasons for the problems in the dairy industry. First of all, it seems to be impossible to completely avoid the presence of *B. cereus* in all milk samples. Secondly the spores are very hydrophobic and will attach to the surfaces of the pipelines of the dairy industry, where they might multiply and resporulate. A third problem is that pasteurisation heating is insufficient to kill the spores, while competition from other vegetative bacteria is eliminated. It seems that several *B. cereus* strains have become psychrotrophic over the years, making possible growth at temperatures as low as 4-6°C (Granum *et al*. 1993a). None of the methods used to control hygiene in the dairy industry so far are able to control *B. cereus.*

1.4. Aims of the present study

Numerous studies have shown that food-borne pathogenic and opportunistic bacteria are able to adhere and to form biofilms on material surfaces found in food processing environments, resulting in an important source of contamination. To reduce or eliminate microorganisms found on food contact surfaces, cleaning and disinfection procedures using physical and chemical methods have been extensively used over the years. It was established that microorganisms attached to surfaces are more resistant to sanitization than free-living cells; moreover, microbial species can become resistant to disinfectants, hence making difficult the suitable cleaning of surfaces. Thus, controlling the adhered microorganisms is an essential step for food safety assurance and towards developing new adhesion control strategies, which should be constantly improved in order to provide alternatives to the food industry. An interesting strategy is the pretreatment of surfaces using microbial surface-active compounds also known as biosurfactants.

This study has been focused on how biosurfactants with different structural traits can modify surface properties of some substrata, thereby influence adherence of bacteria on them. First, two substrata stainless steel and Teflon, which most often used in food industry, were selected. Then, a set of biosurfactants including surfactin, iturin A, mycosubtilin, fengycin and rhamnolipids were chosen for their structural diversity and their ability to be easily produced and purified. They were produced and/or characterized by several analytical methods inculding TLC, HPLC, LC/MS and MALDI/TOF. The influence of these biosurfactants on the hydrophilic/hydrophobic characteristic of the surfaces was thus determined by using contact angle measurement. Adhesion of *B. cereus* 98/4 spores was then investigated on these different conditioned substrata.

In addition, the two substrata were considered for studying the presence, concentrations and spatial organization of lipopeptides by using XPS analyses.

Since there are many similarities between surfactin and lichenysin A, and the latter has shown more potent biosurfactant properties, it was decided to produce and purify it. As its producer strain was unable to synthesize high amounts of lipopeptide, some molecular biology techniques were employed to get a lichenysin overproducing strain by exchanging the native promoter of the lichenysin operon P_{lchA} with a strong and constitutive one, P_{xylA}.

2. Materials and methods

2.1. Culture media

- **Nutrient agar**

Nutrient agar medium is prepared by adding 15 g l^{-1} bacteriological agar type E (Biokar Diagnostics, France) to nutrient broth (Biokar Diagnostics, France) which contains 10 g l^{-1} yeast extract; 10 g l^{-1} peptone and 10 g l^{-1} glucose. It is sterilized by autoclave at 121°C for 20min.

- **Pepton water**

Pepton water solution is used in adhesion tests for diluting. 1.5 g l^{-1} pepton is added to 100 ml distilled water. 1 ml of this solution is added to 1 litre distilled water containing 2% tween 80. Aliquots of 10 ml of this solution are distributed in big tubes (for putting coupons) and then sterilized by autoclave at 121°C for 20 min.

- **Landy medium**

Landy medium (Landy *et al*. 1948) is used for the production of lipopeptides. It contains as follows: glucose, 20 g l^{-1} ; glutamic acid, 5 g l^{-1}; yeast extract, 1 g l^{-1}; K_2HPO_4, 1 g l^{-1} ; $MgSO_4.7H_2O$, 0.5 g l^{-1}; KCl, 0.5 g l^{-1}; $CuSO_4$, 1.6 mg l^{-1}; $Fe_2(SO_4)_3$, 1.2 mg l^{-1}; $MnSO_4$, 0.4 mg l^{-1}. It can be added 3-[N-morpholino]-propane sulfonic acid (MOPS) (1 M) as a buffer. The pH is adjusted to 7 with 5M KOH and the medium is then autoclaved at 110°C for 30 min.

- **Landy modified medium**

It should be added 2.2 g $(NH_4)_2SO_4$ to 1 liter Landy medium.

- **Stock solutions**

The stock concentrated media are prepared as follows and stored at 4°C:

- **Glutamic acid**:

The solution glutamic acid 4× (20 g l^{-1}) is adjusted to pH 7 by a 5M KOH solution and sterilized by filtration through 0.2 µm filter.

- Mineral solutions:

The mineral solution No.1 is made 40× (K_2HPO_4, 40 g l^{-1}; $MgSO_4$, 20 g l^{-1}; KCl, 20 g l^{-1}). To dissolve the salts, it is acidified by addition of H_2SO_4 up to be dissolved the salts and sterilized by autoclave at 110°C for 30 min or sterilized by filtration through 0.2 µm filter.

The mineral solution No. 2 is made 40× ($CuSO_4$, 64 mg l^{-1}; $Fe_2(SO_4)_3$, 48 mg l^{-1}; $MnSO_4$, 16 mg l^{-1}) and acidified by addition of H_2SO_4 up to be dissolved the salts and sterilized either by autoclave at 110°C for 30 min or by filtration through 0.2 µm filter.

The solution 1 M MOPS is prepared by adding 20.9 g to 100 mL distilled water. The pH is adjusted to 7 by KOH 5 M, and sterilized by autoclave at 110°C for 30 min or filter-sterilized (0.2 µm).

- **Preparation of 1L Landy modified medium**

To prepare Landy modified medium, the following solutions are added:

Landy Base: glucose, 20 g; yeast extract, 1 g; $(NH_4)_2SO_4$, 2.2 g; mineral solution No.1 (40×), 25 ml; mineral solution solution No.2 (40×), 25 ml; 650 ml distilled water.

1 M MOPS solution: 100 ml.

Glutamic acid: 250 ml (4×).

- **Luria-Bertani medium (LB)**

The Luria-Bertani medium (Sambrook *et al*. 1989) includes tryptone, 10 g l^{-1}; yeast extract, 5 g l^{-1}; NaCl, 10 g l^{-1}. The pH is adjusted to 7.2. This medium is used to maintain bacteria. It is sterilized by autoclave at 121°C for 20 min.

- **Lindhardt medium**

Lindhardt medium (Lindhardt *et al*. 1989) contains (g l^{-1}) $NaNO_3$, 15; KCl, 1.1; NaCl, 1.1; $FeSO_4.7H_2O$, 0.00028; KH_2PO_4, 3.4; K_2HPO_4, 4.4; $MgSO_4.7H_2O$, 0.5; yeast extract 0.5; $ZnSO_4.7H_2O$, 0.29; $CaCl_2.4H_2O$, 0.24; $CuSO_4.5H_2O$, 0.25; $MnSO_4.H_2O$, 0.17.

Trace elements solution contains (g l^{-1}) $ZnSO_4.7H_2O$, 0.29; $CaCl_2.4H_2O$, 0.24; $CuSO_4.5H_2O$, 0.25; $MnSO_4.H_2O$, 0.17.

5 ml trace elements solution, after autoclaving at 121°C for 15 min or filter-sterilizing is added to 1 litre mineral salts solution (Lindhardt medium) previously autoclaved (121 °C, 15 min). The pH is adjusted to 7 by 1 M KOH.

- **3M medium**

3M medium contains (l^{-1}) $NaNO_3$, 37.5 mg; $MgSO_4.7H_2O$, 22 mg; KCl, 55 mg; NaCl, 55 mg; $CaCl_2.2H_2O$, 2.75 mg; $FeSO_4.7H_2O$, 27.5 μg; $ZnSO_4.7H_2O$, 82.5 μg; $MnSO_4.7H_2O$, 82.5 μg; H_3BO_3, 16.5 μg; $CoCl_2.6H_2O$, 8.3 μg; $CuSO_4.5H_2O$, 8.3 μg; $NaMoO_4.2H_2O$, 5.5 μg; H_3PO_4 (density=1.71 g ml^{-1}), 8.25 μl; Glucose, 18.2 g; The pH is adjusted to 7 by addition of 1 M KOH.

- **SOB medium**

It contains tryptone 20 g l^{-1}, yeast extract 5 g l^{-1}, NaCl 0.5 g l^{-1}, KCl 18.6 g l^{-1}. The pH is adjusted to 7.2 by addition of HCl or KOH. It is sterilized by autoclave at 121°C for 20 min.

- **SOC medium**

To 100 ml of SOB medium, 1.8 ml of 2 M glucose (filter-sterilized) and 0.5 ml of 2 M $MgCl_2$ (sterilized by autoclave) are added.

- **MEB solution (electroporation buffer)**

It contains HEPES 240 mg l^{-1}, $MgCl_2.6H_2O$ 203 mg l^{-1} and ultra pure water. The pH is adjusted to 7.0 by addition of HCl.

It is sterilized by autoclave at 121°C, 20 min and kept at 4°C.

- **B medium**

This medium includes several compositions which should be mixed after sterilizing by autoclave which is often used for doing swarming test (ability of forming spread colonies, Julkowaska *et al*. 2005; Leclere *et al*. 2006). It contains base solution 100 ml, complement (×100) 1 ml, $CaCl_2$ (0.1 M) 2 ml which are sterilized by autoclave at 121°C for 20 min. In addition, glucose 1 ml, glutamic acid 0.3 ml and lysine-HCl 0.2 ml are added after filter-sterilizing (0.2 μm). The composition of different solutions is as follows:

-Base solution: $(NH_4)_2SO_4$, 2 g l^{-1}; $MgSO_4.7H_2O$, 2 g l^{-1}; KCl, 2 g l^{-1}; Na citrate.$2H_2O$, 2 g l^{-1}; Tris-HCl 100 ml (pH 7.5); agar 7 g l^{-1}.

-Complement solution (×100): K_2HPO_4 1.05 g, $FeSO_4.7H_2O$ 2.78 mg, $MnSO_4.H_2O$ 16.9 mg, distilled water 100 ml.

-$CaCl_2$ solution: (0.1 M) 2 ml

-Glucose solution: (×20) 20 g l^{-1}

-Glutamic acid solution: 220 g l^{-1}; the pH is adjusted to 8 by 5 M KOH

-Lysine solution: 78.5 g l^{-1}

- **MS1 medium for 10 ml**

This medium is used for transformation by natural competence. It contains minimum medium (×10) 1 ml, glucose (50%) 100 µl, casein hydrolysate (5%) 40 µl, yeast extract (5%) 200 µl, MgSO4 (1M) 16.7 µl, tryptophan (50 mM) 50 µl, distilled water 9 ml. The pH is adjusted to 7.2 by the addition of KOH.

All solutions are prepared separately and sterilized by autoclave at 121°C for 20 min. Glucose and tryptophan are sterilized by filtration through 0.2 µm filter.

Minimum medium (×10) for 100 ml: It contains $(NH_4)_2SO_4$, 2 g l^{-1}; Na-citrate, 1 g l^{-1}; K_2HPO_4, 14 g and KH_2PO_4, 10 g.

- **MS2 medium for 10 ml**

This medium is used in the second stage of transformation by natural competence and is made of MS1 medium (10 ml) as follows:

$CaCl_2$ (50 mM) 50 µl, $MgCl_2$ (1 M) 25 µl.

All solutions are prepared separately and sterilized by autoclave at 121° C for 20 min.

2.2. Strains

All strains used are summarized in Table 2.1. *B. subtilis* BBG100 (producer of mycosubtilin and surfactin, Leclere *et al*. 2005) and *B. subtilis* ATCC 21332 (producer of surfactin and fengycin) were used for production of mycosubtilin and fengycin, respectively.

Bacillus subtilis S499 supernatant was kindly obtained from Dr Marc Ongena from Gembloux Agro-Bio Tech. to purify iturin A.

P. aeruginosa PTCC 1637 was obtained from Persian Type Culture Collection Biotechnology Center, Iranian Research Organization for Science & Technology (IROST), Tehran, Iran, for rhamnolipid production.

Bacillus cereus 98/4 spores were obtained from INRA, Villeneuve d'Ascq, France, for adhesion tests to different surfaces.

Table 2.1. Strains used for biosurfactants production or adhesion tests

Strain or supernatant	Produced biosurfactant	Source or reference
B. subtilis BBG100	Mycosubtilin, surfactin	Leclere *et al*. 2005
B. subtilis ATCC 21332	Surfactin, fengycin	Lab stock
P. aeruginosa PTCC 1637	rhamnolipid	Mazaheri, IROST, Iran
Bacillus subtilis S499 supernatant	Surfactin, fengycin, iturin A	Gembloux Agro-Bio Tech
B. cereus 98/4 CUETM	-	Faille, INRA, France

2.3. Production and purification of lipopeptides produced by *Bacillus* spp.

2.3.1. Origin of lipopeptides

Surfactin S1 (approx. 98% purity, C14-C15) was purchased from Sigma (St. Louis, MO, USA).
Iturin A (90% purity, C13-C16) and fengycin (94% purity, C13-C18) were kindly provided by Gembloux Agro-Bio Tech, University of Liege, Belgium).
Mycosubtilin (93% purity, C16-C17) and fengycin (much amount as required) were produced by *B. subtilis* BBG100 (Leclère *et al*. 2005; Guez *et al*. 2007) and *B. sutilis* 21331, respectively.
A mixture of surfactin and mycosubtilin were provided by BBG100 under the same condition of cultivation.
Iturin A supernatant was taken from Belgium (produced by *B. sutilis* S499) to be purified.

2.3.2. Inoculum preparation and culture

B. subtilis ATCC 21332 and BGG 100 from the collection of ProBioGEM Laboratory were used. The strain ATCC 21332 was recently proved to be a co-producer of surfactin and fengycin (Gancel *et al*. 2009, Tapi *et al*. 2009).

Inoculum was prepared from a strain conserved at -80°C in 40% glycerol. A tube containing 5 ml of LB medium was inoculated with 0.5 ml bacterial suspension and incubated at 30°C overnight in a rotary shaker at 150 rpm. The strain was then cultivated in a 500 ml flask containing 50 ml modified Landy medium at pH 7 buffered with MOPS 100 mM, overnight at 30°C at 140 rpm. Culture was centrifuged and after washing the cells with sterile NaCl 0.9%, concentrated 10 times in Landy medium. The main culture was inoculated into 1000 mL flask containing 100 mL of Landy modified medium, in order to obtain 0.5 (beginning of exponential phase) as initial optical density (at 600 nm) and incubated at 30°C for 72 h at 140 rpm. Some samples were taken under sterile conditions in order to control culture purity by Gram staining and streaking on LB agar plates, measurement of OD_{600} (growth control), pH and measurement of lipopeptide concentration by HPLC after cell removal by centrifugation.

2.3.3. Extraction

Culture was centrifuged at 13000 × g for 30 min at 4°C for cell removal.

Extraction steps for all LPs were done through C18 Maxi-Clean cartridges (Extract-Clean SPE 500 mg, Alltech, Deerfield, IL), as follows:

After washing C18 cartridge by 20 ml methanol 100% and 8 ml MilliQ water respectively, 1 ml supernatant was passed through C18 cartridge. Then, it is washed by 8 ml MilliQ water. The column was dried by air and LPs were eluted by 8 ml 100% methanol. For extractions in large scale, following stages were performed:

<u>Large scale (50 ml)</u>

-100 ml 100% methanol

-50 ml culture supernatant

-50 ml MilliQ water

-5 min air

-100 ml 100% methanol (elution)

Lipopeptides were eluted with pure methanol (high-performance liquid chromatography grade; Acros Organics, Geel, Belgium). The extract was dried by rotavapor and Speed Vac (Plus SC110A, Savant, GMI, Ramesy, USA) under vacuum and residue was dissolved in 200 µl pure methanol (for 1 ml supernatant). Rotavapor VV2000 (Heidolph Instruments GmbH & Co, Schwabach, Germany) was used under vacuum -1 bar and at 40-50°C for concentration of lipopeptides. It takes 2-4 h (for large volumes) under precaution conditions to avoid making foam.

The extract was then purified by thin layer chromatography (TLC) (silica gel plates: F256) or silica gel column (Razafindralambo *et al*. 1998). Purity and identification of the mycosubtilin, fengycin and iturin A after purification by TLC analysis, (§ 2.11) were controlled by HPLC and mass spectrometry.

2.3.4. Purification: Thin-layer chromatography

Thin-layer chromatography (TLC) analysis (Syldatk *et al*. 1985; Kim *et al*. 2000) was done to detect and purify lipopeptides. Eluted samples were spotted on TLC plates (silica gel plates, 60 F-254, Merck, Germany) and then migrated using chloroforme/methanol/water (65/25/4, v/v/v) as the solvent system. The compositions migrated according to their hydrophobicity. The spots were revealed by spraying with distilled water and looking under UV light. R_f (Retardation Factor) of lipopeptides are 0.09, 0.3 and 0.7 for fengycin, iturin and surfactin, respectively. Lipopeptides were scraped off the TLC plates, then dissolved in pure methanol, and centrifuged to be examined by other experiments.

2.3.5. Determination of lipopeptide concentration and identification

Lipopeptide extracts after extraction and purification by TLC analysis (§ 2.3.4) were analyzed by HPLC to confirm or determine their precise concentrations (for mycosubtilin: Guez *et al*. 2007) using a C18 column (5 µm; 250 by 4.6 mm; VYDAC 218 TP; VYDAC, Hesperia, CA) with the acetonitrile/water/trifluoroacetic acid (ACN/H_2O/TFA) solvent system (40/60/0.5 v/v/v for iturin and mycosubtilin, 80/20/0.5 v/v/v for surfactin and a gradient from 45/55/0.1 v/v/v to 55/45/0.1 v/v/v in 40 min for fengycin) and a flow rate of 0.6 ml min^{-1}. 10-20 µl of purified samples were injected and were compared by the appropriate standard (purified iturin and surfactin were

purchased from Sigma and standard of fengycin was kindly provided by Dr Deleu from Gembloux Agro-Bio Tech, Belgium).

The retention time and second derivatives of UV-visible spectra between 200 and 400 nm (Waters PDA 2996 photodiode array detector) of each peak were analyzed automatically by Millenium Software to identify eluted molecules. All reagents were of analytical grade.

Lipopeptide extracts were further analyzed by mass spectrometry (MS) by Professor Bernard Wathelet from Gembloux Agro-Bio Tech (Belgium). Measurement was performed using a UV laser desorption-time of flight mass spectrometer (Bruker Ultraflex tof; Bruker Daltonics) equipped with a pulsed nitrogen laser (337 nm). The analyzer was used at an acceleration voltage of 20 kV. Samples were measured in the reflectron mode.

2.4. Production and purification of rhamnolipids produced by *Pseudomonas aeruginosa* PTCC 1637

2.4.1. Inoculum preparation and culture

The strain was maintained on nutrient agar slants at 4°C and sub-cultured every two weeks. Every three months a new frozen culture was used to provide slant cultures. These frozen stocks were prepared by transferring a loop of slant culture to a 250 ml Erlenmeyer flask containing 50 ml of Lindhardt medium (§ 2.1) and 2% (v/v) corn oil as carbon source. After growing in shaker incubator at 30°C and 200 rpm for 3 days, 30 ml sterile glycerol was added and mixed thoroughly. Then, 2 ml aliquots were dispensed into sterile vials and stored at -70°C. Frozen cultures were recovered by transferring of whole vial of thawed culture to a 250 ml Erlenmeyer flask containing 50 ml of sterile Lindhardt medium with 6% (v/v) corn oil and incubated on a shaker incubator at 30°C at 150 rpm for 3 days to prepare slant cultures. Corn oil (60 g l^{-1}) was separately sterilized at 121°C for 5 min by autoclave, and then added to already autoclaved Lindhardt medium.

The pre-culture was prepared by transferring a loopful of slant culture to a 250 ml Erlenmeyer flask containing 50 ml of Lindhardt medium and 2% (v/v) corn oil as carbon source growing in shaker incubator at 30°C, 200 rpm for 3 days. Final production medium (7% inoculum size from pre-culture) was grown in 1000 ml Erlenmeyer flasks containing 6% corn oil and 100 ml of sterile Lindhardt medium at 30°C and 150 rpm for 96 h or more as required.

2.4.2. Extraction

Method 1

The cells were removed from the culture broth by centrifugation (12900 ×g, 4°C, 1 h). The supernatant (10ml) was filtered (0.45 µm, Millipore) and acidified to pH 3 with 5 N HCl and kept at 4°C overnight. The resulting rhamnolipid precipitate was recovered by centrifugation (5240×g, 4°C, 1 h). The pellet was dissolved in 1 ml pure methanol by vortexing during 1 min. After centrifugation (12900×g, 4°C, 15 min), it was passed through C18 column as for LPs (§ 2.3.3).

Method 2

The cells were removed from the culture broth by centrifugation (12900×g, 4°C, 1 h). The supernatant (10ml) was filtered (0.45 µm, millipore) and acidified to pH 3 with 2 N HCl and kept at 4°C overnight. The resulting rhamnolipid precipitate was recovered by centrifugation (5240×g, 4°C, 1 h). The glycolipids were extracted by ethyl acetate on mild shaking at room temperature overnight. The solvent was evaporated and the oily residue was dissolved in 0.5 ml pure methanol. This semi-purified sample was used for other analytical methods.

2.4.3. Purification: Thin-layer chromatography

Thin-layer chromatography (TLC) analysis was done to detect and purify rhamnolipids.

Ethyl acetate extracts were purified by TLC. Spots were developed with chloroform/methanol/acetic acid (65/15/2, v/v/v) and visualized with TLC reagents i.e. iodine vapour for lipids and Molish reagent (α-naphtol (15% in methanol)/sulphuric acid/ethanol/water (10.5/6.5/40.5/4)) for sugar detection. Each spot was separately removed and eluted with methanol in order to follow other experiments like HPLC/MS. Rhamnolipid standard contains a mixture of α L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (Rh-C10-C10) and L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (Rh-Rh-C10-C10) with MW 504 and 650, respectively with >99% purity obtained from JBC.

2.4.4. Determination of rhamnolipid concentration

2.4.4.1. Orcinol method

The cells were removed from the culture broth by centrifugation (13000× g, 4°C, 1 h).
The orcinol assay (Chandrasekaran *et al*. 1980) was used to assess the amount of glycolipids in the supernatant. The concentration of rhamnolipids was calculated by comparing the data with those of rhamnose standards between 0 and 50 µg ml^{-1}. All the samples were analysed in triplicate and the linear correlation was demonstrated between the quantity of rhamnolipid and optical density.

2.4.4.2. HPLC

Rhamnolipids after extraction (§ 2.4.2) and purification by TLC (§ 2.4.3) were analyzed by HPLC to determine their precise concentrations. A C18 column 250 by 4.6 mm was used with solvent system acetonitrile/water (70/30, v/v) and at a flow rate 1 ml min^{-1}. 10 µl of purified sample was injected and compared to the appropriate standard (obtained from JBC).

2.4.5. Effect of time, culture medium and sterilization on rhamnolipids

In order to check stability during time on rhamnolipid production in Lindhardt medium, two samples were compared: sample A (12-months old, a dark brown solution, sticky, >150 rpm), sample B (fresh, light brown, <150 rpm) (Table 2.2).

Table 2.2. Comparison of sample A and sample B in different conditions: agitation and time

	Life time	**Condition**
Sample A	one year old	Sticky, dark brown
Sample B	fresh	Normal, light brown

To determine the effect of cultivation time and medium on rhamnolipid production, the samples cultivated in the media 3M and Lindhardt after different days (4, 7 and 9 days) were withdrawn. The samples are designated as follows (Table 2.3):
3M4-, 3M4+, 3M9-, 3M9+, LH4-, LH4+, LH7-, LH9-, and LH9+.

Table 2.3. Effect of cultivation time and medium on rhamnolipid production

Sample	Medium	Time(days)	Autoclave
3M4-	3M	4	-
3M4+	3M	4	+
3M9-	3M	9	-
3M9+	3M	9	+
LH4-	Lindhardt	4	-
LH4+	Lindhardt	4	+
LH7-	Lindhardt	7	-
LH9-	Lindhardt	9	-
LH9+	Lindhardt	9	+

2.4.6. Rhamnolipid identification

2.4.6.1. HPLC/MS

HPLC/MS was done using an Agilent 1100 series (USA), MSD VL ion trap mass spectrometer equipped with an ESI source.

ESI parameters were: ion spray voltage 4000V and source temperature 350 °C. Nitrogen was used as nebulizing and drying gas. Flow rate of drying gas was adjusted at 12 liter min^{-1}.

A reversed phase C-18 column (50 mm × 3.2 mm × 3.5 µm) was used as stationary phase and mobile phase was composed of acetonitrile/water (15/5) containing ammonium formate (0.1 mM) as buffer (pH = 2.6). Flow rate was adjusted at 0.35 ml min^{-1}. 3 µl samples were injected. Negative ion mode was used over the mass range of 150-750 Daltons for monitoring of the separated compounds.

2.4.6.2. Fourier Transform Infrared (FTIR) spectrometry

Infrared (IR) spectra of the rhamnolipid fraction obtained by HPLC (a film of each purified sample on KBr pellet) were obtained using a Perkin Elmer spectrometer. The resulting transmission of radiation is measured over a frequency spectrum from 400 to 4000 cm^{-1}. Fifteen spectra per sample were collected and averaged (Berbenni *et al*. 1995).

2.5. Hemolytic Activity

Hemolytic activity test is performed on blood agar (on the basis of blood agar base medium or LB agar) plates which contains horse/sheep blood 5%.

The isolates (fresh culture) are streaked on blood agar plates and incubated at 30°C. The plates are inspected visually for the presence of clearing zone around the colonies which is indicative of surfactant biosynthesis. The diameter of the clear zones depends on the concentration of the biosurfactants (Mulligan *et al*. 1984). The amount of hemolytic activity was determined by measuring of halo diameter around grown colonies on blood agar using ruler: (−) growth without halo formation, (+) complete hemolysis with a diameter of lysis less than 1 cm, (++) complete hemolysis with a diameter of lysis greater than 1 cm but less than 2 cm, (+++) complete hemolysis with a diameter of lysis greater than 2 cm but less than 3 cm, and (++++) complete hemolysis with a diameter of lysis greater than 3 cm (Jain *et al*. 1991; Youssef *et al*. 2004; Rodrigues *et al*. 2006).

2.6. Surface tension measurement

The surface tension of all solutions used for substratum conditioning was measured by the ring method (Thimon *et al*. 1992; Bonmatin *et al*. 1995) using a Du Nouy tensiometer TD1 (Lauda, Königshofen, Germany). The critical micelle concentration (CMC) was determined by plotting the surface tension as a function of the lipopeptide concentration. The average of three independent measurements was taken.

2.7. Substratum conditioning and contact angle measurement

Stainless steel (304L with a 2R finish, hydrophilic), Teflon (polytetrafluoroethylene, hydrophobic) (PTFE) and glass coupons were provided in the form of 15×45 mm^2 for conditioning experiments. Before each experiment, the coupons were subjected to the cleaning and disinfection protocol as follows:

The coupons were washed in a mild alkaline detergent Galor 7/32 (CFPI, France) 1% and rinsed in distilled water 5 min at a velocity of 0.5 m s^{-1}. They were placed in Galor solution at 60°C for 10 min and rinsed in distilled water 5 min at a velocity of 0.5 m s^{-1}. They were exposed to1% Oxygal (oxidative biocide consisting of 28 g l^{-1} peracetic acid, Europo, France) for 15 min, rinsed in

distilled water 5 min at a velocity of 0.5 m s^{-1}, dried vertically and kept in clean Petri dishes to protect from pollution.

Goniometer (Digidrop, GBX Scientific Instruments, France, www.gbxinstru.com) was used for contact angle measurement. The influence of lipopeptides on the hydrophilic/hydrophobic characteristic of surfaces was determined by using the different concentrations of lipopeptides. For surfactin, iturin, mycosubtilin and rhamnolipid were used the concentrations 1, 10, 25, 50 and 100 mg l^{-1} in 10% (v/v) methanol and the concentrations 0.25, 2.5, 6.25, 12.5 and 25 mg l^{-1} in 10% (v/v) methanol were used for fengycin.

Stainless steel and Teflon coupons were covered by the different concentrations of biosurfactants for 1 h at room temperature. After removing solutions, they were then dried. Four water droplets (5 µl) were applied on each coupon at 20ºC and water contact angles were measured. Each analysis was performed three times. Coupons subjected to 10% methanol were used as control. Ultra high purity MilliQ water was used throughout the experimental procedures.

2.8. Adhesion tests of *B. cereus* 98/4 spores

B. cereus CUETM (Collection Unité EcoToxicologie Microbienne, Villeneuve d'Ascq, France) 98/4, isolated from a dairy processing line, was chosen for adhesion tests to coupons because of its high adherence to various materials (Faille *et al*. 1999). Spores were obtained as previously described (Faille *et al*. 2007) and kept for up to 3 months in distilled water (10^9 spores ml^{-1}) at 4ºC. Stainless steel and Teflon conditioned coupons were vertically immersed in spore suspensions in sterile MilliQ water containing approximately 10^6 spores ml^{-1}, for 4 h at room temperature. The coupons were rinsed by dipping into a beaker of distilled water to remove non-adhered spores. They were then placed into the tubes containing 10 ml of 2% Tween 80 and 10 mg l^{-1} of peptone water (Biokar, Diagnostic, Beauvais, France) to expose to sonication. Adherent cells were detached from the surfaces using an ultrasonic bath (Deltasonic Meaux, France, 40 kHz) for 2.5 min followed by 20 sec vortexing with high speed and again ultrasonic bath for 2.5 min. Detached spores were plated in duplicate using the serial dilution technique on nutrient agar composed of 13 g l^{-1} nutrient broth (Biokar Diagnostics, France) and 15 g l^{-1} bacteriological agar type E (Biokar Diagnostics, France). Enumeration was performed after 48 h incubation at 30°C. Each experiment was performed at least three times.

2.9. Statistical analysis

Data were analyzed by general linear model procedures by means of SAS V8.0 software (SAS Institute, Cary, N.C.). The variance analyses were performed to determine the influence of the conditioning solution concentration on the material surface hydrophobicity and further ability of *B. cereus* 98/4 spores to adhere, taking into account the variability between trials. It was followed by a multiple comparison procedure by Tukey's test (alpha level = 0.05).

2.10. Molecular biology procedures

2.10.1. Chemicals and standard procedures

All enzymes used for DNA manipulation such as restriction enzymes and DNA-ligase were purchased from Fermentas (Fermentas, Burlington, Canada, WWW.fermentas.com), and *Taq* polymerase "Arrow" from Qbiogene (Montreal, Canada).

pGEM-T Easy (Promega, Madison, USA) vector was used to amplify PCR products.

The antibiotics utilized were sterilized by filtration (0.2 μm) and added to the medium at the following concentrations:

Ampicillin sodium salt, 50-100 μg ml^{-1} (Euromedex); kanamycin sulphate, 25-50 μg ml^{-1} (Sigma) and spectinomycin di-hydrochloride, 50-400 μg ml^{-1} (Sigma).

Standard procedures were used for all DNA manipulations (DNA digestions with restriction enzymes, cloning of DNA fragments, and preparation of recombinant plasmid DNA) (Sambrook *et al*. 2001).

2.10.2. Strains and plasmids

The strains and plasmids used in molecular biology section are summarized in Table 2.4. *B. licheniformis* ATCC 14580 was the parental strain for genetic manipulations.

Escherichia coli DH5α and JM109 (commercially available) were the host strains for constructing various recombinant plasmids.

Table 2.4. Strains and plasmids used in molecular biology section

Strain or plasmid	Description[a]	Source or reference
B. licheniformis ATCC 14580	Wild type	Lab stock
E. coli JM109	*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *relA1*, sup*E44*, Δ(*lac-proAB*), [F', *traD36*, *proAB*, *lacI* / *lacZΔM15*]	Promega, Madison, USA
E. coli DH5α	Φ80d*lacZΔM15 recA*1 *endA1 gyrA96 thi*-1 *hsdR17* ($r_k^- m_k^+$) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169 phoA*	Promega, Madison, USA
EBG155	DH5α containing pBG214	This study
EBG156	JM109 containing pBG155	This study
EBG157	JM109 containing pBG156	This study
EBG158	JM109 containing pBG157	This study
EBG159	JM109 containing pBG158	This study
EBG162	JM109 containing pBG160	This study
EBG163	JM109 containing pBG161	This study
EBG165	JM109 containing pBG163	This study
EBG167	JM109 containing pBG162	This study
pBG214	cloning vector, 8100 bp; Spc^r, Kan^r, $Ap^{r,a}$ *	Fickers *et al*. University of Liege, Belgium
pGEM-T Easy	3015 bp, cloning vector, carrying a part of *lac*Z; Ap^r	Promega, Madison, WI
pBG155	pGEM-T Easy::Fragment 1 of PCR	This study
pBG156	pGEM-T Easy::Fragment 2 of PCR	This study
pBG157	pGEM-T Easy::Fragment 3 of PCR	This study
pBG158	pGEM-T Easy::Fragment 4 of PCR	This study
pBG160	7880 bp, pBG214 digested with *Bam*HI+ *Bss*HII; carrying PCR product: fragment 2; Spc^r, Kan^r, Ap^r	This study
pBG161	8021 bp, pBG214 digested with *Bam*HI+ *Bss*HII; carrying PCR product: fragment 4; Spc^r, Kan^r, Ap^r	This study
pBG162	7987 bp, pBG160 digested with *Sac*II+*Sph*I; carrying PCR product: fragment 1; Spc^r, Kan^r, Ap^r	This study
pBG163	8046 bp, pBG161 digested with *Sac*II+*Sph*I; carrying PCR product: fragment 3; Spc^r, Kan^r, Ap^r	This study

a Ap^r, resistant to ampicillin; Kan^r, resistant to kanamycin; Spc^r, resistant to spectinomycin .

***** It originates from pBG113s (Fickers *et al*. 2009) to which *Kan* gene was added.

2.10.3. Production

B. licheniformis ATCC 14580 was cultivated for 96 h under different temperatures in a medium based on Landy with some modifications in carbon and nitrogen source which is summarized in Table 2.5. To verify purity, it was streaked on LB agar and checked by Gram staining. The growth was determined by OD_{600} measurement during 4 days of culture and lichenysin production was measured by ST (§ 2.7) and HPLC (§ 2.3.5). The pH of cultures was also measured during 4 days.

Table 2.5. Different culture conditions for growth of *B. licheniformis* 14580

Agitation	N source (0.4%)	C source (2%)	MOPS	Temperature (°C)
140 rpm/ static	Glutamic acid/ NH_4NO_3	Glucose/ Sucrose	+/-	30/37

0.1 ml of an overnight culture of the strain was grown on LB plate (in triplicate) supplemented by spectinomycin (100 µg/ml) and kanamycin (100 µg/ml) to check the antibiotic resistance pattern of this strain.

2.10.4. Extraction of chromosomal DNA from *B. licheniformis* ATCC 14580

B. licheniformis 14580 was grown overnight in LB at 37°C with agitation at 140 rpm.
Total genomic DNA was then extracted from this strain based on 1 ml of fresh culture using Promega Kit "Wizard® Genomic DNA Purification Kit" (Promega, Madison, USA) according to the protocol supplied by the manufacturer.

2.10.5. Plasmid extraction

Miniprep from kit "QIAprep Spin Miniprep Kit" that is a rapid technique for extraction of plasmids, was used for extractions from 5 ml of culture. To obtain larger amounts of plasmid on the basis of 250 ml of culture, the extraction kit "QIAfilter® Plasmid Maxi Kit" (QIAGEN,

Hilden, Germany) was used according to the protocol. The fragments purified in 0.8% agarose gels were extracted by "QIAquick Gel Extraction Kit" according to the protocol (QIAGEN).
After enzymatic reactions, if necessary, plasmid DNA was purified by "MinElute Reaction Cleanup Kit" according to QIAGEN protocol.
The pBG214 (Index IV) used in this study contains the xylose promoter P_{xylA}, the xyl^R gene and three gene cassettes conferring resistance to kanamycin, ampicillin and spectinomycin.
An overnight culture of *E. coli* DH5α (EBG155) containing pBG214 was obtained at 37°C with agitation at 140 rpm and a 5 ml sample was centrifuged 20 min at 3000×g.
The pellet was taken and plasmid DNA (pBG214) was extracted by Plasmid DNA Purification Protocol using the "QIAprep Spin Miniprep Kit".
pBG214 was double-digested with *Bam*HI-*Bss*HII and *Sac*II-*Sph*I separately to remove the previous ε*fenF* and ε*pbp* cassettes and then produce the linear plasmids pBG215 and pBG216, respectively.

pBG214 (digested with *Bam*HI-*Bss*HII) → pBG215
pBG214 (digested with *Sac*II-*Sph*I) → pBG216

They were purified from agarose gels by QIAquick Gel Extraction Kit (50).

2.10.6. PCR

PCR is a gene amplification method *in vitro* which allows amplifying a large number of a DNA/RNA given sequence on the basis of a small quantity of the nucleic acid and specific oligonucleotides (primers). The primers were designed by the Primer3 software on the basis of published genome of *B. licheniformis* 14580 (PubMed Gene NC-006270) from NCBI site. They were synthesized by Eurogentec (Angers, France).
Protocol of PCR used is standard but the annealing temperature is calculated by software Primer3 according to the Tm after the primers have been determined.
To amplify the cassettes, PCR was performed with a mixture as follows:
Taq polymerase buffer (×10) 5 µl, dNTPs (100 µM) Mix 1 µl, forward primer 2.5 µl (Eurogentec), reverse primer 2.5 µl (Eurogentec), Arrow Taq polymerase 0.4 µl (5 U/ µl), ultra pure water 37.5 µl, template DNA 1 µl (1-10 ng).

The PCR (Eppendorf, Hamburg, Germany) program was based on the Primer3 software results and consisted of denaturation: 3 min at 94°C, annealing: 30 s at 58°C (for cassettes of 1 and 3) or 54°C (for cassettes 2 and 4), and elongation: 2 min at 72°C during 29 cycles and then 5 min at 72°C. Finally, reactions were kept at 4°C.

2.10.7. Cloning in plasmid

Plasmid cloning allows inserting a DNA fragment into a vector. These fragments originate from either PCR or enzymatic digestion of other plasmids by restriction enzymes.

To obtain sufficient amount of the four PCR fragments containing the sequences upstream and downstream from *lch* promoter, they were inserted in a commercial vector, pGEM-T Easy. This linear plasmid has deoxythymidine at its 5' ends, permitting direct cloning of PCR products with deoxyadenosine at 3´ ends. This plasmid is replicative only in *E. coli*. It contains a cassette of resistance to ampicillin and a multiple cloning site (MCS) upstream from a gene encoding the enzyme β-galactosidase. Insertional inactivation of this gene allows recombinant clones to be directly identified by colour screening on indicator plates. The pGEM-T Easy vector contains multiple restriction sites within the MCS. These restriction sites allow for the release of the insert by digestion with restriction enzymes such as *Eco*R I, *Bst*Z I, *Not* I or other combinations (Figure 2.1).

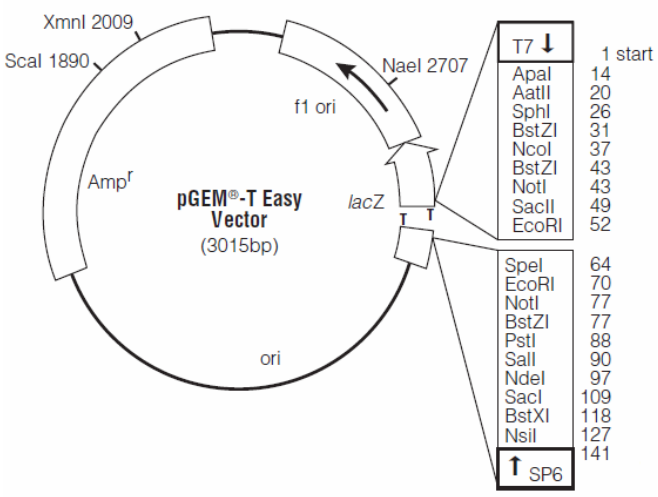


Figure 2.1. The pGEM-T Easy vector circle map. The pGEM-T Easy vector MCS contains recognition sites for various restriction enzymes.

After ligation of PCR products to pGEM-T Easy Vector (Table 2.6), the resulting plasmids were inserted in *E. coli* JM109 by thermal shock to generate the strains EBG156, EBG157, EBG158, and EBG159 containing the plasmids pBG155, pBG156, pBG157 and pBG158 respectively. The white colonies were selected on LB agar supplemented by ampicillin (100 μg ml^{-1}), X-Gal (8 mg l^{-1}) and IPTG (80 μg l^{-1}). The plasmids designated pBG155, pBG156, pBG157 and pBG158 containing fragments of 1, 2, 3 and 4 respectively, were extracted and purified by "QIAprep Spin Miniprep Kit".

The presence of recombinant plasmids was examined in a 0.7% agarose gel. The recombinant plasmids were digested by restriction enzymes and the presence of the PCR fragments was confirmed on a 1% agarose gel. The resulting fragments were then ligated to plasmid pBG214 (a plasmid kindly obtained from Fickers *et al*. (University of Liege, Belgium) carrying Spc^r, Km^r and Ap^r) containing the cohesive ends complemetary to those of the PCR fragments. To optimise ligation, the latter fragments and plasmid were quantified on an agarose gel. Regarding the size of linearized vector that is larger than the insert one, therefore, a ratio of 1/3 (vector/insert) was used for

Table 2.6. Ligation of PCR fragments in pGEM-T Easy (volumes are in μl)

	Fragment 1	**Fragment 2**	**Fragment 3**	**Fragment 4**
Ultra pure water	2	1	0.5	2
Buffer (×2)	5	5	5	5
pGEM-T Easy (50 ng/μl)	1	1	1	1
Purified PCR fragments	1	2	2.5	1
Ligase	1	1	1	1

ligation. This ratio has been got experimentally and is calculated as follows:

50 ng (vector) × (size of insert/size of vector) × 3 = ng of insert for a ratio of 1/3 (vector/insert)

2.10.8. Main plasmid constructions: pBG162 and pBG163

The fragments 1 and 3 were inserted into pBG216 (§ 2.10.4) and the fragments 2 and 4 were inserted into pBG215 (§ 2.10.4) to generate hybrid plasmids pBG216-1, pBG216-3, pBG215-2 (pBG160) and pBG215-4 (pBG161), respectively. The molar proportion of 1/3 (vector/insert) was considered for ligation. These new plasmids were transferred into *E. coli* JM109 according to the following protocol:

50 µl of competent cells of *E. coli* JM109 were added to sterile Eppendorf tubes. To each tube was added 5 and 10 µl (different ratios were used) of pBG216-1, pBG216-3, pBG215-2 and pBG215-4 respectively. They were slowly mixed and then placed on ice for 20 min. They were exposed to a heat shock at 42°C for 90 sec and placed immediately on ice for 2 min. 950 µl of SOC medium (room temperature) were added to all tubes which were placed under agitation at 140 rpm for 90 min at 37°C. In the next step, every tube was spread onto several LB plates containing ampicillin (50 µg ml^{-1}) and spectinomycin (100 µg ml^{-1}) separately.

All grown colonies were again inoculated in LB broth containing ampicillin (50 µg ml^{-1}) and then grown on spectinomycin (100 µg ml^{-1}) and kanamycin (50 µg ml^{-1}) to verify if the desired hybrid plasmids conferred resistance to all three antibiotics. All hybrid plasmids were extracted and purified as previously mentioned (§ 2.10.4).

2.10.9. Gel electrophoresis

Gel electrophoresis in agarose gels is performed to quantify extracted DNAs (plasmidic or chromosomal) by using a size marker to estimate concentration of genetic material in a sample. 0.7% agarose gels were used for fragments greater than 1 kb and 1-1.5% ones for fragments smaller than 1 kb. Migration was done at 110 V during 1 h in TBE buffer (Tris/Boric acid/ EDTA) 0.5× pH 8.3 prepared from a 5× stock solution (1 litre: 445 mM Tris; 445 mM boric acid; 10 mM EDTA). 3 µl loading buffer (sucrose, 50%; EDTA, 50 mM; bromophenol blue 0.01%; Urea, 4 M) + 10 µl samples were placed in each well. Markers used were O'GeneRuler (sizes of marker O'GR are presented in index) 1 kb DNA Ladder (Fermentas), 100 bp Smart Ladder (Eurogentec), λ *Hin*dIII and λ *Hin*dIII+*Eco*RI (stock of laboratory). The results were determined by GelDoc from Bio-Rad. Analysis was performed by using the software Quantity One (version 4.1.1) and the photos were captured in format TIFF.

2.10.10. Transformation techniques

2.10.10.1. Transformation by thermal shock

It is one of the most often used techniques for transferring a plasmid into *E. coli*. The tubes containing *E. coli* JM109 (competent cells that are stored at -70°C) are thawed on ice during 10 min. Then, it is added 2-3 µl (it depends on plasmid concentration) of the desired plasmids or ligation mixtures to 50 µl of the competent cells; mix quietly and keep on ice for 20 min. They are exposed to 42°C for 90 sec in a water bath. Then, they are immediately transferred on ice for 2 min. 950 µl SOC medium were added to them and the cultures were incubated at 37°C, 150 rpm for 90 min. The transformants are selected on LB containing the required antibiotics and incubated at 37°C for 24-48 h.

2.10.10.2. Electroporation method 1

There are many transformation methods, one of which is electroporation, a simple and widely used technique, for various bacterial species. This technique uses an electric pulse treatment of cells to induce a membrane potential which causes breakdown of the cell membrane permeation barrier to allow the entry of DNA into the cells (Tsong, 1992). In principle, the induced membrane potential, and hence efficiency of DNA entry into bacterial cells, increases with the strength of an applied electric field. However, the percentage of cell death caused by electrical damage also increases with the applied field strength. As a result, the transformation efficiency is the combined effect of these two factors under a given transformation condition.

B. licheniformis ATCC 14580 was transformed according to the following protocol:

An overnight culture in LB (10 ml) of this strain is prepared and incubated at 37°C at 140 rpm. 2 ml of this culture are inoculated to 100 ml of fresh LB and incubated at 37°C at 140 rpm up to an OD_{600} of 0.5-1.0 (approximately after 3 h).

20 ml of this culture is cooled on ice-water for 10 min and centrifuged in a sterile tube at 5240×g for 10 min at 4°C in a pre-chilled centrifuge.

The supernatant is discarded and the cells are suspended in 20 ml of cold MEB buffer and recentrifuged.

Then, previous step is repeated. The supernatant is discarded, 1 ml of cold MEB buffer is added and the cells are resuspended. They are transferred into a sterile and cold 1.5 ml Eppendorf tube and centrifuged at 5240×g for 5 min at 4°C in a pre-chilled centrifuge.

The supernatant is discarded and the cells are suspended in 100 µl of cold MEB buffer. About 150 ng of plasmidic DNA are added and after mixing, the tube is placed on ice for 5 min.

The cells are transferred into a pre-chilled electroporation cuvette and exposed to a single electrical pulse for 3 ms using a Bio-Rad Gene Pulser set at 2.5 kV, 25 µF and 200 Ω.

900 µl SOC medium (room temperature) are immediately added to the cuvette and mixed slowly and transferred into a sterile tube and incubated 1 h at 37°C at 140 rpm.

The cells are spread in LB plates containing 100 µg ml^{-1} spectinomycin at 37°C for 24 h.

The resulting isolated transformants were expected to contain pBG162- and pBG163-generated chromosomal insertions which were selected on the following LB plates:

LB + blood; LB + blood + xylose; LB + kanamycin 50 µg ml^{-1}.

Transformants are enumerated after overnight incubation at 37°C.

2.10.10.3. Electroporation method 2

To prepare electro-competent cells using the optimized high-osmolarity protocol, an overnight culture of *B. licheniformis* was diluted 20-fold in growth medium (LB containing 0.5 M sorbitol) and was grown at 37°C up to an OD_{600} of 0.85-0.95. The cells were cooled on ice-water for 10 min and harvested by centrifugation at 4°C and 5240×g for 5 min. Following four washes in ice-cold electroporation medium (0.5 M mannitol and 10% glycerol), the cells were suspended in 1/40 of the initial culture volume of the electroporation medium, giving a cell concentration of 1-1.3 $\times 10^{10}$ cfu ml^{-1}. The competent cells can be stored at -80°C until use with some decrease in transformation efficiency. For electroporation, 60 µl of the competent cells were mixed with 1 µl (50 ng/µl) of DNA and then transferred into an ice-chilled electroporation cuvette (1 mm electrode gap). After incubation for 1-1.5 min, the cells were exposed to a single electrical pulse using a Gene Pulser set at 2.5 kV, 25 µF and 200 Ω, resulting in time constants of 4.5-5 ms. Immediately following the electrical discharge, 1 ml of recovery medium (LB containing 0.5 M sorbitol and 0.38 M mannitol) was added to the cells. After incubation at 37°C for 3 h, the cells were plated on LB plates

containing spectinomycin 400 μg ml^{-1}, blood and xylose. Transformants were enumerated after overnight growth at 37°C.

2.10.10.4. Natural competence

This method is often used for transformation of *B. subtilis* which is a naturally competent species. An overnight culture of *B. licheniformis* 14580 was obtained in 5 ml MS1 medium at 37°C and 140 rpm (on the basis of isolating of a fresh culture). OD_{600} was measured and the volume was calculated to obtain an OD_{600} of about 0.7. The culture was centrifuged and was added MS1 medium to get an OD_{600} 0.7 and then incubated at 37°C, 140 rpm for 5 h. The culture was diluted 10-fold with MS2 medium (0.5 ml of the culture was added to 4.5 ml of MS2). It was incubated in a water bath at 37°C 140 rpm for 90 min. About 150 ng of plasmidic DNA was added to 100 μl of competent cells. The mixture was incubated in a water bath at 37°C for 30 min and was then plated on LB containing spectinomycin, blood and xylose for selection of transformant cells containing pBG162- or pBG163-generated chromosomal insertions as follows:

LB + 100 μg ml^{-1} spectinomycin
LB + 100 μg ml^{-1} spectinomycin + 1% xylose
LB + blood + 100 μg ml^{-1} spectinomycin
LB + blood + 100 μg ml^{-1} spectinomycin + 1% xylose
Transformants were enumerated after overnight incubation at 37°C.

2.10.11. Verification of genetic constructions

There are different strategies for verification of genetic constructions. When a plasmid was constructed, its presence is verified by electrophoresis on 0.7% agarose gel after extraction as previously mentioned (§ 2.10.4).
To determine the orientation of the fragments inside a plasmid, it is digested by different restriction enzymes (in this study it was used *Sac*II and *Hin*dIII).
All constructions were verified by PCR and DNA sequencing by Cogenics Genome Express (Meylan, France).

2.11. XPS analyses

XPS analyses were performed on a Kratos Axis Ultra spectrometer (Kratos Analytical, Manchester, UK) equipped with a monochromatized aluminium X-ray source (powered at 10 mA and 15 kV) and an eight channeltrons detector. The spectrometer was interfaced with a Sun Ultra 5 workstation. Instrument control and data acquisition were performed with the Vision2 program.
Stainless steel coupons were coated with various lipopeptides dilutions and rinsed, as previously described (§ 2.7). The samples were allowed to air dry in a laminar air flow cabinet.
The samples were fixed on a standard stainless steel multispecimen holder by using a piece of double sided isolative tape.
The pressure in the analysis chamber was about 10^{-6} Pa. The angle between the sample surface and the direction of photoelectrons collection was about 0°. In another experiment, the angle for surfactin was changed to 60°. The X-ray bombarded area was approximately 2000 µm × 800 µm. Analyses were performed in the hybrid lens mode, a combination of magnetic and electrostatic lenses, with the slot aperture and the iris drive position was set at 0.5. The resulting analyzed area was 700 µm × 300 µm. The pass energy of the hemispherical analyzer was set at 160 eV for the wide scan and 40 eV for narrow scans. In the latter conditions, the full width at half maximum (FWHM) of the Ag $3d_{5/2}$ peak of a standard silver sample was about 0.9 eV.
Charge stabilisation was achieved by using the Kratos Axis device. An electron source mounted co-axially to the electrostatic lens column and a charge balance plate used to reflect electrons back towards the sample. The magnetic field of the immersion lens placed below the sample acts as a guide path for the low energy electrons returning to the sample. The electron source was operated at 1.9 A filament current and a bias of -1.1 eV. The charge balance plate was set at -3.3 V.
The following sequence of spectra was recorded:
On stainless steel: survey spectrum, C1s, O1s, Cr 2p, Fe 2p, Ni 2p, Mo 3d, N 1s, Na 1s, Ca 2p, P 2p, S 2p, Si 2p, and C1s again to check for charge stability as a function of time and the absence of degradation of the sample during the analyses.
On PTFE: survey spectrum, C1s, F1s, O 1s, N 1s, S 2p, Si 2p and F 1s and C 1s again to check for charge stability as a function of time and the absence of degradation of the sample during the analyses.

Results

3. Production, purification and characterization of biosurfactants

3.1. Introduction

Lipopeptides surfactin S1, iturin A, mycosubtilin and fengycin and also rhamnolipids were bought or produced and/or purified in our laboratory. To determine lipopeptide concentrations in supernatants and the presence of different variants, HPLC analysis was done. MALDI-TOF/MS or LC/MS-ESI (as required) was performed to characterize their structures and to verify their purities.

The surface tension of the solutions used for the next experiments, as well as the CMC of the lipopeptides in these conditions were also determined for each family.

3.2. Lipopeptides

3.2.1. Surfactin

Surfactin S1 was purchased from Sigma. The powder was disolved in pure methanol at a concentration of 500 mg l^{-1}. Analytical HPLC analysis was done and eleven different peaks were separated (Figure 3.1). The 2nd derivative of the UV spectrum of each peak was obtained and compared to a reference (Figures 3.2). All of them showed the typical 2nd derivative of the UV spectrum of surfactin. To verify surfactin homologues, LC/MS was performed for each peak. The ion masses $[M+Na]^+$ 1058, 1072.9 and 1086.9 with high intensity in two latters were observed between 4.5 to 7.7 min (Table 3.1 and Figure 3.3).

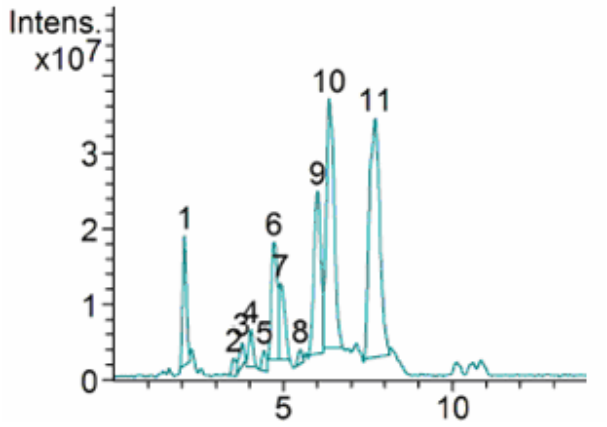


Figure 3.1. Chromatogram of analytical HPLC (absorbance at 214 nm) for surfactin from Sigma.

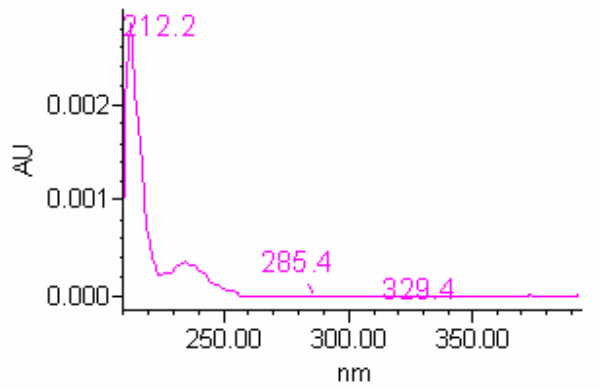


Figure 3.2. The 2nd derivative of the UV spectrum of surfactin.

Table 3.1. Ion mass for different surfactin homologues in Sigma sample

Number of peak	Retention time (min)	Ion mass $[M+Na]^+$
1	2.1	301
2	3.6	353.3, 1022.8
3	3.8	1044.8
4	4.1	1044.8
5	4.5	1058.9
6	4.7	1058.9
7	4.9	1058.9
8	5.5	1058.9
9	6.0	1072.9
10	6.4	1072.9
11	7.7	1086.9

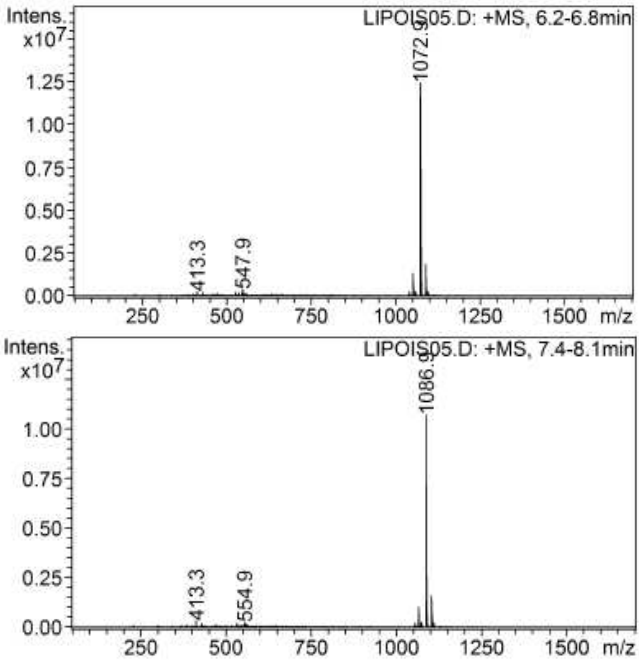


Figure 3.3. MS spectra of surfactin S1 (Sigma).

In literature, the mixture of surfactin homologues mainly contains C14 and C15 with ion masses $[M+Na]^+$ of 1044 and 1058, repectively. Surprisingly, in this surfactin sample from Sigma, the homologues C16 and C17 (with ion masses 1072.9 and 1086.9) were the main compounds, while homologue C13 was not observed.

ST (§ 2.6) and CMC for this surfactin solution were determined as 31.5 mN m^{-1} (standard deviation 0.05) and ~10 mg l^{-1}, respectively (Figure 3.4).

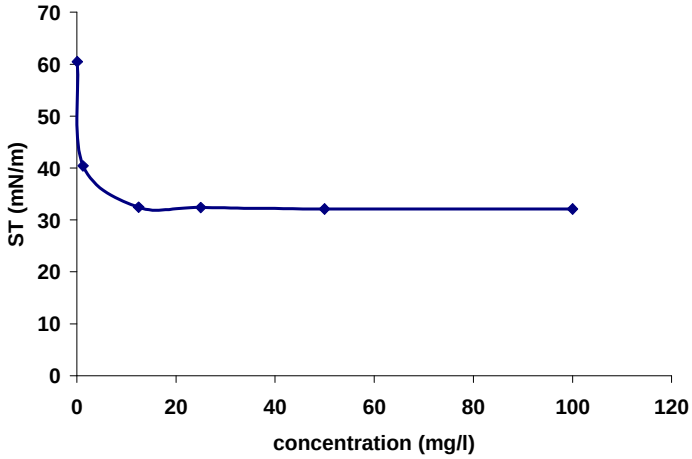


Figure 3.4. The surface tensions of the serially diluted surfactin S1.

3.2.2. Iturin A

Iturin A was kindly provided by Dr Magali Deleu from Gembloux Agro-Bio Tech, Belgium.
In addition, to obtain larger amounts of required lipopeptide, iturin A was extracted on C18 column and purified by TLC (§ 2.3.4) from the supernatant of a culture of *B. subtilis* S499 in optimized medium (Akpa *et al*. 2001) (kindly provided by Dr Marc Ongena from Gembloux Agro-Bio Tech, Belgium).

HPLC analysis of supernatant (§ 2.3.5) detected 100 mg l^{-1} iturin A in 300 ml supernatant (Figure 3.5). Figure 3.6 shows the 2nd derivative of the UV spectrum of iturin A. After extraction by C18 cartridge, its concentration was determined 20 mg by HPLC, while 6 mg iturin A was finally obtained after purification by TLC (Table 3.2). Recovery efficiency (18%) was very small due to the high concentrations of nutrients present in optimized medium compared to Landy medium (§ 2.1) and the fact that a large amount of lipopeptide was lost during extraction procedures. The purity of final product was determined 90-95%.

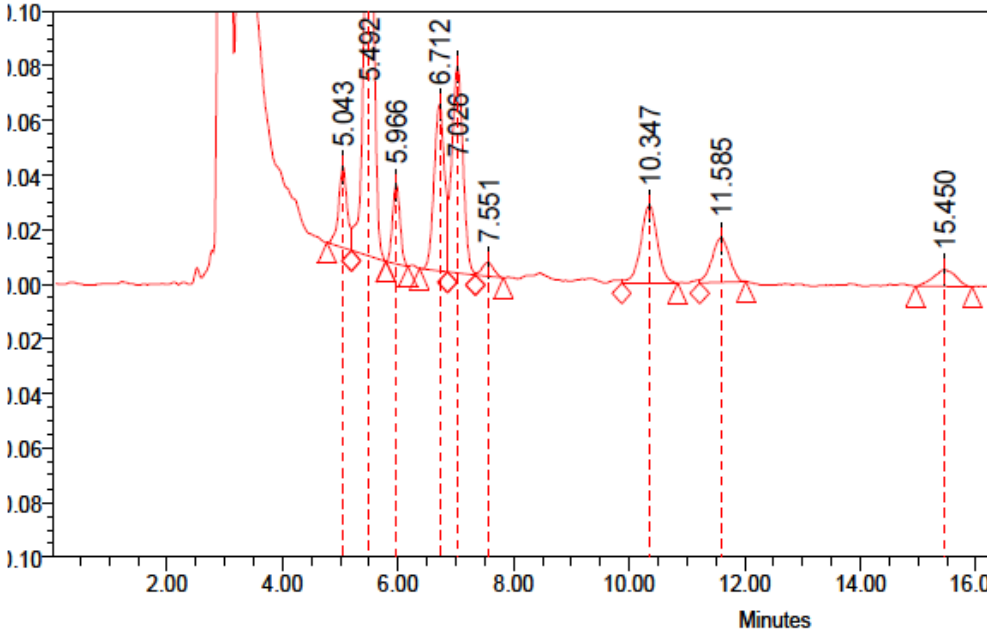


Figure 3.5. HPLC chromatogram of iturin A.

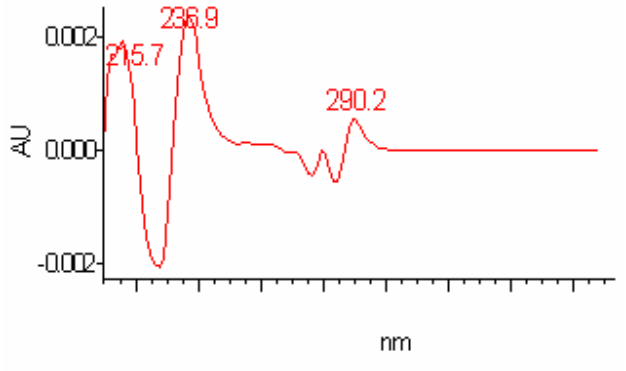


Figure 3.6. The 2nd derivative of the UV spectrum of iturin A.

Table 3.2. Amount of iturin A in different steps of purification

Supernatant (mg)	After extraction (mg)	Final product (mg)	Recovery (%)	Purity of final product (%)
30	20	6	18	90-95

MALDI-TOF/MS analysis of both samples allowed identification of several homologues of iturin A (Figure 3.7).

Ion masses attributed to protonated forms of iturin A purified in our laboratory and their Na^+ and K^+ adducts are summarized in Table 3.3. In the sample taken from Belgium (Belgium sample), the high intensity signals at *m/z* 1043.7 and other low intensity signals such as at *m/z* 1057.7, 1065.0 and 1105.6 were observed. The ion mass 1043.7 corresponds to the protonated homologue C14 (in majority). Other ion masses (minor) correspond to the protonated H^+ (C15) and Na^+ (C14) ions of iturin A (Table 3.3).

In the sample purified in our laboratory (Lab sample), the signal at 1043.7 was similar to that of the sample obtained from Belgium. On the other hand, in this sample signals at *m/z* 1057.7, 1065.7 and 1079.7 were observed with a high intensity. Ion masses 1057.7 and 1079.7 correspond to homologue C15 ($[M+H]^+$ and $[M+Na]^+$, respectively) of iturin A. Regarding ion masses, the ratio C14/C15 was 1.9 for the Lab sample and 4.6 for the Belgium sample. It means that the proportion C15 is higher in the Lab sample.

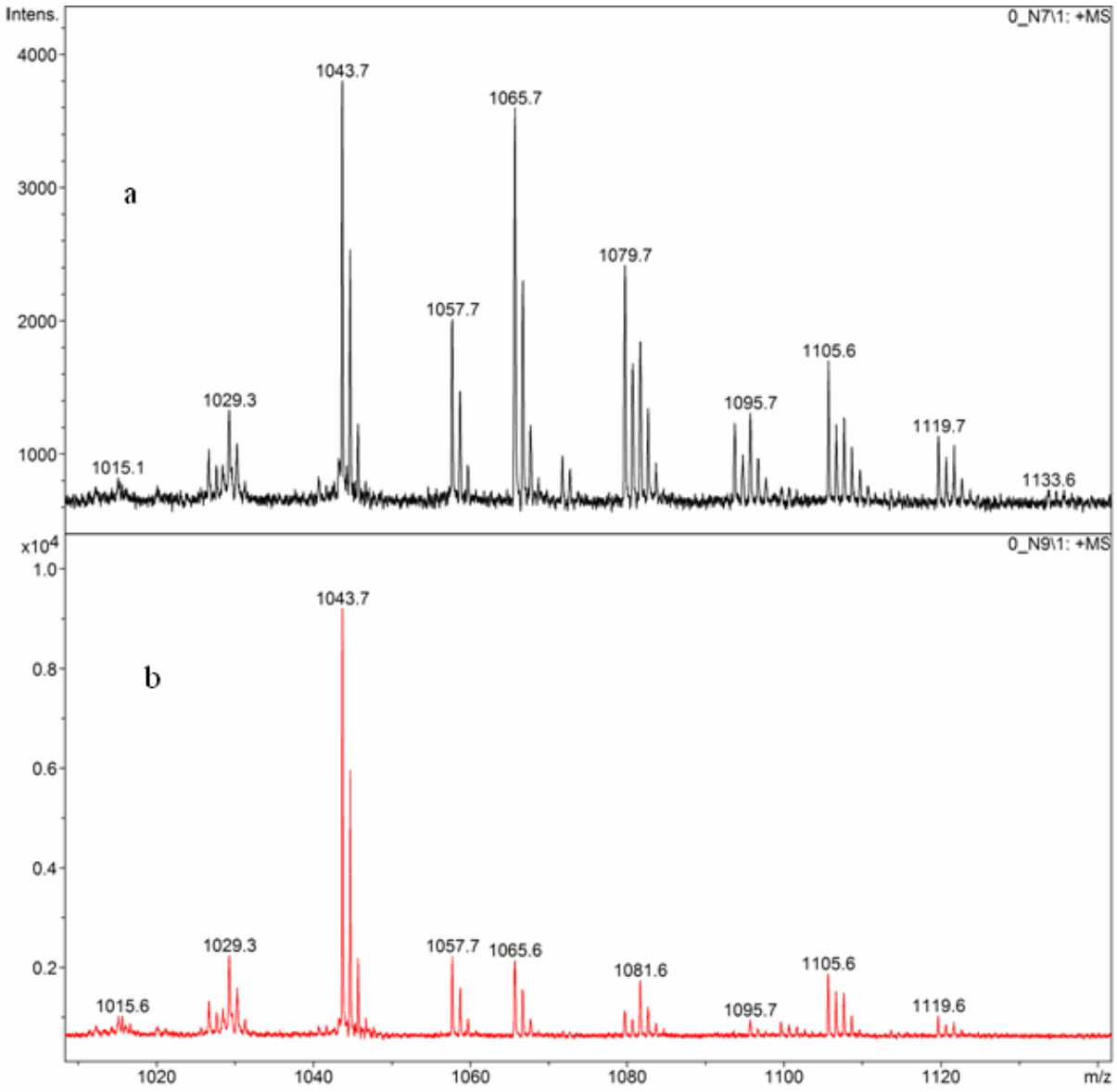


Figure 3.7. MALDI-TOF/MS spectra of iturin A produced by *B. subtilis* S499. a: The spectrum of iturin A purified in the Lab, b: The spectrum of iturin A taken from Belgium. Intens: intensity.

Table 3.3. Ion masses obtained from iturin A

Carbon atoms	**$[M+H]^+$**	**$[M+Na]^+$**	**$[M+K]^+$**
C14	1043.7	1065.7	1081.7
C15	1057.7	1079.7	1095.7

ST and CMC of the two samples of iturin A purified were also determined (Figure 3.8, Table 3.4). There are differences among ST of these samples. As mentioned previously (§ 1.1.5.3), iturin A is produced as a mixture of different homologues and the proportion of these homologues depends on culture conditions which causes to change ST in different samples. The Belgium sample contains more homologue C14 and has higher ST than the Lab sample.

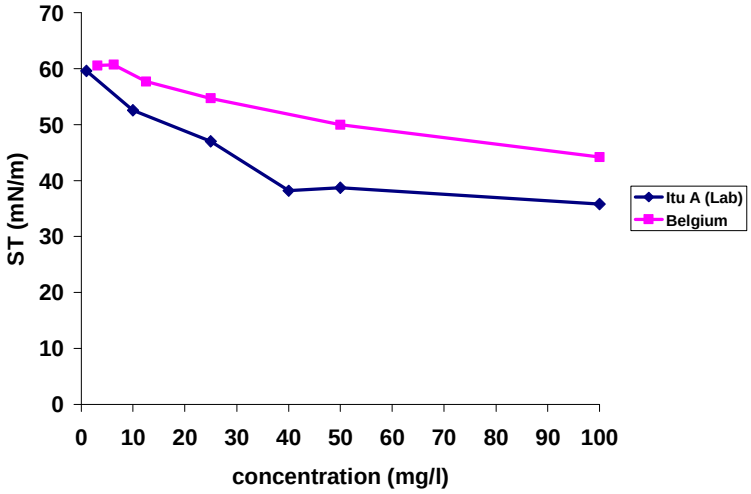


Figure 3.8. The surface tensions of the serially diluted iturin A: Lab sample and Belgium sample.

Table 3.4. Results of ST and CMC determination for iturin A

Iturin A	**ST (mN m^{-1})**	**CMC (mg l^{-1})**
Literature*	54	43
Lab sample	36 ± 0.35**	38
Belgium sample	44.2 ± 0.6**	>48

* Deleu 2000, **Standard deviation

3.2.3. Mycosubtilin

Mycosubtilin was produced by *B. subtilis* BBG100 in batch condition in Landy modified at 30°C for 72h at 140 rpm. It was extracted and purified as previously mentioned (§ 2.3.3). HPLC analysis (§ 2.3.5) showed that *B. subtilis* BBG100 produced 230 mg l^{-1} mycosubtilin in supernatant (Figure 3.9).

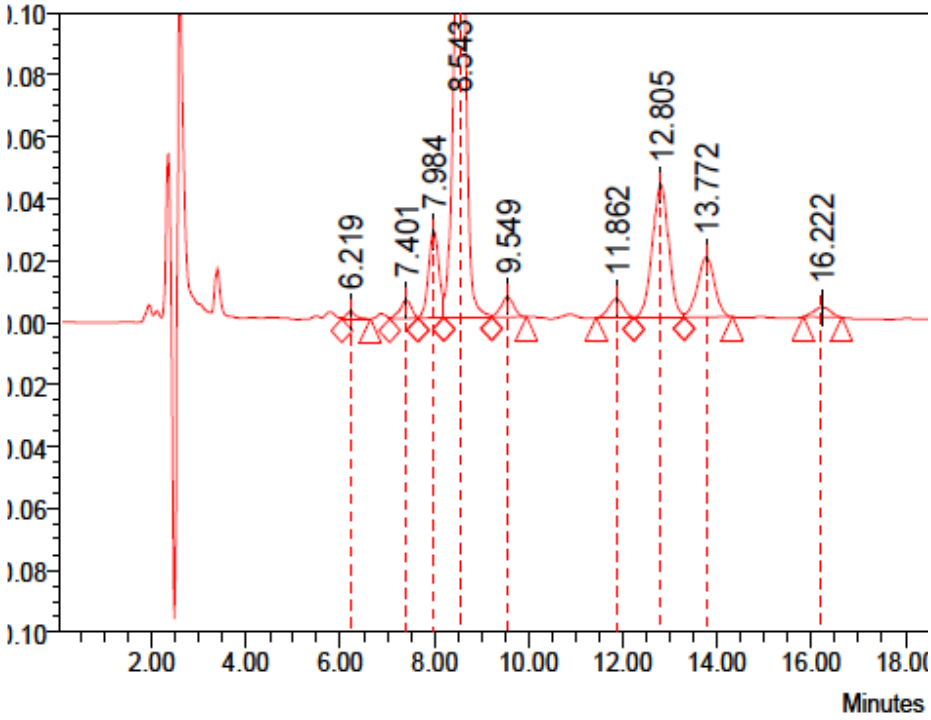


Figure 3.9. HPLC chromatogram of mycosubtilin.

MALDI-TOF/MS analysis of lipopeptide (obtained from supernatant) allowed identification of several homologues of mycosubtilins (Figure 3.8). The high intensity signals at *m/z* 1109.5 and 1123.5 correspond to the ion $[M+K]^+$ of homologues C16 and C17 (Table 3.5), respectively.

The ion 1137 could be homologue C18 from the ion $[M+K]^+$ or an isoform in which amino acid moiety is modified (e.g. Gln(1) instead of Asn(1)).

Table 3.5. The mass values of $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ ions corresponding to identified homologues of mycosubtilin in culture extracts from *B. subtilis* BBG 100

Mycosubtilin	**$[M+H]^+$**	**$[M+Na]^+$**	**$[M+K]^+$**
C16	1071.58	1093.56	1109.54
C17	1085.6	1107.58	1123.55

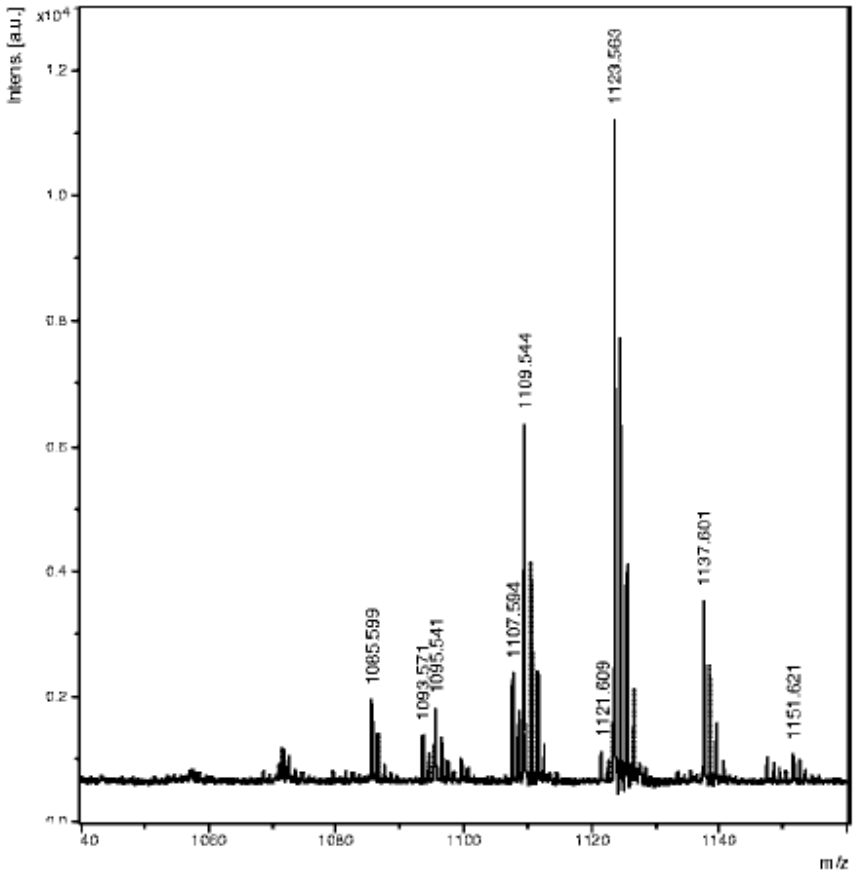


Figure 3.8. MALDI-TOF/MS spectra of mycosubtilins produced by *B. subtilis* BBG100.

ST and CMC of purified mycosubtilin were also determined (Figure 3.9, Table 3.6).

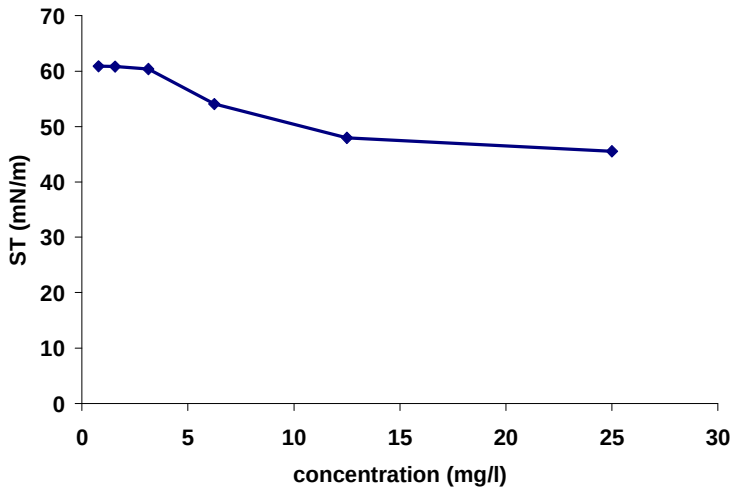


Figure 3.9. The surface tensions of the serially diluted mycosubtilin.

Table 3.6. Results of ST and CMC determination for mycosubtilin

Mycosubtilin	ST (mN m^{-1})	CMC (µM)
Literature*	55	37
Lab sample	45 ± 0.36**	~15

* Thimon *et al.* 1992, **Standard deviation

3.2.4. Fengycin

Fengycin was produced by *B. subtilis* ATCC 21332 in Landy modified medium at 30°C for 72 h at 140 rpm. Then, it was extracted and purified as previously mentioned (§ 2.3.3). *B. subtilis* ATCC 21332 produced about 600 mg l^{-1} fengycin in supernatant. 54 mg product from 200 ml culture was obtained. The recovery of the purification was 45%.

HPLC analysis (§ 2.3.5) gave peaks between 8 and 18 min (Figure 3.10). Peaks were selected through second derivative spectra (Figure 3.11) which gave 2 major peaks at 213 and 236 nm associated with a minor peak at 290 nm.

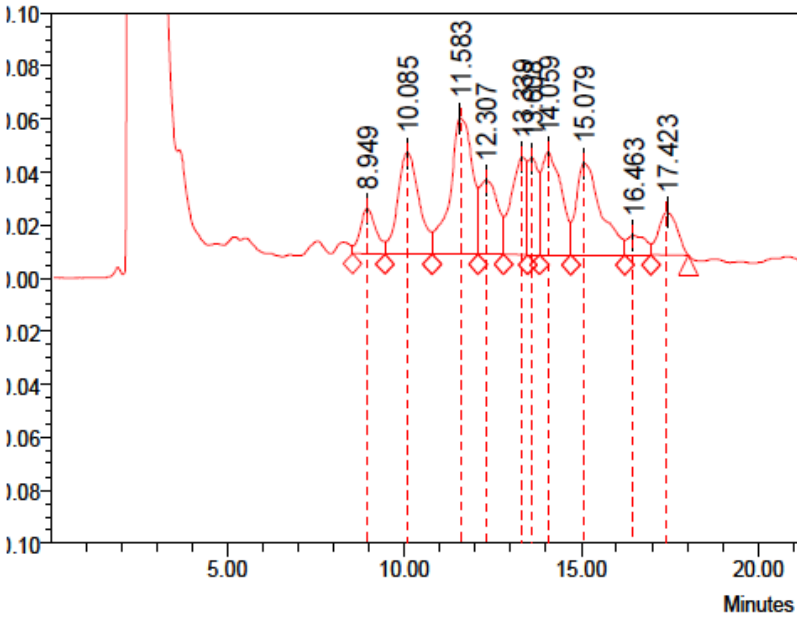


Figure 3.10. HPLC chromatogram of fengycin.

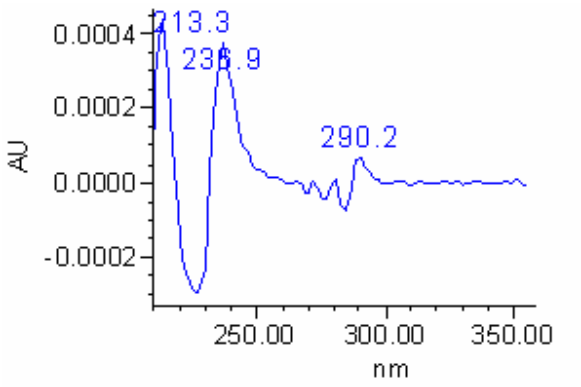


Figure 3.11. The 2nd derivative of the UV spectrum of fengycin.

ST and CMC of fengycin were determined (Figure 3.12 and Table 3.7).

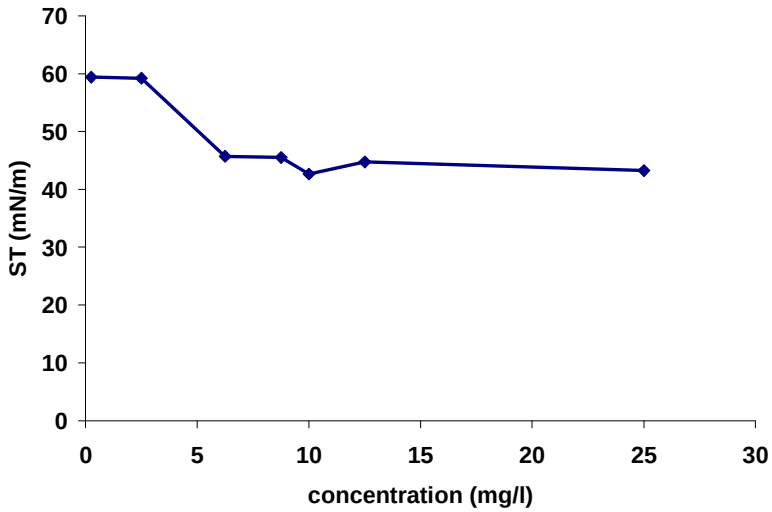


Figure 3.12. The surface tensions of the serially diluted fengycin.

Table 3.7. Results of ST and CMC determination for fengycin

Fengycin	**ST (mN.m^{-1})**	**CMC (µM)**
Literature*	42	4.6
Lab sample	43 ± 0.05**	4.3

* Deleu 2000, **Standard deviation

3.3. Discussion

The results of all analyses obtained in this study are summarized at the Table 3.7.

The critical micelle concentration is slightly different for each homologous compound (Deleu *et al*. 2003). The different solutions of lipopeptides used in this study contained several homologous compounds from the same family, so ST and CMC of these molecules are a little different from those of the literature.

Regarding recovery of purification of final product, the extraction methods should be improved. The purity of biosurfactants was determined between 90-95% by HPLC and MALDI-TOF/MS.

Table 3.7. Summary of different analyses on lipopeptides

Lipopeptide	**Recovery (%)**	**Purity (%)**	**CMC (mg l^{-1})**	**ST (mN m^{-1})**
Surfactin	obtained from Sigma	98	~10	31
Iturin A (Lab)	18	90	38	36
Iturin A	obtained from Belgium	90	>48	44.2
Mycosubtilin	44	93	~15	45
Fengycin (Lab)	45	94	6.25	43

3.4. Rhamnolipids

3.4.1. Production

P. aeruginosa 1637 was cultivated under agitation 150 rpm in flask in Lindhardt medium (LH) with 6% corn oil (§ 2.1) at 30°C for 96 h. The presence of biosurfactants was proved by different analytical methods.

Rhamnolipid concentration in supernatant was determined ~20 g l^{-1} by the orcinol assay (§ 2.4.4.1). The value was calculated from a standard curve (Figure 3.13) prepared with L-rhamnose concentrations as a function of optical density. The concentration was expressed as rhamnolipid

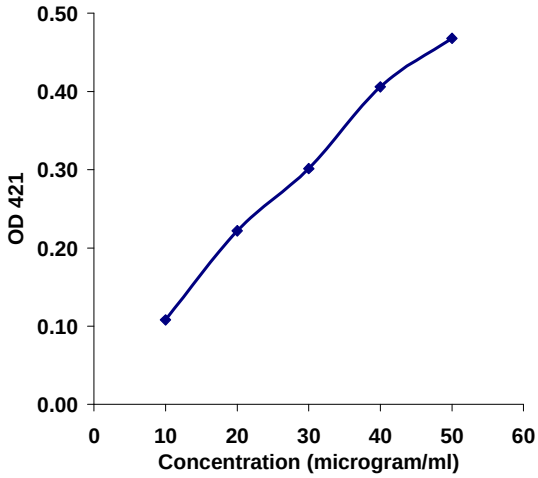


Figure 3.13. Rhamnose standard curve.

values by multiplying rhamnose values (obtained from the curve) by a coefficient of 3.4 (obtained from the correlation of pure rhamnolipids/rhamnose). The concentration confirmed by HPLC (§ 2.4.4.2) was 23 g l^{-1} in sample.

Biosurfactant was studied using sample A and sample B to determine stability of rhamnolipid during time. The sample A was a dark brown sticky solution that had been kept for one year under refrigeration condition (4ºC). The sample B was a fresh one and light brown. ST and TLC were determined for both samples. The storage of sample A had not affected rhamnolipid quality significantly when it was compared with sample B (ST and TLC demonstrated the same result). Different culture media (Lindhardt and 3M) were used in different culture times (4, 7 and 9 days) (§ 2.4.5). Neither the different production media (Lindhardt and 3M), nor the different times of culture influenced ST measurements and TLC spots, but higher amount of rhamnolipid were

produced in Lindhardt medium than in 3M medium: 20 g l^{-1} against 15 g l^{-1}. In addition, no change in these measurements was observed after autoclaving the samples: This confirms that this particular rhamnolipid is resistant to heat.

Furthermore, it was used two methods of extraction for rhamnolipids (§ 2.4.2). No difference could be observed according to the method used (after TLC and ST measurements).
The results of ST and CMC are observed in Table 3.8 and Figure 3.14.

Table 3.8. ST and CMC of rhamnolipids produced in LH and 3M medium after 96 h

Medium	Production (g l^{-})	ST (mN m^{-1})	CMC (mg l^{-1})
LH	20	29.4 ± 0.1*	95
3M	15	26.8 ± 0.1*	75

* Standard deviation

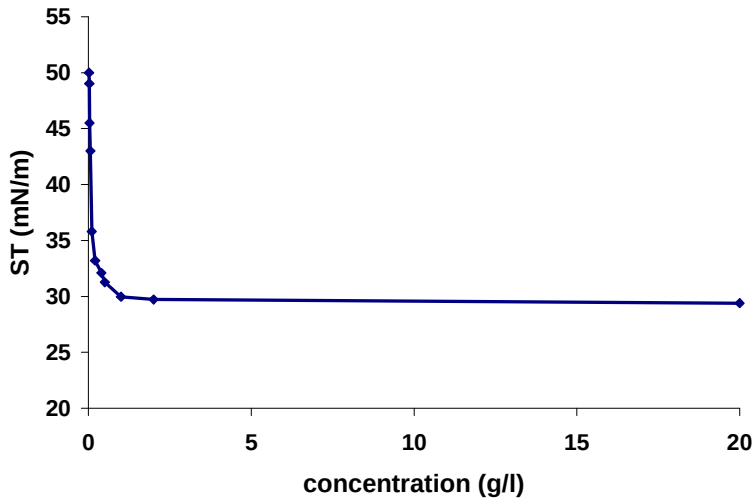


Figure 3.14. The surface tensions of the serially diluted rhamnolipids in LH medium.

3.4.2. TLC

The produced rhamnolipids were separated on TLC plates (§ 2.4.3). A commercial rhamnose and a standard from JBC (a mixture of mono- and di-rhamnolipids) were used as control. The TLC plates were visualized with Molisch reagent (§ 2.4.3) and two predominant spots were observed which corresponded to spots of standard. The lower spot with R_f 0.3 belongs to the di-rhamnolipid

structures and the higher spot with R_f 0.7 belongs to mono-rhamnolipid molecules. The commercial rhamnose sample had a characteristic spot close to the first spot of Lab samples (R_f value 0.23).

The R_f values of two observed spots are in agreement with the literature (Arino *et al*. 1996; Amiriyan *et al*. 2004; Gunther *et al*. 2005 and Wang *et al*. 2008) in which they are referred to RL3 (di-rhamnolipid) and RL1 (mono-rhamnolipid), respectively.

3.4.3. HPLC analysis

HPLC was performed for purified rhamnolipids as previously mentioned (§ 2.4.4.2). The chromatogram demonstrated 2 peaks corresponding to mono- and di-rhamnolipids in comparison with the standard with retention times 13 and 24 min (Figure 3.15).

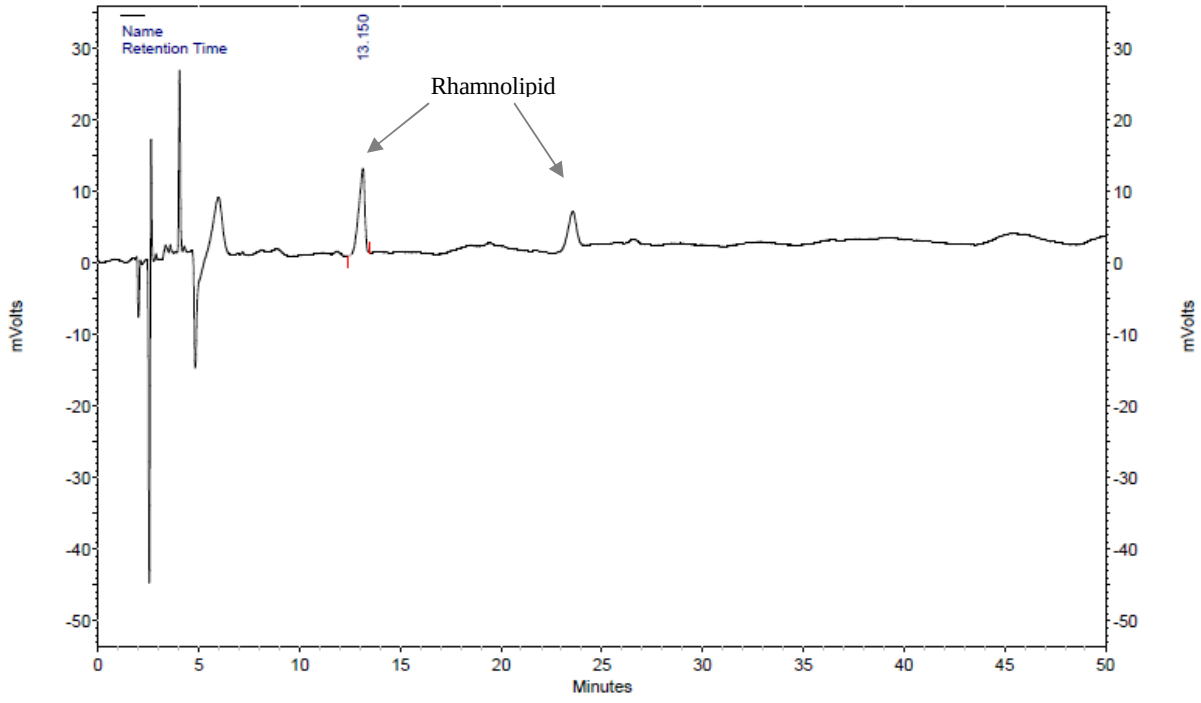


Figure 3.15. HPLC chromatogram of rhamnolipids that shows 2 peaks correspond to mono- and di-rhamnolipids in comparison with the standard.

3.4.4. HPLC/MS Analyses

The different samples were analyzed by HPLC/MS analysis to further confirm the structure of rhamnolipids. The samples designated as A (one year storage), LH7 (cultivated in Lindhardt medium for 7 days) and 3M (cultivated in 3M medium for 7 days) were submitted to HPLC/MS. The peak profile of these chromatograms in all samples were similar. With negative electrospray ionisation and under the operating parameters used, fragmentation of the pseudomolecular ions occurred. Most ions between m/z 275 and 507 are fragment ions produced by cleavage (Deziel *et al*. 2000). This procedure exhibited an ion mass at *m*/*z* 479, common in three samples (Table 3.9 and Index III). The product corresponds to the fragment of Rh-Rh-C10 of di-rhamnolipids. Ion mass at *m*/*z* 329 found in the sample LH7 is unknown. The two spots of TLC obtained from LH sample were also analyzed with HPLC/MS. Spot 1 showed a major ion mass at *m*/*z* 339 which corresponds to fragment C10-C10.

Table 3.9. Homologues and some rhamnolipid fragments detected by HPLC/MS

Sample	Pseudomolecular /Fragment ion	Retention time (min)	Putative compound
A, 3M7	479	9.6	Rh-Rh-C10
LH7	479.3 329.3	9.9 17.5	Rh-Rh-C10 ?
LH7 TLC: spot 1	339	1.4	C10-C10
LH7 TLC: spot 2	337 451	1.3	C8-C12:1, C12:1-C8 Rh-Rh-C8

The spot 2 also demonstrated one predominant ion mass at *m*/*z* 337 that corresponds to the fragment C8-C12:1 or C12:1-C8 and a minor ion mass at m/z 451 that corresponds to the fragment Rh-Rh-C8. Figure 3.16 can explain possibly fragmentation patterns produced for rhamnolipid Rh-Rh-C10-C10.

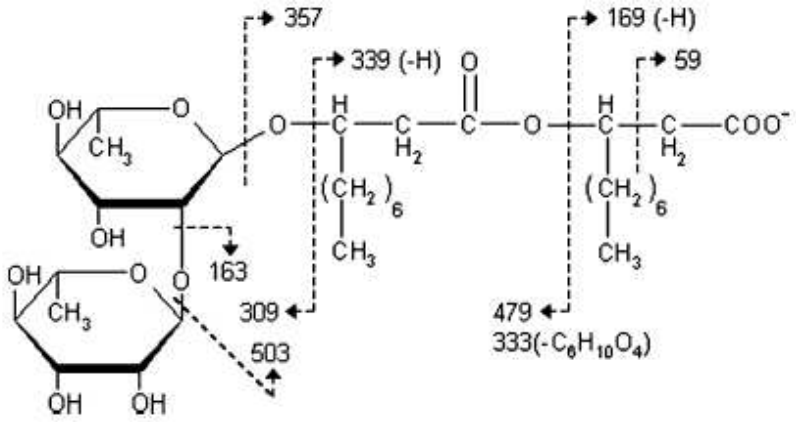


Figure 3.16. Possible fractionation sites of rhamnolipid Rh-Rh-C10-C10 (Heyd *et al.* 2008).

3.4.5. FTIR

The rhamnolipid fractions obtained from HPLC were analyzed by FTIR in order to confirm the structure of rhamnolipids produced by *P. aeruginosa* PTCC 1637.

Infrared analysis of rhamnolipids produced by *P. aeruginosa* 1637 revealed a pattern similar to that of rhamnolipid standard, indicating that the biosurfactant is a glycolipid. A characteristic FTIR transmittance spectrum of Rh-Rh-C10-C10 is shown in Figures 3.17 and 3.18.

The rhamnolipid infrared spectrum based on the band characteristics information indicates the presence of wavenumber 3368, OH; wavenumber 2923, alkyl chain C-H; wanenumber 1732, C=O; wavenumber 1050, C-O-C; and wavenumber 1456, COO stretching modes (Table of wanenumbers is shown in Index IV).

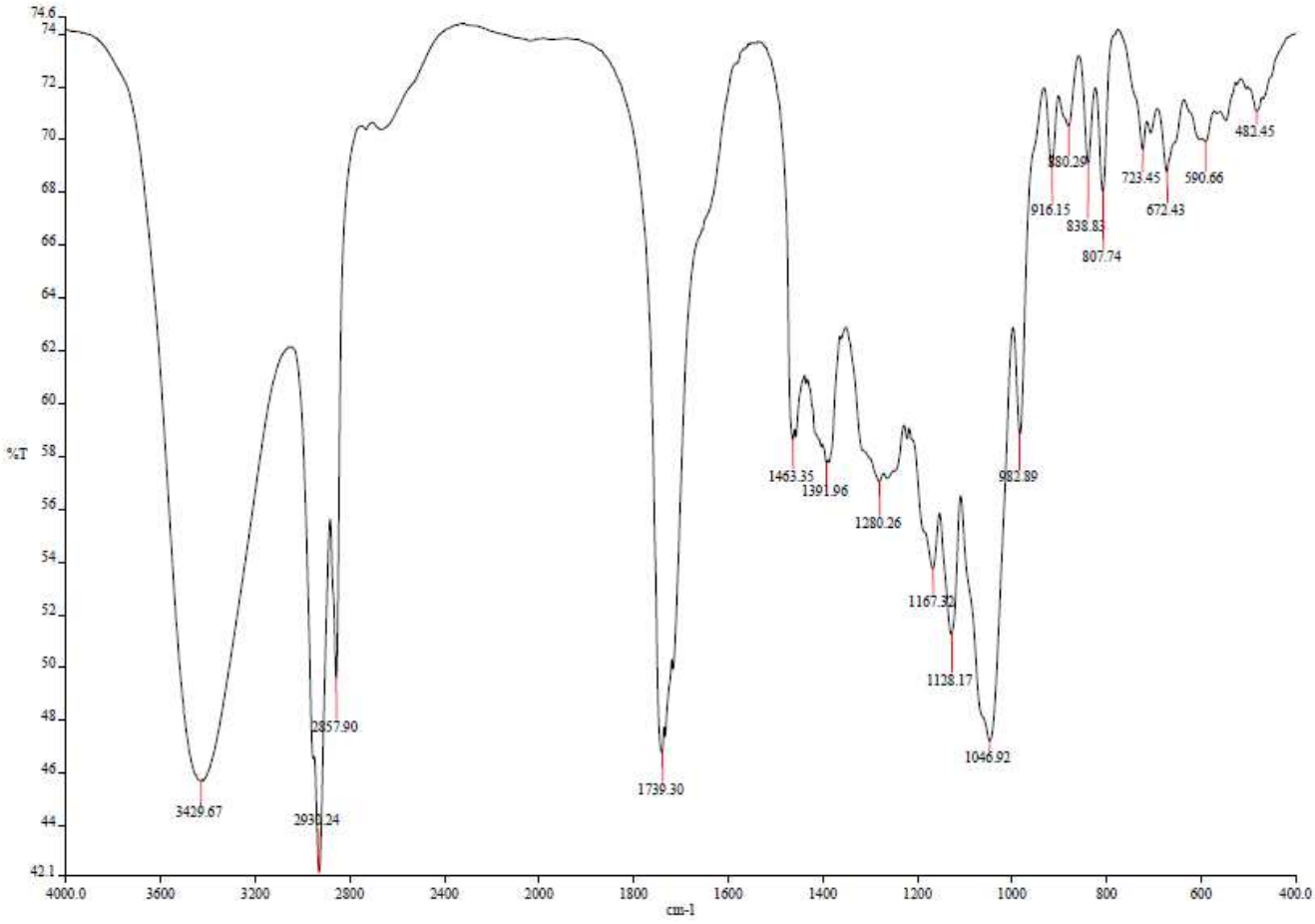


Figure 3.17. FTIR spectrum analysis of rhamnolipid cultivated in Lindhardt medium for 7 days. Major absorption valleys are assigned in the chart.

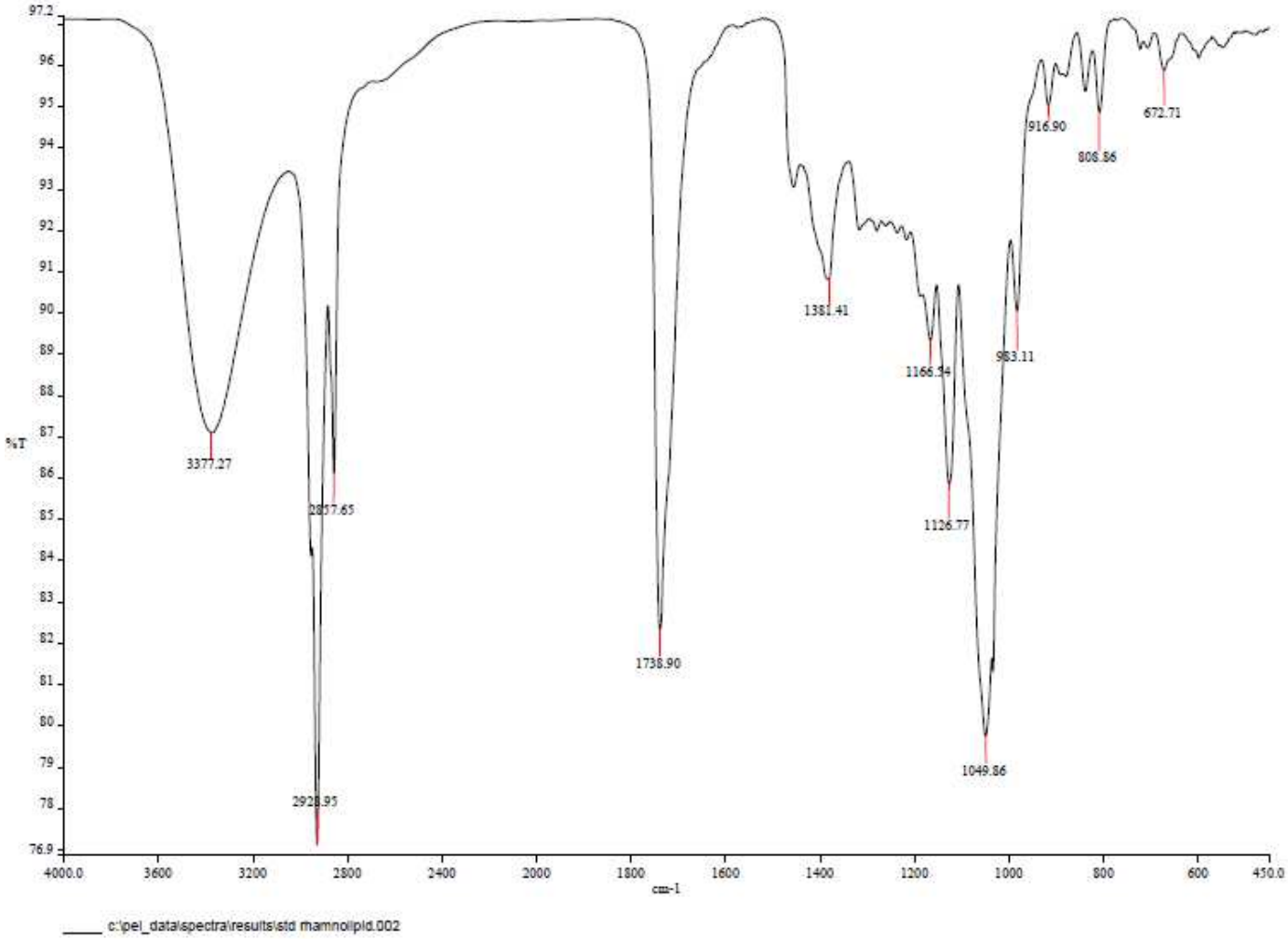


Figure 3.18. FTIR spectrum analysis of rhamnolipid standard. Major absorption valleys are assigned in the chart.

3.5. Discussion

Rhamnolipids were first isolated from *P. aeruginosa* and described by Jarvis and Johnson in 1949. These compounds are predominantly constructed from the union of one or two rhamnose sugar molecules and one or two β-hydroxy (3-hydroxy) fatty acids (Lang *et al*. 1999). Rhamnolipids with one sugar molecule are known as mono-rhamnolipids and those with two sugar molecules are di-rhamnolipids. The length of the carbon chains found on the β-hydroxyacyl portion of the rhamnolipid can vary significantly. In this study, *P. aeruginosa* 1637 produced rhamnolipids by using corn oil as sole carbon source (the previous studies in the Lab showed this

carbon source is the best one for higher yield) in LH medium. 3M medium was also used for production, but higher amount of rhamnolipids were produced in LH medium than in the previous one: 20 g l^{-1} against 15 g l^{-1}. In LH medium, ST and CMC were determined as 29 mN m^{-1} and 95 mg l^{-1}, respectively.

Two different methods were used for purification with similar results. The storage and autoclaving of rhamnolipid did not affect quality significantly.

TLC results exhibited two spots in which the lower spot with R_f 0.3 belongs to the di-rhamnolipid structures and the higher spot with R_f 0.7 belongs to mono-rhamnolipid molecules.

For a better understanding of the chemical structures of rhamnolipids, the composition of mixtures, and their surface-active properties, detailed structural analyses such as MS, infrared (IR) spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy are necessary. For confirmation of the structure, rhamnolipids were further analyzed by HPLC and HPLC/MS which confirmed the presence of rhamnolipids.

In addition, the rhamnolipid fractions purified by HPLC were analyzed by NMR (Tahzibi *et al*. 2004) and FTIR in our Lab. These analyses confirmed the structure of rhamnolipids produced by *P. aeruginosa* PTCC 1637 (Index IV: FTIR spectrum of rhamnolipid).

Manso Pajarron *et al*. (1993) and Rendell *et al*. (1990) isolated rhamnolipids by thin-layer chromatography and HPLC and then analyzed the various fractions by FAB (Fast Atom Bombardment). Manso Pajarron *et al*. (1992) also analyzed a rhamnolipid preparation obtained from a commercial source. The preparation contained Rh-Rh-C10-C8, Rh-Rh-C8-C10, Rh-Rh-C10-C10, Rh-Rh-C12-C10 and Rh-Rh-C10-C12. In these compounds, Rh-Rh-C10-C10 was the most abundant.

Arino *et al*. (1996) separated a mixture of rhamnolipids by thin-layer chromatography in several fractions and hydrolyzed these fractions into their respective sugar and 3-hydroxy fatty acids. The sugar was determined to be rhamnose and the fatty acids were analyzed by GC/MS. By determining the nature and number of each component in each fraction, they were able to deduce the structure of these rhamnolipids. They also reported the rhamnolipid profile of *P. aeruginosa* strain GL1 isolated from hydrocarbon-contaminated soils. When bacteria were grown on glycerol as carbon source, they observed a variety of mono- and di-rhamnolipids containing one or two 3-hydroxy fatty acid residues. In this study, rhamnolipids with two fatty acids and one or two

rhamnoses represented 90% of all rhamnolipids. In addition, the fatty acids were predominantly C10, along with some C8, C12:1, and C12.

De Koster *et al*. (1994) observed the same saturated C8 to C12 mono- and di-rhamnolipids already mentioned. Finally, Bosch *et al*. (1898) analyzed the rhamnolipids produced by a *Pseudomonas* sp. after column and thin-layer chromatography. They only observed Rh-C10-C10 and Rh-Rh-C10-C10. Moreover, Deziel *et al*. (1999) showed rhamnolipids produced by *P. aeruginosa* 57RP differ both in quantity and in structure depending on the carbon source used. With mannitol, essentially all the rhamnolipids contained two fatty acid moieties, with Rh-Rh-C10-C10 being by far the most abundant, while with naphthalene, nearly 80% of the total rhamnolipids contained only one fatty acid moiety. In most cases, where some quantifification results were presented, the predominant rhamnolipid was Rh-Rh-C10-C10.

Our results present many similarities with the above-mentioned reports. This study showed that each of these structural types was composed of several individual compounds which differed from one another by the nature of the hydroxy fatty acid moiety.

4. Effect of different biosurfactants on surface hydrophobicity and adhesion of *B. cereus* 98/4 spores to stainless steel and Teflon

4.1. Introduction

The synthesis of extracellular molecules such as biosurfactants has major consequences on bacterial adhesion. These molecules may be adsorbed on surfaces and modify their hydrophobicities.

In this study, stainless steel and Teflon coupons were selected for conditioning experiments. The influence of lipopeptides on the hydrophilic/hydrophobic characteristic of the surfaces was determined by using contact angle measurement as mentioned in materials and methods (§ 2.7). Adhesion of *B. cereus* 98/4 spores was then investigated on these different conditioned substrata.

4.2. Influence of biosurfactants on surface properties

4.2.1. Stainless steel

The influence of lipopeptides on the hydrophilic/hydrophobic characteristic of surfaces was determined by using the different concentrations of lipopeptides i.e., 1, 10, 25, 50 and 100 mg l^{-1} in 10% methanol for surfactin, iturin (obtained from Dr Deleu), mycosubtilin and rhamnolipid and also the concentrations 0.25, 2.5, 6.25, 12.5 and 25 mg l^{-1} in 10% methanol for fengycin.

Stainless steel, Teflon and glass coupons were covered by the different concentrations of lipopeptides as mentioned in materials and methods (§ 2.7).

Treating stainless steel with 10% methanol gave contact angle values in the range of 30-35°, close to values obtained for untreated surfaces (data not shown) showing that methanol in contact with surfaces did not affect their hydrophobic/hydrophilic character of the surfaces.

When the glass coupons were conditioned by lipopeptides, hydrophobicity did not changed, so these coupons were removed in next experiments.

Conditioning with the various lipopeptides resulted mostly in a more or less marked increase of the hydrophobic character. However the changes varied among lipopeptides.

When stainless steel was conditioned with fengycin, contact angles were significantly higher (p-value < 0.0001) for concentrations ranging from 2.5 to 12.5 mg l^{-1}(intermediate concentrations). The

maximal value of 61.73° was obtained at the concentration 6.25 mg l^{-1}, which corresponds to the CMC of the mixture of compounds used (Figure 4.1). At higher concentrations, contact angle decreased up to 100 mg l^{-1}.

The small modifications induced by the conditioning with iturin A and mycosubtilin were not significant (*p*-value = 0.3022).

Conversely, when surfactin S1 was used, a significant increase (p-value < 0.0001) in the contact angles was observed following conditioning with concentrations over 1 mg l^{-1} (Figure 4.2). This effect increased further along with increasing the lipopeptide concentration (even for concentrations over the CMC) and reached a value of 81.1° at 100 mg l^{-1}: the highest value obtained among the tested biosurfactants.

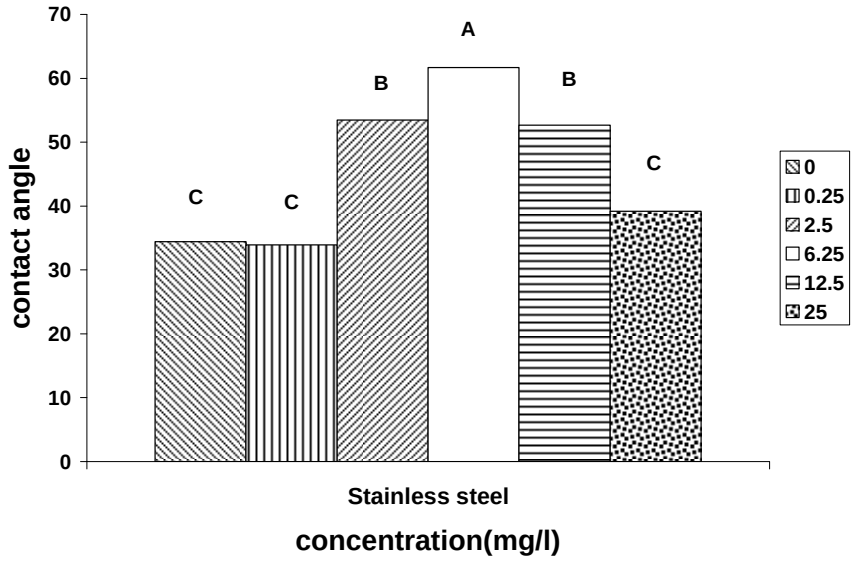


Figure 4.1. Effect of conditioning stainless steel with fengycin. Hydrophobicity was estimated by the water contact angle values. Coupons treated with 10% methanol solution were used as control. The results were analysed by a multiple comparison procedure by Tukey's test (alpha level = 0.05). Bars marked with the same letter are not statistically different from each other.

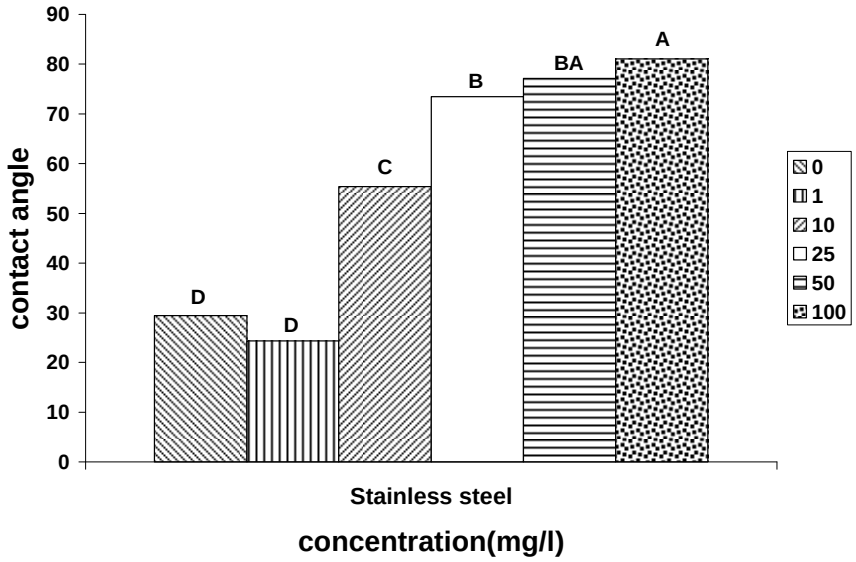


Figure 4.2. Effect of conditioning stainless steel with surfactin. Hydrophobicity was estimated by the water contact angle values. Coupons treated with 10% methanol solution were used as control. The results were analysed by a multiple comparison procedure by Tukey's test (alpha level = 0.05). Bars marked with the same letter are not statistically different from each other.

Contact angle measurements showed rhamnolipid increased hydrophobicity on stainless steel with concentrations from 1 mg $l^{-1}$and the highest value was observed at 100 mg l^{-1} (Figure 4.3).

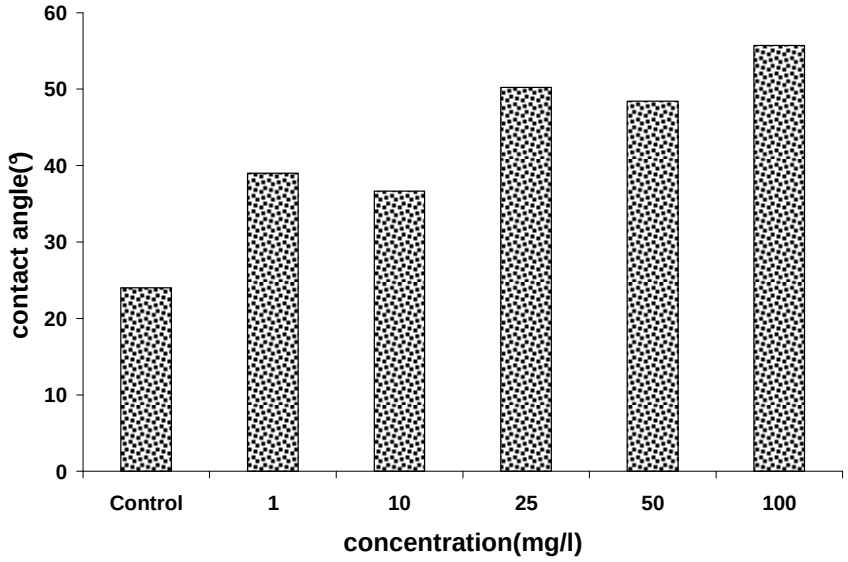


Figure 4.3. Effect of different concentrations of rhamnolipid on hydrophobicity of stainless steel. Hydrophobicity was estimated by the water contact angle values. Coupons treated with 10% methanol solution were used as control.

4.2.2. Neutralizing of surfactin charges and effect on hydrophobicity of it

Similar experiments were performed with surfactin dissolved in 10% methanol or in 10% methanol with 0.1 M $NaHCO_3$ in order to investigate the role of the lipopeptide charge in the substratum conditioning. After neutralizing of surfactin charges using 10% methanol with 0.1 M $NaHCO_3$, it was observed that surfactin can no longer change surface hydrophobicity on stainless steel (Figure 4.4).

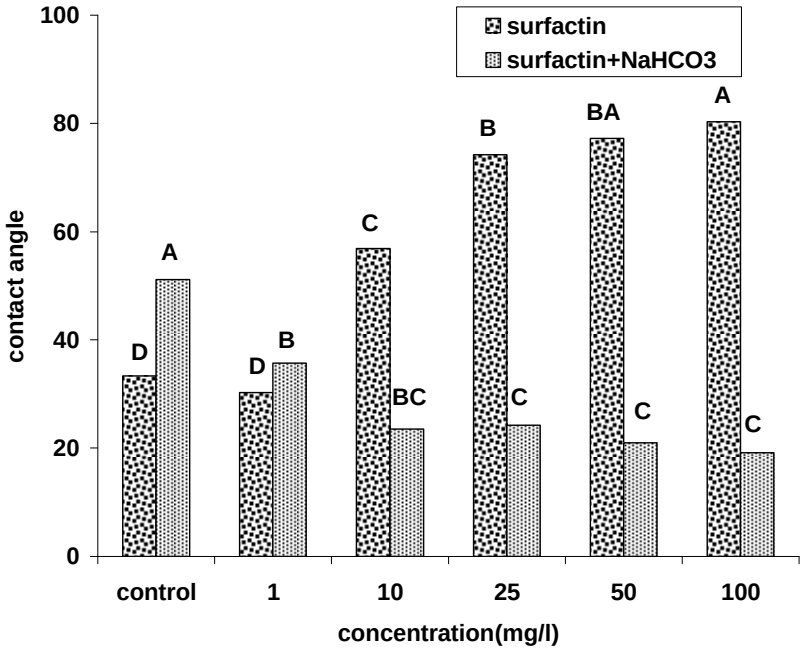


Figure 4.4. Effect of surfactin and neutralized surfactin on stainless steel hydrophobicity, which was evaluated by goniometry. Coupons treated with 10% methanol or 10% methanol with $NaHCO_3$ 0.1 M solutions were used as control. The results were analysed by a multiple comparison procedure by Tukey's test (alpha level = 0.05). Bars marked with the same letter are not statistically different from each other.

4.2.3. Teflon

In the presence of 10% methanol, Teflon exhibited a very high hydrophobic character (water contact angle around 115°), close to the value of 120° obtained without any conditioning step. Whatever the lipopeptide used, Teflon conditioning resulted in a reduction of the contact angle.

Conditioning of Teflon with fengycin (Figure 4.5) or surfactin (Figure 4.6) resulted in a slight but significant decrease (*p*-values < 0.0001) of the water contact angle, and Teflon remained highly hydrophobic (contact angle 95° and 100° for surfactin and fengycin, respectively) even at 100 mg l^{-1}.

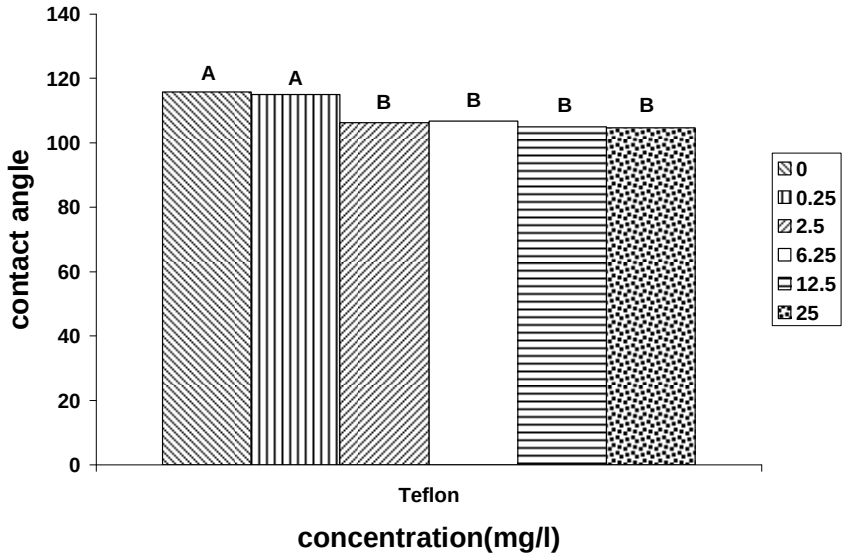


Figure 4.5. Effect of conditioning on Teflon with fengycin. Hydrophobicity was estimated by the water contact angle values. Coupons treated with 10% methanol solution were used as control. The results were analysed by a multiple comparison procedure by Tukey's test (alpha level = 0.05). Bars marked with the same letter are not statistically different from each other.

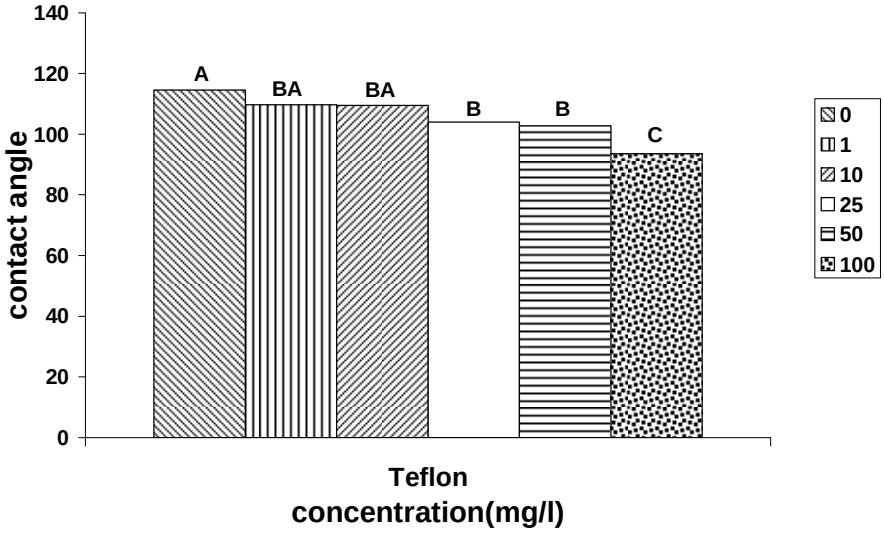


Figure 4.6. Effect of conditioning Teflon with surfactin. Hydrophobicity was estimated by the water contact angle values. Coupons treated with 10% methanol solution were used as control. The results were analysed by a multiple comparison procedure by Tukey's test (alpha level = 0.05). Bars marked with the same letter are not statistically different from each other.

With iturin A and mycosubtilin, a remarkable decrease (p-value < 0.0001) in the contact angles was observed at concentrations over 1 mg l^{-1}, and the lowest value was observed at 100 mg l^{-1}. At this concentration, conditioned Teflon was rather hydrophilic, with water contact angle around 58° and 44.9° for iturin A (Figure 4.7) and mycosubtilin (Figure 4.8), respectively. As observed in Figure 4.8 the decrease was more marked with mycosubtilin: at intermediate concentration, e.g. 10 mg l^{-1}, the contact angle was significantly lower with mycosubtilin than with iturin A.
Conversely, at higher concentrations (100 mg l^{-1}), a similar water contact angle was observed for both lipopeptides.

Contact angle measurements showed that rhamnolipid did not affect Teflon hydrophobicity.

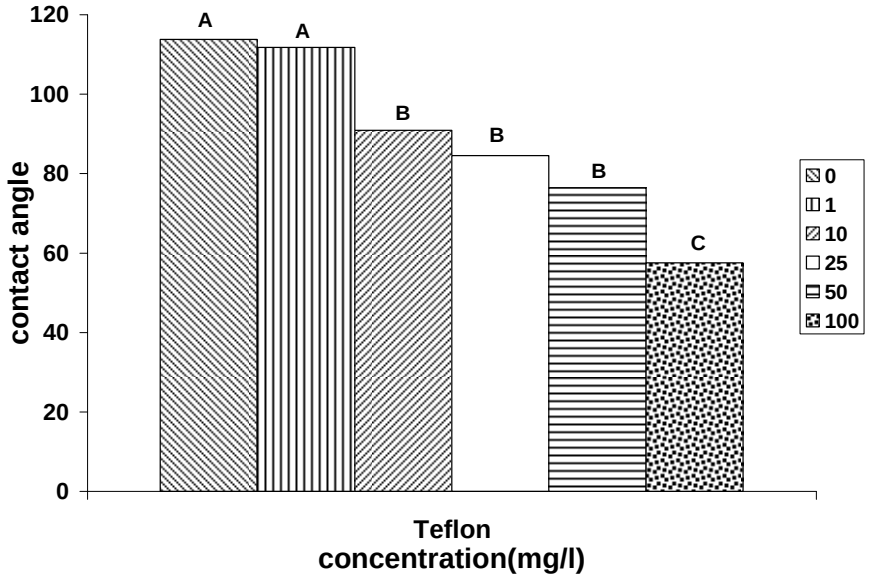


Figure 4.7. Effect of conditioning Teflon with iturin A. Hydrophobicity was estimated by the water contact angle values. Coupons treated with 10% methanol solution were used as control. The results were analysed by a multiple comparison procedure by Tukey's test (alpha level = 0.05). Bars marked with the same letter are not statistically different from each other.

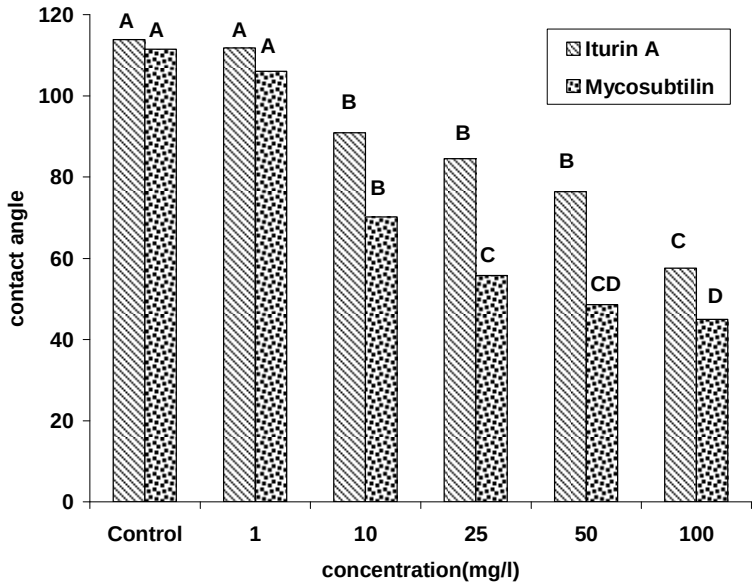


Figure 4.8. Effect of conditioning Teflon with iturin A and mycosubtilin. Hydrophobicity was estimated by the water contact angle values. Coupons treated with 10% methanol solution were used as control. The results were analysed by a multiple comparison procedure by Tukey's test (alpha level = 0.05). Bars marked with the same letter are not statistically different from each other.

4.3. Influence of biosurfactants on adhesion of *B. cereus* 98/4 spores to substrata

As previously mentioned (§ 1.3), one of the commonly considered factors involved in adhesion of bacteria (vegetative cells and spores) to substrata is bacterial cell surface properties, especially hydrophobicity (Faille *et al*. 2002).

B. cereus 98/4 was chosen for adhesion tests to coupons because of its high adherence to various materials (Faille *et al*. 1999). The hydrophobic character of spores from strains belonging to the *B. cereus* species has often been reported to play an important part in their ability to firmly adhere to stainless steel and other inert surfaces (Husmark *et al*. 1992; Faille *et al*. 1997). They were obtained as previously described (Faille *et al*. 2007).

The consequences of adhesion of the spores of *B. cereus* 98/4 were then investigated on both substrata conditioned by biosurfactants. Only biosurfactants capable of modifying surface properties were tested: surfactin, fengycin and rhamnolipids, on stainless steel; iturin, mycosubtilin and surfactin, on Teflon.

Stainless steel and Teflon conditioned coupons (§ 2.7) were vertically immersed in spore suspensions in sterile MilliQ water containing approximately 10^6 spores ml^{-1}, for 4 h at room

temperature. Adherent cells were detached from the surfaces as mentioned in materials and methods (§ 2.8) and were enumerated after 48 h incubation at 30°C.

Interestingly, despite great differences in the effects of lipopeptides on hydrophobicity of stainless steel and Teflon, their adhesion profiles were close to the water angle profiles as will be shown below.

4.3.1. Stainless steel

Conditioning with fengycin significantly affected spores adhesion (*p*-value = 0.0047). The maximal number of spores (1.9×10^4) was found at the intermediate fengycin concentrations of 2.5 and 6.25 mg l^{-1} (Figure 4.9).

For surfactin (Figure 4.10), significant differences were also observed between concentrations, with the number of adherent spores varying from around 5×10^3 at 0 and 1 mg l^{-1} to around 2×10^4 at 50 and 100 mg l^{-1} (*p*-value=0.0017). However, iturin A and mycosubtilin did not significantly affect spore adhesion.

As observed, these changes are consistent with the results of contact angle.

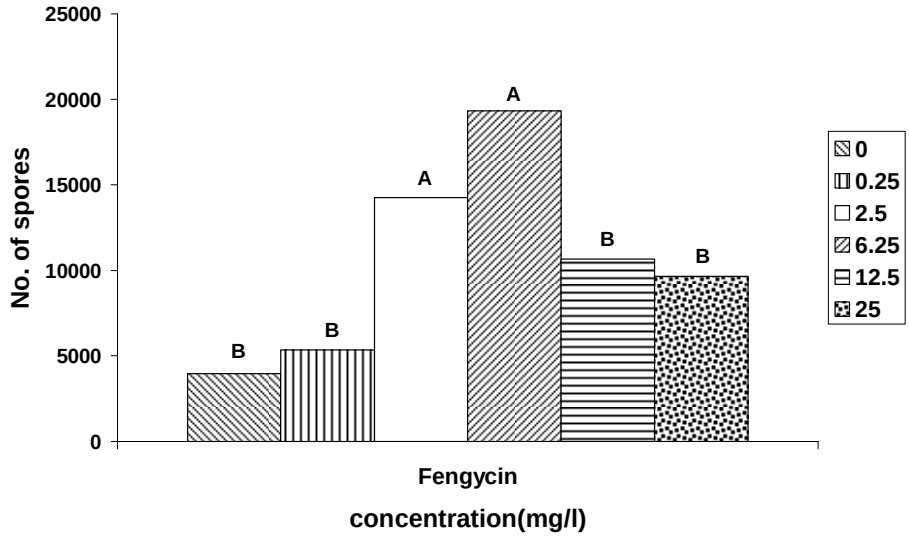


Figure 4.9. Effect of conditioning with fengycin on *B. cereus* 98/4 spores attachment to stainless steel coupons. Coupons treated with 10% methanol solution were used as control. The results were analyzed by a multiple comparison procedure by Tukey's test (alpha level = 0.05). Bars marked with the same letter are not statistically different from each other.

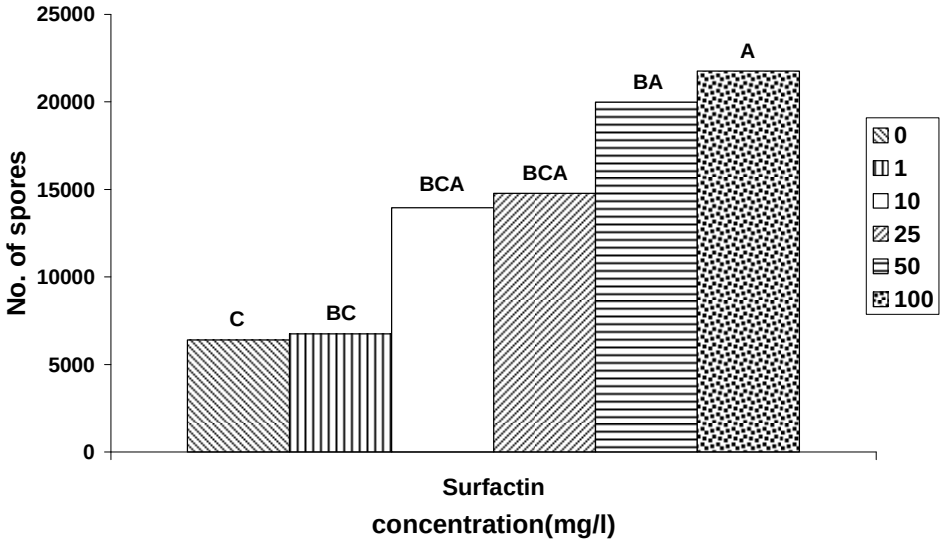


Figure 4.10. Effect of conditioning with surfactin on *B. cereus* 98/4 spores attachment to stainless steel coupons. Coupons treated with 10% methanol solution were used as control. The results were analyzed by a multiple comparison procedure by Tukey's test (alpha level = 0.05). Bars marked with the same letter are not statistically different from each other.

As the same for lipopeptides, the adhesion profile of *B. cereus* 98/4 spores on stainless steel following conditioning with rhamnolipids was related to profile obtained by water contact angles and a marked and significant (p-value < 0.0001) increase (4-fold) in the number of adherent spores was observed (Figure 4.11).

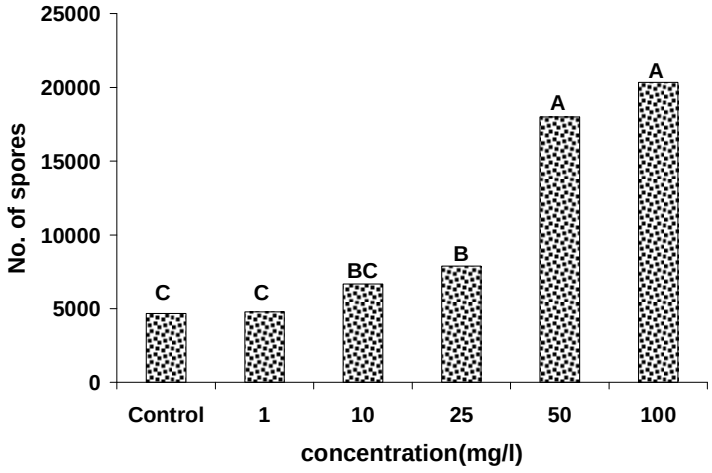


Figure 4.11. Effect of conditioning rhamnolipid on *B. cereus* 98/4 spores attachment to stainless steel coupons. Coupons treated with 10% methanol solution were used as control. The results were analyzed by a multiple comparison procedure by Tukey's test (alpha level = 0.05). Bars marked with the same letter are not statistically different from each other.

4.3.2. Teflon

On Teflon, the adhesion profiles were clearly related to the profiles observed in water contact angles, and a more or less marked decrease in the number of adherent spores was observed following every conditioning with lipopeptides.

However, the effects with mycosubtilin and iturin A were more remarkable and the number of adherent spores decreased 2.5- and 6.5-fold at concentration 100 mg l^{-1}, respectively (Figures 4.12 and 4.13). This result was highly significant (*p*-value < 0.0001).

Conversely, conditioning of Teflon with any concentration of fengycin (data not shown) or surfactin (Figure 4.14) had little effect on *B. cereus* 98/4 spores adhesion, and the number of adherent spores decreased < 2-fold.

However, statistical analysis confirmed the influence of surfactin on *B. cereus* 98/4 spore adhesion (*p*-value = 0.0083).

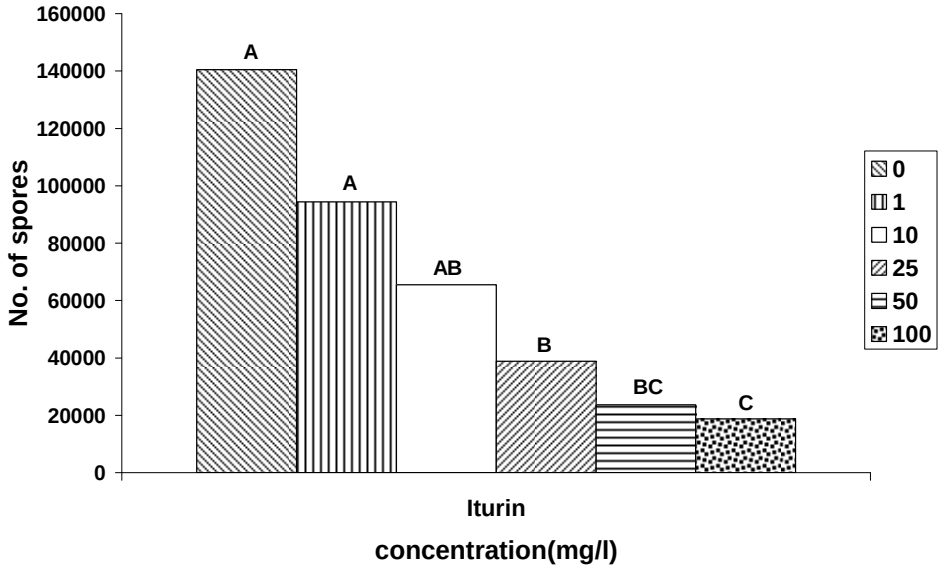


Figure 4.12. Effect of conditioning with iturin A on *B. cereus* 98/4 spores attachment to Teflon coupons. Coupons treated with 10% methanol solution were used as control. The results were analyzed by a multiple comparison procedure by Tukey's test (alpha level = 0.05). Bars marked with the same letter are not statistically different from each other.

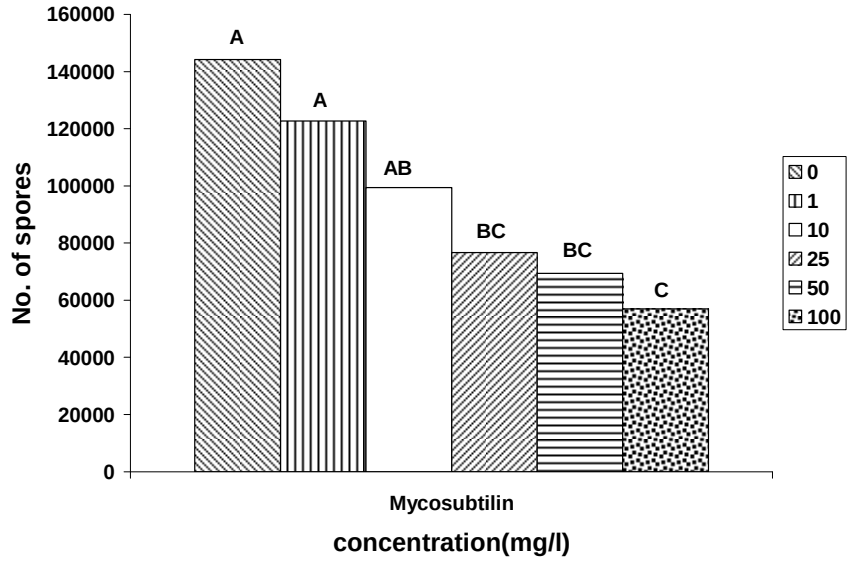


Figure 4.13. Effect of conditioning with mycosubtilin on *B. cereus* 98/4 spores attachment to Teflon coupons. Coupons treated with 10% methanol solution were used as control. The results were analysed by a multiple comparison procedure by Tukey's test (alpha level = 0.05). Bars marked with the same letter are not statistically different from each other.

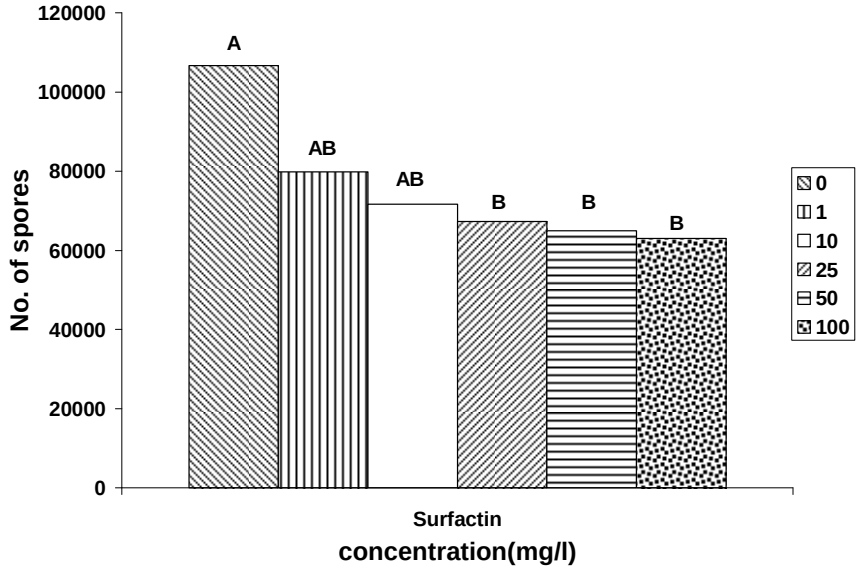


Figure 4.14. Effect of conditioning with surfactin on *B. cereus* 98/4 spores attachment to Teflon coupons. Coupons treated with 10% methanol solution were used as control. The results were analyzed by a multiple comparison procedure by Tukey's test (alpha level = 0.05). Bars marked with the same letter are not statistically different from each other.

4.3.3. Relationship between surface hydrophobicity and spore adhesion

In order to investigate the relationship between substratum hydrophobicity and *B. cereus* spores adhesion, numbers of adherent spores were plotted against water contact angles for each conditioning with both substrata (stainless steel and Teflon). Linear regressions were performed for the whole set of data, and for each conditioning, including 10% methanol (Table 4.2). Results indicated that, following conditioning with lipopeptides, changes in hydrophobicity of substrata could explain most of the variations in the ability of *B. cereus* 98/4 spores to adhere to them. When each conditioning procedure was analyzed separately, fengycin was not behaving like the other ones, despite a significant influence on the substratum hydrophobicity. Indeed, the number of adherent spores was poorly affected by the substratum hydrophobicity (slope = 0.0062) as compared to the other lipopeptides (slopes ranging from 0.0132 to 0.0162).

Table 4.2. Relationship between substratum hydrophobicity and spore adhesion for the various conditionings

Conditioning	Slope of the linear regression	R^2
10% methanol	0.0139	0.8807
Fengycin	0.0062	0.6205
Iturin A	0.0159	0.9485
Mycosubtilin	0.0162	0.7873
Surfactin	0.0132	0.9755
All data	0.0107	0.6384

4.4. Discussion

Lipopeptides have an important role in adhesion to various surfaces by hydrophobic interactions. Ahimou *et al*. (2000) demonstrated lipopeptide molecules adsorb on *Bacillus subtilis* after their excretion in extracellular medium and induce changes of the cell surface hydrophobicity illustrated by alterations of the water contact angle.

After contact with a surface, lipopeptides can form a conditioning film, thereby varying surface properties like hydrophobicity (Rosenberg and Ron 1999). This effect on surface hydrophobicity

arises from amphiphilic structure of these molecules by orientation of peptide cycle and fatty acid chain in relation to the hydrophobic/hydrophilic character of the surface. One explanation is that on the hydrophilic surface, lipopeptide molecules are probably oriented in such a way that the peptide cycle is adsorbed onto the surface and the hydrocarbon chains are exposed to the surrounding medium. Therefore, the surface becomes more hydrophobic (Ahimou *et al*. 2000).

The spatial organization of the lipopeptide molecules could be influenced by their concentration and the surface environment (Ahimou *et al*. 2000). Manne *et al*. (1994) proposed an adsorption model for ionic surfactant molecules at the graphite-aqueous solution interface in which at low concentration (almost 10% of CMC), molecules are adsorbed with alkane chains extended on the substrate plane, and this chain is gradually desorbed with an increase in the concentration.

At concentrations near the CMC, the surfactant molecules were oriented perpendicular to the substratum plane, with the hydrophilic head groups in contact with the aqueous phase (Ahimou *et al*. 2000).

On the contrary, fatty acid chains can interact with the hydrophobic surfaces and the polar peptidic moieties can be exposed to the environment. In these conditions, a decrease in the hydrophobicity of the surface can be observed.

In this study, the modifications induced by different types of biosurfactants (lipopeptides and rhamnolipids) on the surface properties of two substrata, stainless steel and Teflon were studied using contact angle measurement. The water contact angle provides a suitable evaluation of surface hydrophobicity (Ahimou *et al*. 2001).

Two families of lipopeptides increased the hydrophobicity of stainless steel, *viz* surfactin and fengycin. Iturin A and mycosubtilin had no effect. The difference between the families can be mainly attributed to the variability in the primary structure of the peptide cycle which is responsible for the 3D structure at the interface (Deleu *et al*. 1999). The 3D structure of peptide combined with the presence of the lipidic chain could be a crucial parameter in changing surface properties.

The study of 3D structure of surfactin, that is the structural basis of its important surface activities, showed that the carboxylic groups of both glutamate and aspartate form a minor hydrophilic domain and the nonpolar residues in position 4 and to a lesser extent, positions 2 and 7 from peptidic part along with the lipid tail, form major hydrophobic domains. The presence of these two domains is important for surface activity (Youssef *et al*. 2005). The importance of the negative charges of Glu

and Asp in adhesion of surfactin to the stainless steel surface was proved when these charges were neutralized by 0.1 M $NaHCO_3$. In this case, as shown in Figure 4.4, it was observed that surfactin can no longer change the surface hydrophobicity on stainless steel.

The spatial organization of fengycin is not known but this compound also contains charged amino acid residues (Glu) in the peptidic moiety. These charged residues could interact with charges present on stainless steel. The absence of charged amino acid in the peptide moiety of iturin A and mycosubtilin could explain the absence of effect of such compounds on stainless steel.

The surfactin peptide cycle is more hydrophobic than that of iturin A and fengycin (Deleu *et al*. 1999), which can explain the higher contact angle observed with the former. Indeed, the change in the hydrophobicity depends on the whole hydrophobic character of the lipopeptide film adsorbed onto the surface, resulting from both lipid and peptide parts. The presence of five hydrophobic amino acids in the peptide moiety of surfactin increases its whole hydrophobic character.
However, at concentrations higher than CMC, different results were observed for fengycin and a decrease in the contact angle occurred. At concentrations higher than CMC, fengycins might form a fully interdigitated bilayer or micelle, where each hydrocarbon tail spans the entire hydrocarbon width of the bilayer, and interacts laterally, through hydrophobic forces, with the hydrocarbon tail of fengycin molecules from the opposing lamellar leaflet. Such a hypothesis was suggested for the organization of iturin in solution (Grau *et al*. 2001).

Rhamnolipids have also an amphiphilic structure in which one or two rhamnosyl groups establish a hydrophilic part and fatty acid chain, in turn, confer a hydrophobic character to the molecule. After contact with a surface, they can form a conditioning film; thereby varying surface hydrophobicity. As stainless steel is a hydrophilic surface, rhamnolipids interact with it by their rhamnose moiety and the hydrophobic moiety is exposed to the surrounding medium. Therefore, stainless steel becomes more hydrophobic. On the contrary, rhamnolipids could not influence Teflon surface properties.

All lipopeptides decreased the hydrophobicity on Teflon. The higher hydrophobicity decrease was observed for iturin A and mycosubtilin compared to surfactin and fengycin. As previously mentioned, the iturin A peptide moiety has a lower hydrophobicity than the surfactin peptide moiety (on one side, presence of Leu (4) and Val (1) in surfactin and, on the other side, the presence of polar

groups on the hydroxyl amino acid residues, Ser and Tyr in iturin A) and it could be one of the reasons why differences were observed between these families.

Moreover, the length of the fatty acid chain could be a determinant too. This hypothesis was confirmed by the results obtained with another group of biosurfactants, *viz* mycosubtilins. Mycosubtilin differs from iturin A by the sequence inversion of two adjacent Ser6-Asn7 residues. In addition, it has a longer carbon atom chain (16-17) in comparison with iturin A (14-15) in the alkyl chain and as shown in Figure 4.7, this longer fatty acid chain led to an enhanced effect on Teflon.

A mixture of surfactin and mycosubtilin at ratio of 50/50 was also used for determination of surface properties. The mixture reduced hydrophobicity on Teflon from concentration 10 mg l^{-1}, but the effect after concentration of 40 mg l^{-1} was noticeable.

It was then determined which biosurfactants would modify the adhesion of *B. cereus* 98/4 spores to the conditioned surfaces. According to the previous studies, pre-treatment of stainless steel by surface-active compounds produced by *P. fluorescens* 495 gave rise to a substantial reduction (90 %) in the number of adherent cells of *Listeria monocytogenes* LO28 (Meylheuc *et al.* 2001). Similarly, pre-treatment of silicone rubber with *S. thermophilus* surfactant inhibited the adhesion of *Candida albicans* by 85% (Busscher *et al.* 1997) and a biosurfactant of *Lactococcus lactis* 53 inhibited adhesion of four bacterial and two yeast strains to silicon rubber (Rodrigues *et al.* 2004). Furthermore, biosurfactants from *Lactobacillus fermentum* and *Lactobacillus acidophilus* adsorbed on glass, reduced by 77% the number of adhering uropathogenic cells of *Enterococcus faecalis* (Velraeds *et al*.1996).

Pre-treatment of substrata with some biosurfactants caused a change (reduction or increase depending on the lipopeptide type and substrata) in the number of adherent spores on stainless steel and Teflon. These modifications perfectly fit with the hydrophobicity changes measured by contact angle. As *B. cereus* 98/4 spores are so hydrophobic, they adhere to hydrophobic surfaces with a higher affinity (Faille *et al.* 2002). Therefore, according to the results, more hydrophobic surfaces attract a greater number of spores. The lower correlation observed with mycosubtilin in Table 4.2 could be due to the impurities of the samples used. The effect of fengycin was completely different from the other lipopeptides, especially on stainless steel, as an increase in the

hydrophobicity with the fengycin concentration was followed by a decrease. Such behavior, which could be linked to the formation of a bilayer or micelle as previously suggested, could explain the less good correlation coefficient observed with this lipopeptide.

4.5. Results valorization

Results of this study led to an artice pulished in Biofouling and three communications in national and international congresses as follows:

Shakeri Fard P, Gancel F, Faille C and Jacques P. 2009. Effect of different *Bacillus subtilis* lipopeptides on surface hydrophobicity and adhesion of *Bacillus cereus 98/4* spores to stainless steel and Teflon. Biofouling 25(6): 533-541

Shakeri Fard P, Gancel F, Faille C and Jacques P. June 2009. Effect of different *Bacillus subtilis* lipopeptides on surface hydrophobicity and adhesion of *Bacillus cereus 98/4* spores to stainless steel and Teflon. 3rd Congress of FEMS held in Gothenburg, Sweden

Shakeri Fard P, Jacques P, Faille C, Mazaheri Assadi M. June 2009. Biosurfactant production by *Pseudomonas aeruginosa* PTCC 1637 and its effect on hydrophobicity of stainless steel and Teflon. 3rd Congress of FEMS held in Gothenburg, Sweden

Shakeri Fard P, Gancel F, Faille C and Jacques P. June 2007. Influence of lipopeptides of *Bacillus subtilis* on the surface hydrophobicity of stainless steel and Teflon. SFM, Nantes, France

5. Molecular analysis of lipopeptide behaviour on different surfaces by X-ray photoelectron spectroscopy (XPS)

5.1. Introduction

X-ray photoelectron spectroscopy (XPS) is a quantitative spectroscopic technique that measures the elemental composition within a material (§ 1.2.2). This also includes the domain of biomaterials, the performances of which rely strongly upon the interactions between the surface and cells or biological fluids.
Several strategies have been developed to extract information from data sets of XPS spectra: peak decomposition, quantification, background analysis, statistical approaches, etc (Evoy *et al*. 2008).

The strategy followed in the present study was to use peak decomposition and quantification to analyze the data.
In chapter 4, the influence of different lipopeptides in modification of surfaces was investigated. If they are able to adsorb to the surfaces, they are expected to modify the surface chemical composition which can be determined by XPS analyses.

The substrata stainless steel (SS) and Teflon were considered for studying the presence, concentrations and spatial organization of lipopeptides by using XPS analyses. The substrata were washed according to the previously mentioned protocol (§ 2.7) and were conditioned with different concentrations of lipopeptides. They were then exposed to XPS analysis. To avoid contamination, it should be noticed many precise precautions, as the method is very exact and sensitive. The substrata without presence of lipopeptides were analyzed as blank since they can determine main elements of stainless steel and Teflon. Attention was mainly focused on the decomposition of C1s, N1s, O1s, Cr2p, Fe2p and Ni2p peaks for stainless steel, and C1s, N1s, O1s and F1s peaks for Teflon, to analyze chemical functions.

5.2. Stainless steel substrate

The native stainless steel contains a lot of different elements mainly C, O, Cr, Fe, but also Ni, Ca, Zn, Mo, Si, S and P. Table 5.1 presents the elemental composition determined by XPS of SS treated or not with different lipopeptides.

The carbon peak has been decomposed into four components, keeping a constant full width at half maximum (FWHM about 1.40 eV). The components are assigned as follows: carbon only

Table 5.1. Elemental composition of surface relevant to stainless steel uncoated (blank) and coated with different lipopeptides. All numbers represent mole fraction in %.

Substrate (SS)	**Ni**	**Fe**	**Cr**	**O**	**N**	**C**	**Mo**	**Si**
Blank*	1.76	9.00	10.61	33.94	2.19	38.88	0.57	0.85
Surfactin 10 mg l^{-1}	2.08	9.28	12.59	32.90	3.42	35.98	0.64	0.55
Surfactin 100 mg l^{-1}	0.45	3.25	4.52	21.82	6.79	59.85	0.04	0.42
Blank*	0.80	8.71	8.88	42.47	0.23	34.84	bdl**	1.26
Fengycin 2.5 mg l^{-1}	0.84	8.99	8.68	41.82	0.36	35.42	bdl	1.12
Fengycin 6.25 mg l^{-1}	0.84	6.84	8.35	37.00	1.08	42.22	bdl	0.69
Fengycin 25 mg l^{-1}	0.52	6.21	6.38	36.68	4.16	40.78	bdl	0.87
Iturin A 10 mg l^{-1}	0.69	7.09	8.54	39.89	1.24	39.43	bdl	0.79
Iturin A 100 mg l^{-1}	0.53	4.13	5.41	32.67	4.38	49.26	bdl	0.48

*Since the experiment were performed in different days, so there are two different blanks in Table 5.1

** below detection limit.

bound to carbon and hydrogen (C–(C,H), at a binding energy of 284.8 eV), carbon making a single bond with oxygen or nitrogen (C–(O,N), at 286.3 eV, attributed to ether, alcohol, amine, or amide), carbon making two single bonds or one double bond with oxygen (O–C–O, C=O, at 287.9 eV, attributed to ester, acetal, hemiacetal, amide, carbonyl, and carboxylate), and carbon making one double bond and one single bond with oxygen (O=C–OH, O=C–OR, at 289.2 eV, due to carboxyl or ester functions). The nitrogen peak was decomposed into two contributions (FWHM about 1.70 eV) characteristic of nonprotonated nitrogen (N_{nonpr}, at 399.8 eV, typical of amide and amine) and protonated nitrogen (N_{pr}, at 402 eV, typical of ammonium or protonated amine). The oxygen peak was decomposed into two components (FWHM about 1.74 eV): one (531.1 eV) attributed mainly to oxygen making a double bond with carbon (including amide and carboxyl) and to oxygen of carboxylate, the other (532.7 eV) attributed to oxygen making single bonds with hydrogen or carbon (C–O–H of alcohol and carboxyl, C–O–C of acetal and hemiacetal).

The carbon only bound to carbon and hydrogen C–(C, H), may originate from lipids or from the side chains of amino acids. Carboxylate and carboxyl functions may be due to proteins. The amide is expected to represent peptidic bonds and protonated amine may be due to basic amino acids.

Treatment of stainless steel coupons with 10 mg l^{-1} of surfactin led to few increase of typical elements of stainless steel (Ni, Cr, Fe and Mo) and few decrease of O and C. The higher accessibility of typical elements could result from desorption of organic contaminant compounds by surfactin as confirmed by some decrease of O and C. In other words, the contaminant organic compounds could be replaced by a small amount of surfactin as observed by the increase of N.
With 100 mg l^{-1} of surfactin, typical elements of stainless steel clearly decreased, and N and C increased indicating the coverage of the surface by lipopeptidic molecules. O decreased progressively, as total amount of it in molecule of lipopeptide is less than that of stainless steel. The N attributed to surfactin (i.e. after subtraction of N from stainless steel found at the same binding energy) is of the order of 1.23 and 4.6% at 10 and 100 mg l^{-1}, respectively. The ratio of carbon, nitrogen and oxygen after treatment with 100 mg l^{-1} can be correlated to the structure of lipopeptide indicating mainly that surfactin is detected at the surface of stainless steel.

With the lowest concentration of fengycin, the typical elements of stainless steel showed few increase except Cr and they decreased following more adsorption of fengycin on the surface. Similar changes were observed for O and N, so that O decreased from 42.47 to 36.68 and N signal increased from 0.23 to 4.16 concomitantly with the increase in concentration of fengycin.
The C signal after adsorption of fengycin increased up to the concentration 6.25 mg l^{-1} (i.e. CMC of molecule) and then decreased at the concentration 25 mg l^{-1}.

The typical elements of stainless steel, in both concentrations 10 and 100 mg l^{-1} of iturin A decreased considering that the decrease in 100 mg l^{-1} was more remarkable. The changes of O and N were similar to fengycin, but C in both concentrations increased progressively.

5.3. Teflon substrate

The native substrate Teflon behaves as expexted. It is composed of 66.6% F and 33.3% C (it should be noticed that hydrogen is not detected by XPS). There are slight traces of C and O contamination on Teflon (Figures 5.1 and 5.2, Table 5.2).

The peak 689.11 eV is attributed to fluorine making a single bond with carbon.

When surfactin adheres to the surface, as shown in Table 5.2, there is an increase in the nitrogen, oxygen and carbon signals and a concomitant decrease in fluorine, the specific signal of the substrate, from 65 to 35 (Figure 5.1, Table 5.2). This is more marked at 100 mg l^{-1} compared to 10 mg l^{-1}.

The shape of the C peak (position of the components) also shows the presence of surfactin without ambiguity (Figures 5.2). The component at 292.2 eV is indeed typical of Teflon, while the other components are typical of surfactin. The small peak recorded at 284.8 eV on the native Teflon is attributed to contaminants. The O and N signals after adsorption of surfactin increase (Figures 5.1 and 5.2). When surfactin concentration reaches from 10 mg l^{-1} to 100 mg l^{-1}, peak surface increases remarkably. The nitrogen attributed to surfactin is of the order of 0.7 and 3.6% at 10 and 100 mg l^{-1} respectively, which is close to the amounts measured on SS.

Table 5.2. Elemental composition of surface relevant to Teflon uncoated (blank) and coated with different lipopeptides. All numbers represent mole fraction in %.

Substrate (Teflon)	**F**	**O**	**N**	**C**
Blank*	64.76	0.49	bdl**	34.58
Surfactin 10 mg l^{-1}	55.19	2.32	0.75	41.43
Surfactin 100 mg l^{-1}	34.88	7.01	3.61	54.22
Blank*	66.12	0.28	0.03	33.47
Fengycin 2.5 mg l^{-1}	62.48	0.87	0.1	34.28
Fengycin 25 mg l^{-1}	61.59	1.45	0.61	36.16
Iturin A 10 mg l^{-1}	63.16	1.05	0.2	35.41
Iturin A 100 mg l^{-1}	61.00	1.69	0.59	36.55

* Since the experiments were performed in different days, so there are two different blanks in Table 5.2.

** below detection limit.

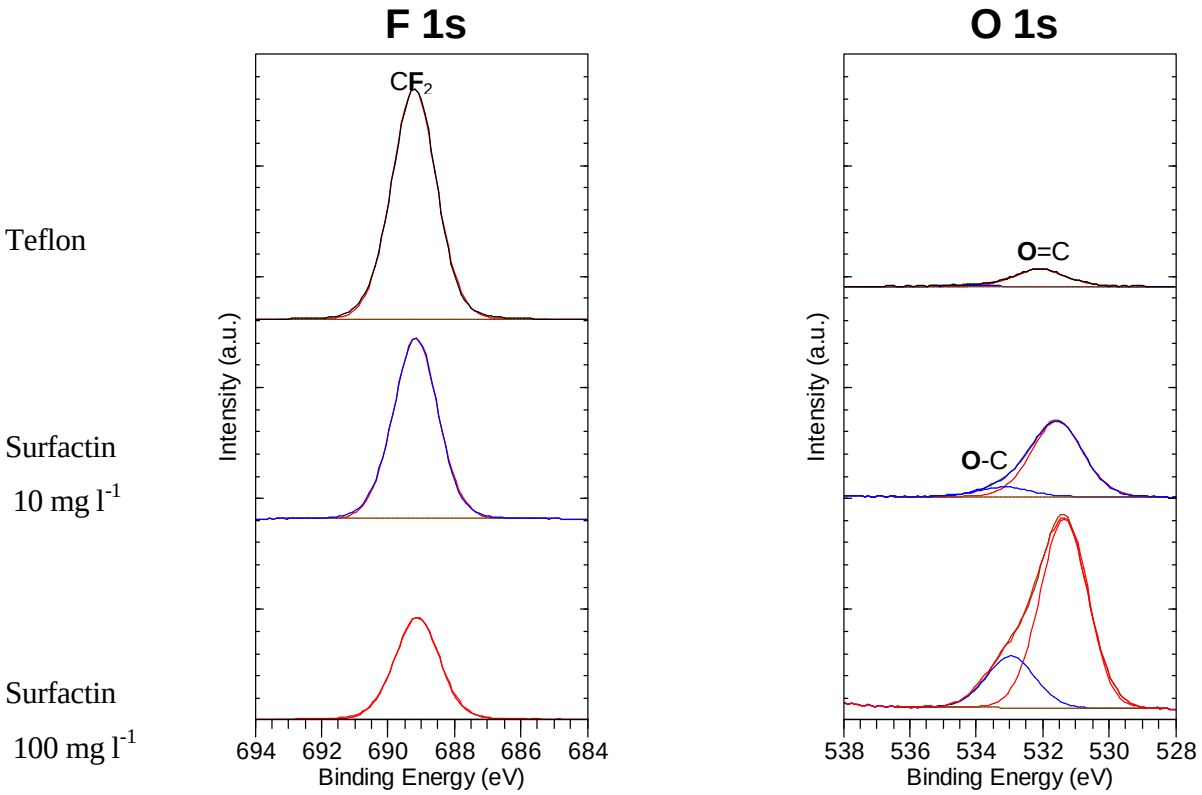


Figure 5.1. O and F peak before (top peak) and after (down peak) adsorption of surfactin 10 and 100 mg l^{-1}, respectively on Teflon.

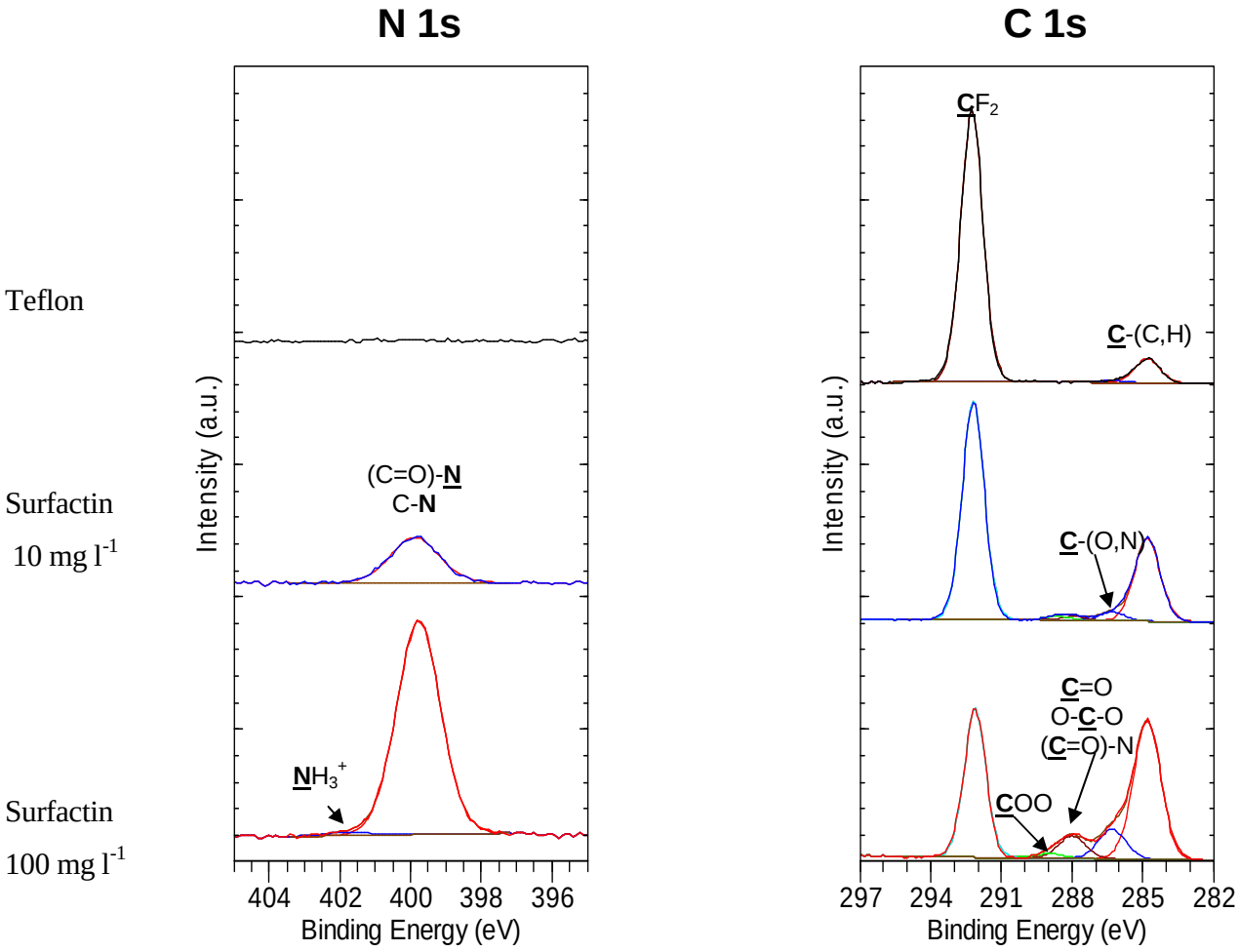


Figure 5.2. N and C peak before (top peak) and after adsorption of surfactin 10 and 100 mg l^{-1}, respectively on Teflon.

The results for iturin A and fengycin were similar to surfactin, as all determinant signals of O, N and C increased after adsorption of lipopeptides (Table 5.2).

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5.4. Discussion

At the highest concentration of lipopeptides on stainless steel, the typical elements of the stainless steel decreased, and N and C signals increased (Table 5.1).

After adsorption of surfactin on stainless steel at 10 mg l^{-1}, the C signal decreased and the typical elements of stainless steel (Ni, Cr, Fe and Mo) slightly increased. At higher concentration of surfactin i.e. 100 mg l^{-1}, the C signal increased and the typical elements of stainless steel decreased. The primary decrease of C signal could be attributed to the displacement of carbon-containing contaminants by surfactin (which are observed on other systems, for example for albumin adsorption on glass).

The N signal after surfactin adsorption on stainless steel increased at 10 and 100 mg l^{-1}, respectively. This amount is close to the amounts measured on Teflon. In addition, the N content after adsorption at 100 mg l^{-1} (3.6%) is very close to the N signal recorded on a surfactin layer obtained by the Langmuir-Blodgett method on a mica surface (3.5%, Deleu *et al*. 1999), which seems to be compatible with a monolayer of surfactin. However, it is difficult to make the difference between a complete monolayer and an incomplete layer with some aggregates.

The C signal after adsorption of fengycin on stainless steel increased up to the concentration 6.25 mg l^{-1} (i.e. CMC of molecule). It then decreased at the concentration 25 mg l^{-1}. These changes are similar to changes of hydrophobicity measured by goniometer following adhesion of fengycin on stainless steel. Since fengycin adheres to stainless steel by its polar head, it is expected to increase the C content up to its CMC. After this concentration, it could then form micelles or bilayers that cause to decrease C content from 42.22 to 40.78. N content increased from 0.36 to 4.16 continuously following increase in fengycin concentration as observed in Table 5.1. Other typical elements of stainless steel reduced when the concentration of fengycin was increased. This is attributed to the adsorption of the molecule to the surface.

The C and N signals increased after adsorption of iturin A on stainless steel and other typical elements of stainless steel reduced progressively.

This result is not in accordance with the results obtained by goniometry in which at higher concentrations no modification of hydrophobicity was observed.

It is supposed iturin molecules form a bilayer at higher concentrations on stainless steel and under

these conditions they are unable to change hydrophobicity.

When surfactin adhered to Teflon (Table 5.2), it caused to increase N, O and C signals that were more marked at 100 mg l^{-1} compared to 10 mg l^{-1}. This can be also correlated to the results obtained by goniometry for surfactin in which it reduced hydrophobicity on Teflon. In addition, surfactin has surface area greater than fengycin, so it can extend on the surface better than the latter that corresponds to XPS results in which C signals were greater than the two others.

There are similar changes for fengycin and iturin A on Teflon, although in less extent compared to surfactin, so that N, O and C signals increased at higher concentrations of these lipopeptides. This result is not in accordance with the results obtained by goniometry for fengycin and iturin A. Indeed , hydrophobicity did not change at higher concentrations of fengycin but increased noticeably in the case of iturin A.

As it was mentioned previously, fengycin at higher concentrations forms micelles or bilayers, so it can no longer change hydrophobicity on Teflon in these concentrations.

Regarding surface area of iturin A (300 $Å^2$) that is clearly greater than the two others (262 and 181 $Å^2$ for surfactin and fengycin, respectively), it seems that small amounts of molecule introduces high modification of surface properties.

6. Overproduction of lichenysin: a biosurfactant produced by *Bacillus licheniformis* ATCC 14580

6.1. Introduction

Lichenysin A, produced by *B. licheniformis* strains ATCC 10716, BAS50 and BNP29, is a cyclic lipoheptapeptide characterized as one of the highest biosurfactant activities reported (Yakimov *et al*. 1995), although it is produced in much lower amounts than surfactin (Yakimov *et al*. 1996).

Lichenysin A differs structurally from surfactin as mentioned previously (§ 1.1.5.2.2), in two constituent amino acids (it has glutamine instead of glutamate as the first residue, and isoleucine instead of leucine as the last one). It is likely that the Glu/Gln difference is the most relevant to the difference in activity between the two lipopeptides.

Our strategy during this research focused on effects of biosurfactants on surface properties. Since these two molecules have many similarities and lichenysin has shown potent biosurfactant properties (§ 1.1.5.2.2), it was decided to produce and purify it. Looking for an available strain producing such a lipopeptide, *B. licheniformis* ATCC 14580 was selected because its genome has been completely sequenced and contains an operon that encodes lichenysin synthetases. On the other hand, the *comP* gene (necessary to stimulate surfactin production in *B. subtilis* through *comA* gene product phosphorylation) is punctuated in this strain by an insertion sequence element (Randy, 2004). Therefore, *B. licheniformis* ATCC 14580 is unable to produce large amounts of lichenysin.

The availability of a complete genome sequence permit a thorough comparison of the biochemical pathways and regulatory networks in *B. subtilis* and *B. licheniformis*, thereby offering new opportunities and strategies for the improvement of lipopeptide production. Bioinformatic analyses were employed to compare the different genes involved in the biosynthesis of surfactin in both *B. subtilis* 168 and *B. amyloliquefaciens* FZB42 with those coding for lichenysin synthesis in *B. licheniformis* ATCC 14580.

Since the strain has the genes of lichenysin synthesis, but produces low amounts of product, some genetic manipulations were planned to increase the yield of lichenysin. The genetic manipulations aimed at the replacement of the native promoter of lichenysin synthetase P_{lchA} by the promoter P_{xylA} (inducible by xylose), using homologous recombination.

6.2. Bioinformatic analyses

The only non-ribosomal peptide synthetase operon identified in the genome of *B. licheniformis* is the one responsible for lichenysin biosynthesis. Lichenysin structurally resembles surfactin in *B. subtilis* (Grangemard *et al*. 2001), and their respective biosynthetic operons are highly similar. In pairwise BLAST comparisons, 66% of the predicted *B. licheniformis* ATCC 14580 genes have orthologs in *B. subtilis* 168 (GenBank® Accession No. NC-000964) (Rey *et al*. 2004).

The gene sequences responsible for lichenysin and surfactin production were analyzed by GenBank database search tools provided by the National Center for Biotechnology Information (NCBI, USA), Needle software (Needleman and Wunsch, 1970) and NRPS Predictor software.

We first decided to confirm the presence of the gene *sfp* (2455 bp downstream of *lch* gene in *B. licheniformis* ATCC 14580) which encodes phosphopantetheinyl transferase to change the apo enzyme (synthetases) to the active form or holo enzyme. Table 6.1 shows nucleic acid and protein sequence analysis for this gene in *B. licheniformis* ATCC 14580 (GenBank® Accesion No. NC-006270), *B. subtilis* 168 and *B. amyloliquefaciens* FZB42 (GenBank® Accesion No. NC-009725). This gene in *B. licheniformis* ATCC 14580 shows about 62% similarity at the nucleic level with the *sfp* gene present in *B. subtilis* 168 and *B. amyloliquefaciens* FZB42, and 52% or 56% similarity at the protein level in *B. subtilis* 168 and *B. amyloliquefaciens* FZB42, respectively.

Table 6.1. Comparison of *sfp* genes in different *Bacillus* strains at nucleic and proteic level. Data taken from NCBI site

	***B. licheniformis* ATCC 14580- *B. subtilis* 168**	***B. licheniformis* 14580- *B. amyloliquefaciens* FZB42**	***B. subtilis* 168- *B. amyloliquefaciens* FZB42**
Size (nt)	681-666	681-675	666-675
Nucleic identity (%)	61.5	61.8	69.3
Size	226-220	226-224	220-224
Proteic identity (%)	52	56.2	67.9

In addition, in order to ensure the lichenysin gene functions properly, some comparison analyses of the proteic or nucleic sequences were performed at the ORF level of the synthetase operon.

The genetic organization of the lichenysin A synthetases (*lchA* gene, in *B. licheniformis* ATCC 14580) and surfactin synthetases (*srfA* gene, in *B. subtilis* 168 and *B. amyloliquefaciens* FZB42) are shown in Figure 6.1. As expected, surfactin and lichenysin A operon have similar modular architectures (§ 1.1.5.5).

The surfactin/lichenysin operons contain four open reading frames (ORFs) coding for surfactin or lichenysin synthetases, which are designated *srfA-A, srfA-B, srfA-C* and *srfA-D* or *lchAA, lchAB, lchAC* and *lchAD*, respectively (Yakimov *et al*. 1998; Peypoux *et al*. 1999). They present a linear array of seven modules (one module per residue).

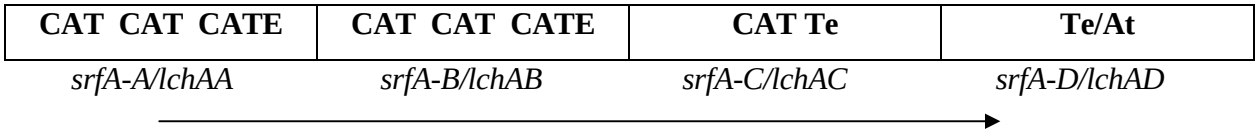

Figure 6.1. Schematic representation of operons (ORFs and domains of NRPS) encoding lichenysin A (*lchAA-C*) and surfactin (*srfAA-C*) synthetase.

Three modules are present in the products of *srfA-A/ lchAA* and *srfA-B*/*lchAB*, and one module in the product of *srfA-C/lchAC*. The fourth gene *srfA-D/lchAD* codes for a thioesterase/acyltransferase (Te/At) domain (Steller *et al*. 2004).

As shown in Table 6.2, similarities at proteic or nucleic levels are higher between *B. subtilis* 168 and *B. amyloliquefaciens* FZB42 than between *B. licheniformis* ATCC 14580 and the two other strains.

Regarding the presence of *sfp* gene and RBS (ribosomal binding site) region (Index VII) (data not shown) and similarity between ORFs in *B. licheniformis* ATCC 14580 with the two other strains, it was expected that *B. licheniformis* ATCC 14580 has the capacity to produce lichenysin lipopeptide, unless at least a detrimental point mutation had occurred in this operon. In addition, informatic analyses demonstrated that there is a high similarity in the -35/-10 region between *B. licheniformis* ATCC 14580 and *B. subtilis* 168 (data not shown) and there is also a *comA* box similar to one of *B. subtilis* 168 *comA* boxes in upstream from -35 box (Yakimov and Golyshin 1997).

Table 6.2. Comparative analysis of NRPS synthetases of *Bacillus* strains (*amyloliquefaciens* FZB42, *subtilis* 168 and *licheniformis* ATCC 14580) using Needle (Global) software

Sequence origins	Lipopeptides NRPS genes (%)	Protein similarity (%)	DNA similarity (%)
B. licheniformis 14580- *B. subtilis* 168	*lch AA-srfAA*	77.2	65.8
	lch AB-srfAB	77.1	66.5
	lch AC-srfAC	75.3	64.3
B. subtilis 168- *B. amyloliquefaciens* FZB42	*srfAA*	86.1	73.3
	srfAB	87.1	74.0
	srfAC	93	86.5
B. licheniformis 14580- *B. amyloliquefaciens* FZB42	*lch AA-srfAA*	78	66.1
	lch AB-srfAB	77	66.8
	lch AC-srfAC	75.8	64.6

In the literature, the main differences between surfactin and lichenysin are the first and seventh amino acid in which Glu and Leu are substituted by Gln and Ile, respectively in surfactin. In addition, some differences could be observed in the length and isomery of fatty acid chain incorporated into the lipopeptides.

A comparison between adenylation domains of module 1 and 7 and condensation domain of module 1 (involved in the fatty acid chain binding to the first amino acid) was thus performed. Results are shown in Tables 6.3 and 6.4.

Table 6.3. Comparative analysis of the first condensation (C) and adenylation (A) domains in the first (M1) and seventh (M7) modules in *Bacillus* strains (*amyloliquefaciens* FZB42, *subtilis* 168 and *licheniformis* ATCC 14580) by the software Needle at nucleic level (Data from NCBI)

Domain	***B. amyloliquefaciens* FZB42 *B. subtilis* 168**	***B. subtilis* 168 *B. licheniformis* 14580**	***B. amyloliquefaciens* FZB42 *B. licheniformis* 14580**
C (1st)			
Similarity (%)	69.6	60.1	61.6
Gaps (%)	3.4	8.6	8.9
A (M1)			
Similarity (%)	68.7	62.3	63.7
Gaps (%)	5.4	6.5	5.5
A (M7)			
Similarity (%)	68.4	57.9	46.5
Gaps (%)	20.9	13.1	29.7

Table 6.4. Comparative analysis of the first condensation (C) and adenylation (A) domains in the first (M1) and seventh (M7) modules from *Bacillus* strains (*amyloliquefaciens* FZB42, *subtilis* 168 and *licheniformis* ATCC 14580) by the software Needle at protein levels (Data from NCBI)

Domain	***B. amyloliquefaciens* FZB42 *B. subtilis* 168**	***B. subtilis* 168 *B. licheniformis* 14580**	***B. amyloliquefaciens* FZB42 *B. licheniformis* 14580**
C (1st)			
Similarity (%)	83.4	71.5	72.8
Gaps (%)	0.3	2.6	3.0
A (M1)			
Similarity (%)	82.5	75.8	76.2
Gaps (%)	3.8	4.2	2.8
A (M7)			
Similarity (%)	83.8	69.3	70.8
Gaps (%)	13.5	9.5	6.4

As previously shown, similarities at proteic or nucleic levels are still higher between *B. subtilis* 168 and *B. amyloliquefaciens* FZB42 than between *B. licheniformis* ATCC 14580 and the two other strains. Interestingly, a lower conservation was found for the adenylation domain of module 7.

Then NRPS Predictor, a software that predicts amino acid activated by adenylation domain, was used to confirm the potential peptidic structure of lichenysin produced by *B. licheniformis* ATCC 14580. An identical analysis was done for surfactin from *B. subtilis* 168 and *B. amyloliquefaciens* FZB42 (Table 6.5).

Table 6.5. Amino acids predicted by NRPS Predictor software in different synthetases: SrfAA (in *B. subtilis* 168), LchAA (in *B. licheniformis* ATCC 14580) and SrfAA` (in *B. amyloliquefaciens* FZB42)

Srf AA	**Identity**	**Lch AA**	**Identity**	**Srf AA`**	**Identity**
Glu	100	Gln	100	Glu	100
Leu	100	Leu	100	Leu	100
Leu	100	Leu	100	Leu	100
Srf AB		**Lch AB**		**Srf AB`**	
Val	100	Val	100	Val	100
Asp	100	Asp	100	Asp	100
Leu	100	Leu	100	Leu	100
Srf AC		**Lch AC**		**Srf AC**	
Leu	100	Ile	100	Leu	100

Differences were observed only for the amino acid residue activated in module 1 and in module 7. Table 6.6 shows the amino acid composition of the binding pocket (residues at 8 Å) of the different adenylation domains as well as the NRPS code. These results confirm the lowest conservation of the adenylation domain of module 7 followed by module 1 compared to the others.

A higher similarity was observed again between proteic sequences from *B. subtilis* and *B. amyloliquefaciens*. As observed in Tables 6.6 all amino acids encoded (in modules of 2, 3, 4, 5 and 6, respectively) are the same in all three strains and have common NRPS code except module 2 that encodes Leu, in which a M (Met) is replaced by a L (Leu). It seems that this modification has no effect on the amino acid recognition. In modules 1 and 7, three variations are observed in the NRPS code in which K (Lys) is replaced by Q (Glu), A (Ala) by G (Gly), and C (Cys) by V (Val). These changes result in the substitution of Glu to Gln and Leu to Ile in *B. licheniformis* lipopeptide. In this regard, Stachelhaus *et al*. (1999) have shown that the single point mutation His322 to Glu in the aspartate-specific activating domain from surfactin synthetase SrfAB completely altered the specificity to asparagine.

Table 6.6. Residues 8 Å around the substrate and NRPS code for module 1 of synthetase.* Module

***Bacillus* strain**	**Residues 8 Å around the substrate**	**NRPS code**	**Amino acid, M***
B. subtilis 168	LSLAFDASVKQADGLIGGETNVYGPTETCVDASV	DAKDLGVVDK	Glu, 1
B. amyloliquefaciens FZB42	LSLAFDASVKQADGLIGGETNVYGPTETCVDASV	DAKDLGVVDK	Glu, 1
B. licheniformis ATCC 14580	LGLAFDASVQQTDGLVGGETNVYGPTETCVDASS	DAQDLGVVDK	Gln, 1
B. subtilis 168	TETSFDAFMFDGMIMFGGELHMYGPSESTVFATY	DAFMMGMVFK	Leu, 2
B. amyloliquefaciens FZB42	TNTSFDAFMFDGMIMFGGELHMYGPSESTVFATY	DAFMMGMVFK	Leu, 2
B. licheniformis ATCC 14580	TNASFDAFMFDGMILFGGELHMYGPSESTVFTTY	DAFMLGMVFK	Leu, 2
B. subtilis 168	LWHAFDASIWEPFLLTGGDVNNYGPTENTVVATS	DAWFLGNVVK	Leu, 3
B. amyloliquefaciens FZB42	LWHAFDASIWEPFLLVGGDINNYGPTENTVVATS	DAWFLGNVVK	Leu, 3
B. licheniformis ATCC 14580	LWHAFDASVWEPFLLTGGDVNNYGPTENTVVATS	DAWFLGNVVK	Leu, 3
B. subtilis 168	LNAGFDAGTFEGWLIIGGDWNGYGPTENTTFSTS	DAFWIGGTFK	Val, 4
B. amyloliquefaciens FZB42	LHSGFDAGTFEGWLIIGGDWNGYGPTENTTFSTS	DAFWIGGTFK	Val, 4
B. licheniformis ATCC 14580	LNAGFDASTFEGWLIIGGDWNGYGPTENTTFSTC	DAFWIGGTFK	Val, 4
B. subtilis 168	YWFSFDLGYTCPKLVLGGEINHYGPTEATIGAIA	DLTKVGHIGK	Asp, 5
B. amyloliquefaciens FZB42	YWFSFDLGYTAPKLVLGGEINHYGPTETTIGAIA	DLTKVGHIGK	Asp, 5
B. licheniformis ATCC 14580	YWFSFDLGYTSPKLVLGGEINHYGPTETTIGAIA	DLTKVGHIGK	Asp, 5
B. subtilis 168	LWDAFDASIWEPFLLTGGDVNNYGPTENTVVATS	DAWFLGNVVK	Leu, 6
B. amyloliquefaciens FZB42	LWHAFDASIWEPFLLVGGDINNYGPTENTVVATS	DAWFLGNVVK	Leu, 6
B. licheniformis ATCC 14580	LWHAFDASVWEPFLLTGGDVNNYGPTENTVVATS	DAWFLGNVVK	Leu, 6
B. subtilis 168	LHVSFDAFTFDAFALFGGEINCYGPTEGTVFATA	DAFFLGCVFK	Leu, 7
B. amyloliquefaciens FZB42	LHVSFDAFTFDAFALFGGEINCYGPTEGTVFATA	DAFFLGCVFK	Leu, 7
B. licheniformis ATCC 14580	VETSFDGFTFDGFVLFGGEIHVYGPTETTVFATF	DGFFLGVVFK	Ile, 7

6.3. Production

In respect to the presence of whole operon of lichenysin in *B. licheniformis* ATCC 14580, the production of this molecule was investigated. Different conditions were tested, and the strain was cultivated in different culture media derived from Landy medium: 1) with and without the presence of MOPS; 2) with different nitrogen sources (glutamic acid and NH_4NO_3); 3) with different carbon sources (glucose and sucrose); 4) in aerobic and anaerobic conditions (§ 2.10.3). The strain grew under the different culture conditions, but a significant decrease of the supernatant ST could not be detected (Table 6.7).

Table 6.7. The results of culture of *B. licheniformis* ATCC 14580 in different culture media. ST, OD_{600} and area determined by HPLC are shown

Culture conditions	1**	2**	3**	4**	5**	6**
ST (mN m^{-1})*	54.4±0.2	49.0±0.1	50.7±0.2	63.3±0.2	54.0±0.1	53.7±0.15
OD_{600}	0.41	ND	ND	0.34	ND	ND
Area (HPLC)	127157, 1301640	NM	NM	98670, 3080022	30942, 1747138	48820, 2674864

* Mean of three experiments, ± standard deviation.

**1.Glutamic acid+MOPS (ST of medium before cultivation: 66.1 mN m^{-1}), aerobic; 2. Glutamic acid+MOPS anaerobic; 3. Glutamic acid, anaerobic; 4. NH_4NO_3+MOPS (ST of medium before cultivation: 70.5 mN m^{-1}), aerobic; 5. NH_4NO_3+MOPS, anaerobic; 6. NH_4NO_3, anaerobic.

ND: not determined, as it was culltivated under anaerobic conditions, NM: not measurable

The MALDI-TOF/MS analysis of *B. licheniformis* ATCC 14580 colony revealed one molecular ion at *m/z* 1059.5 that could correspond to $[M+K]^+$ of one lichenysin homologue compound. This would confirm the presence of a small amount of lichenysin (Figure 6.2). HPLC analysis of the different supernatants was carried in the conditions of surfactin detection. Two peaks were detected in all samples (Figure 6.3). These peaks were eluted with a retention time similar to surfactin but their second derivatives (Figure 6.3) appeared different. Since we did not have a lichenysin standard, surfactin standard was used for calculaton of product concentration. As these two molecules are mainly different in two amino acids i.e. Glu/Gln and Leu/Ile in their structure (§ 1.1.5.2.2), they have somehow similar

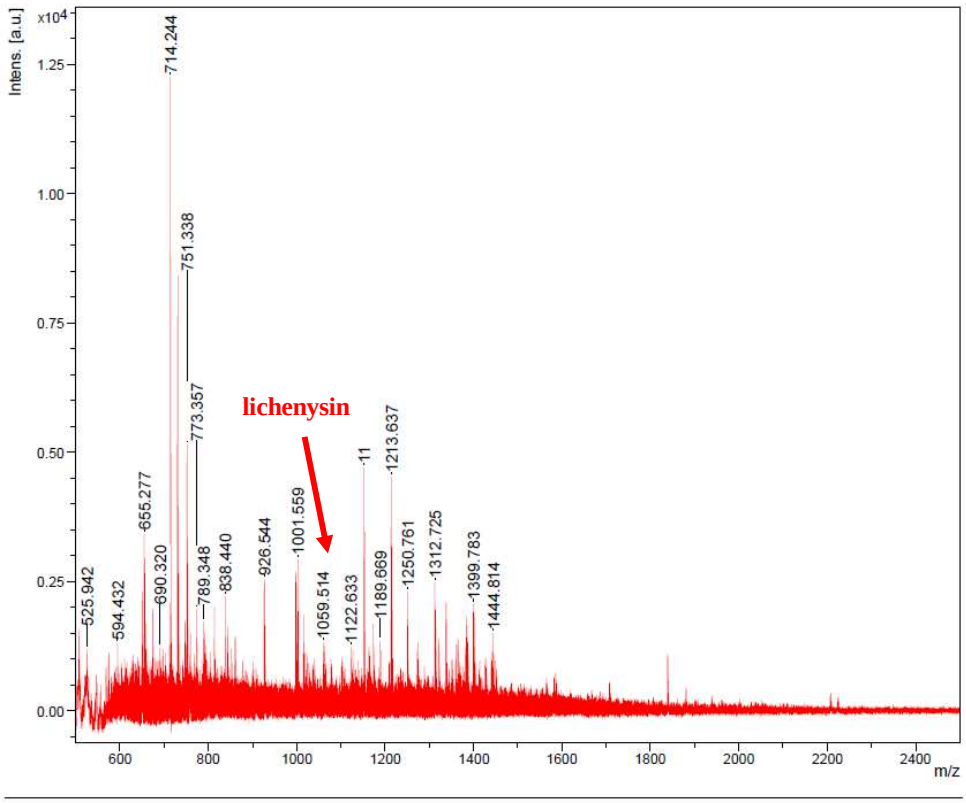

Figure 6.2. MALDI-TOF/MS analysis of *B. licheniformis* ATCC 14580 grown on blood agar

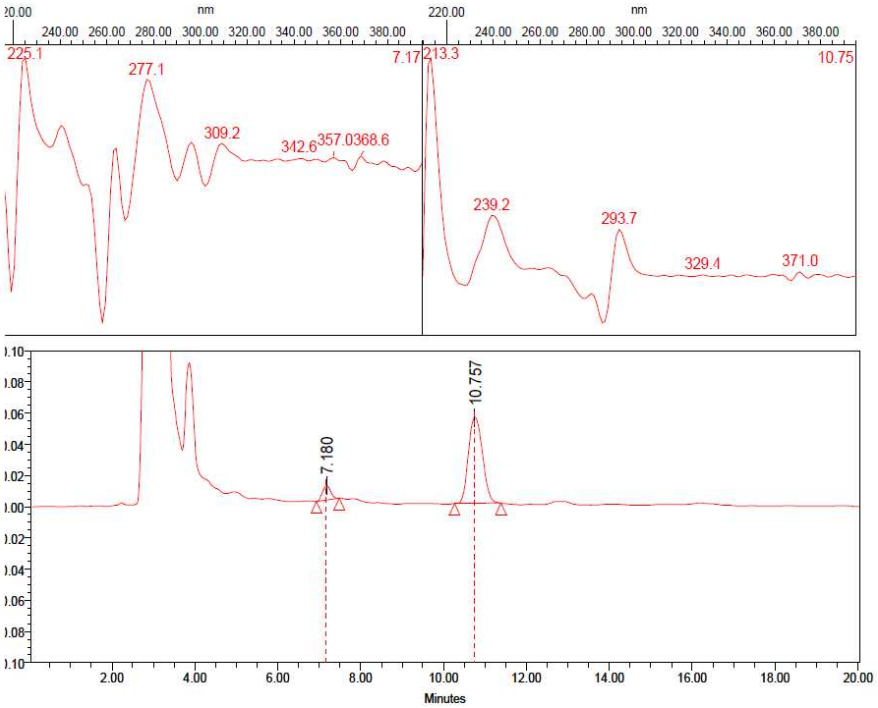

Figure 6.3. HPLC chromatogram of *B.licheniformis* ATCC 14580 supernatant

retention times in HPLC.

It is thus confirmed that the strain has the capacity to synthesize lichenysin. Regarding to bioinformatic analyses, some genetic manipulations were performed with the aim to increase the yield of lipopeptide.

6.4. Strategy of molecular optimization of lichenysin synthesis

The strategy was to replace the native promoter of lichenysin synthetase P_{lchA} by the promoter P_{xylA}, inducible by xylose, using homologous recombination. The promoter replacement of *B. licheniformis* ATCC 14580, whose genome is completely sequenced, is performed with the aim of obtaining a high-yield lichenysin producer.

The strategy was as follows:

- PCR amplification of the cassettes of two sides of lichenysin promoter (four primers to be designed);
- Construction of a plasmid containing the sequences of two sides of lichenysin promoter flanking P_{xylA} originating from *Bacillus megaterium* xylose isomerase;
- Replacement of the native promoter of lichenysin synthetase P_{lchA} in *B. licheniformis* by P_{xylA} , using homologous recombination.

Genomic DNA was extracted as previously written (§ 2.10.4). PCR amplification was performed using the primers listed in Table 6.8. All PCR-generated cassettes were purified and quantified in 1 % agarose gel as previously described (§ 2.10.9). The different plasmids replicated in different *E. coli* transformants were extracted (§ 2.10.5). The transformations of *E. coli* were performed by thermal shock. All constructed plasmids were purified and quantified in 0.7% agarose gels.

B. licheniformis ATCC 14580 was transformed by either the natural competence method (§ 2.10.10.4, assuming that both impairment of the *comP* gene and apparent lack of the *comS* gene had no effect on competence) or electroporation (§ 2.10.10.2/3).

The transformants were selected on LB agar containing the required antibiotics and different compounds (such as blood or xylose). They were incubated at 37°C for 48 h. The genetic modifications were investigated by PCR amplification.

6.4.1. Evaluation of antibiotic resistance of the wild-type strain

To verify antibiotic resistance pattern, the strain was cultivated on spectinomycin 100 μg ml^{-1} and kanamycin 100 μg ml^{-1}. A few colonies were observed on plates containing spectinomycin while there were no colonies on kanamycin ones. So we changed the concentrations to 150 and 200 μg ml^{-1} for spectinomycin and 25 and 50 μg ml^{-1} for kanamycin. At those concentrations, growth on all plates after 48 h was negative (the plates were kept at room temperature for 1 week more to be sure about the absence of growth).

6.4.2. Primer designing and PCR amplification of four cassettes from *B. licheniformis* ATCC 14580

The sequence of *lch* promoter (P_{lchA}) of *B. licheniformis* ATCC 14580 was determined by database obtained from NCBI site (PubMed Gene NC-006270). Two set of cassettes situated upstream and downstream from the promoter region were identified (Figure 6.4). The cassettes of 1 and 3 contain the end of *lam* gene (binding protein dependent on transport system in inner membrane protein) and the beginning of *lch* promoter. The cassette 2 consists of the region after -10 box and and the beginning of *lchAA*. The cassette 4 contains a part of the promoter region (-35 and -10 boxes) (Index VII) and the beginning of *lchAA* (Figure 6.4 and Table 6.8). To increase transformation possibility in *B. licheniformis* ATCC 14580, four cassettes were considered.

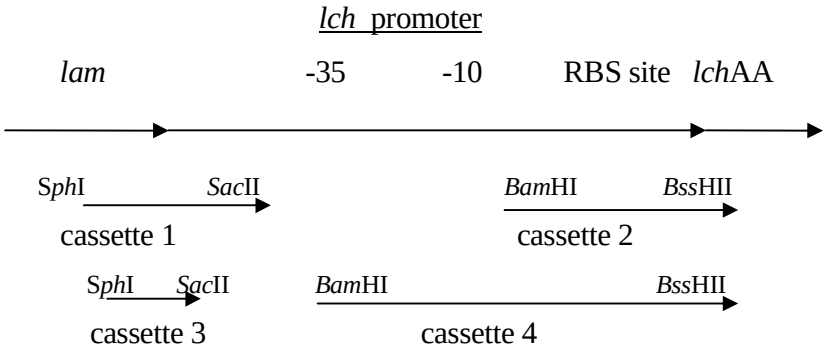

Figure 6.4. *lch* promoter and designed cassettes.

Table 6.8. Cassettes obtained from *B. licheniformis* ATCC 14580 and different oligonucleotide primers. Underlying bases indicate substituted bases to generate terminal sites which are shown in boldface type

Cassette 1 (671 bp)	the end of *lam* gene and the upstream from P_{lchA} Forward primer: *lam-Sph*I (26 bp) 5′-AAGCTTGAACTGGCAT**GCATGC**ATGA-3′ Reverse primer : *lch-Sac*II (22 bp) 5′-**CCGCGG**GATACCGAATGTTGTA-3′
Cassette 2 (567 bp)	the downstream from P_{lchA} (after-10) and the upstream from *lchAA* Forward primer: -10-*Bam*HI (20 bp) 5′-**GGATCC**GCAGGGACGCAAAG-3′ Reverse primer: *lchAA-Bss*HII (24 bp) 5′- TGCGAACAACGGCAAATTTAAACA-3′ *
Cassette 3 (580 bp)	the end of *lam* gene and the upstream from P_{lchA} Forward primer : *lam-Sph*I (21 bp) 5′-AACTGGCAT**GCATG**AATCTCG-3′ Reverse primer : *lch-Sac*II (20 bp) 5′- ATCGTTTAAAC**CCGCGG**AAA-3′
Cassette 4 (720 bp)	the promoter region and the upstream from *lchAA* Forward primer: -35-*Bam*HI (21 bp) 5′-G**GGATC**CCGCATTAACATTCA-3′ Reverse primer: *lchAA-Bss*HII (22 bp) 5′- CGGCAAATTTAAACAGCGGACT-3′ *

*Natural *Bss*HII sites are situated in the amplicons, before the primer sequence.

The primers were designed by software Primer 3 and the oligonuleotides were ordered to Eurogentec (Angers, France). After PCR amplification (§ 2.10.6), the resulting bands were observed using the GelDoc device from Bio-Rad with Quantity One software (Bio-Rad Laboratories, Hercules, USA) and then purified using QIAquick Gel Extraction Kit Protocol (QIAGEN) and quantified in 1% agarose gel. The fragments between 570 and 720 bp of *lch* promoter region were obtained in this way (Figure 6.5)..

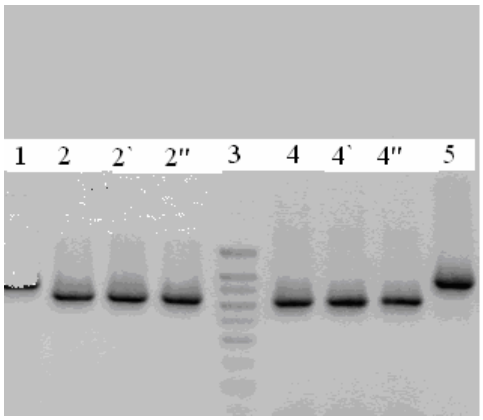

Figure 6.5. PCR fragments. Lane 1: Fragment 1 (671 bp); lane 2, 2′, 2″ Fragment 2 (567 bp); lane 3: Smart ladder (marker: 1000, 800, 600, 500,… from up to down); lane 4, 4′, 4″: Fragment 3 (580 bp); lane 5: Fragment 4 (720 bp).

6.4.3. PCR fragments cloning into pGEM-T Easy plasmid

To obtain sufficient amount of the four fragments containing the sequences upstream and downstream from *lch* promoter, they were cloned in a commercial vector, pGEM-T Easy (Promega). The plasmids designated pBG155, pBG156, pBG157 and pBG158 (Table 2.4) contain fragments of cassettes 1, 2, 3 and 4 respectively. To verify these new plasmids, they were digested with *Eco*RI. Table 6.9 shows the size of expected fragments after digestion with *Eco*RI. These fragments are observed in Figure 6.6.

Table 6.9. The fragments resulting from digestion of different plasmids (containing 4 different fragments of PCR) with *Eco*RI

Plasmid	Number of cut sites	Number of sites obtained	Fragments sizes (bp)
pBG155	2	2	700, 2986
pBG156	2	2	641, 2941
pBG157	2	2	615, 2980
pBG158	2	2	784, 2951

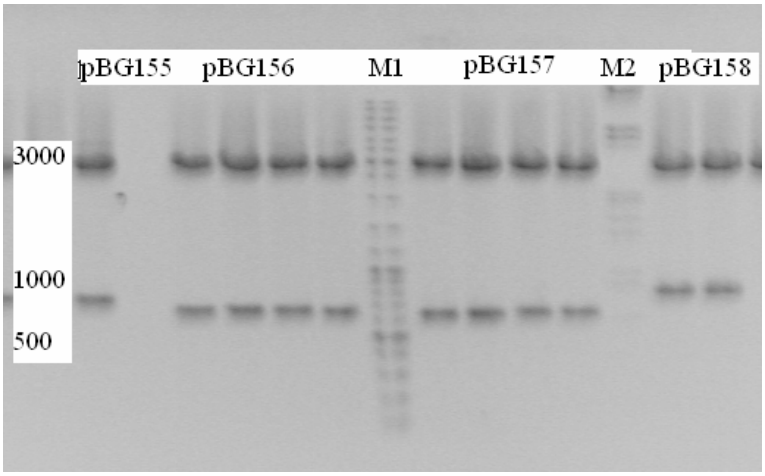

Figure 6.6. pBG155, pBG156, pBG157 and pBG158 after digestion with *Eco*RI (1% agarose gel). M1 is marker O'GR and M2 is lambda DNA digested by *Eco*RI+*Hin*dIII (Index VI).

To further verify, plasmids were digested by *Hin*dIII (plasmids containing fragments 1 and 3) and *Ssp*I (plasmids containing fragments 2 and 4) (data not shown).

To determine the direction of fragments, they were digested by *Hin*dIII and *Sac*II (data not shown).

To isolate the PCR fragments, pBG155 and pBG157 were then double-digested with *Sph*I and *Sac*II, and pBG156 and pBG158 were also double-digested with *Bam*HI and *Bss*HII. They were then loaded on 1% agarose gel to examine the fragments. Purification by Gel Extraction Kit was used to obtain the fragments 1 to 4.

DNA sequencing confirmed the identity of all 4 PCR fragments inserted into pGEM-T Easy.

6.4.4. Construction of pBG162 and pBG163

The fragments 1 and 3 were inserted into pBG216 separately (it originates from pBG214 (containing P_{xylA}) double-digested by *Sac*II-*Sph*I to remove ε*pbp* cassette from it and to produce the linear plasmid pBG216) (§ 2.10.7) to generate hybrid plasmids pBG216-1 and BG216-3, respectively. The fragments 2 and 4 were also inserted into pBG215 separately (it originates from pBG214) double-digested by *Bam*HI-*Bss*HII to remove the ε*fenF* cassette from it and to produce the linear plasmid pBG215) to generate the hybrid plasmids pBG215-2 and pBG215-4, respectively. These new plasmids were inserted into *E. coli* JM109 as previously mentioned (§ 2.10.8). Regarding the restriction maps of the hybrid plasmids, the plasmids pBG216-1 and

pBG216-3 were digested with *Hin*dIII, *Nsi*I, *Ssp*I, *Nde*I and *Nsp*I and the plasmids pBG215-2 and pBG215-4 were also digested with *Ssp*I. The restriction pattern was as expected.

In addition, pBG214 was digested with *Hin*dIII, *Nsi*I and *Ssp*I to be compared as control with the other hybrid plasmids.

The fragments 1 and 3 were ligated into pBG215-2 (pBG160) and pBG215-4 (pBG161) respectively, previously digested with *Sph*I and *Sac*II, to give rise to the plasmids pBG162 and pBG163, respectively (Figures 6.7 and 6.8) as follows:

pBG160 + (*Sph*I and *Sac*II) → pBG160′ (linear) + fragment 1 → pBG162

pBG161 + (*Sph*I and *Sac*II) → pBG161′ (linear) + fragment 3 → pBG163

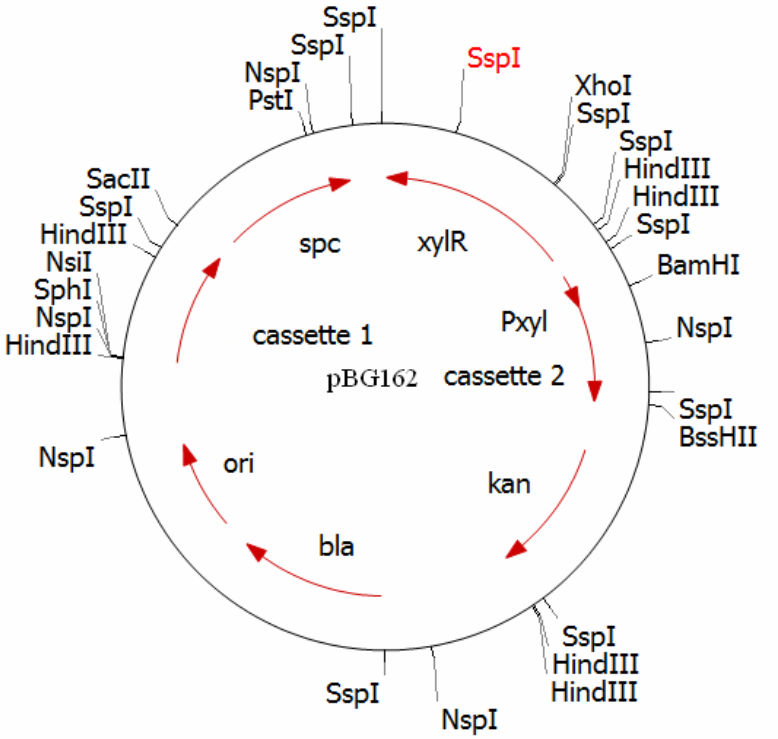

Figure 6.7. Genetic map of pBG162 (7704 bp)

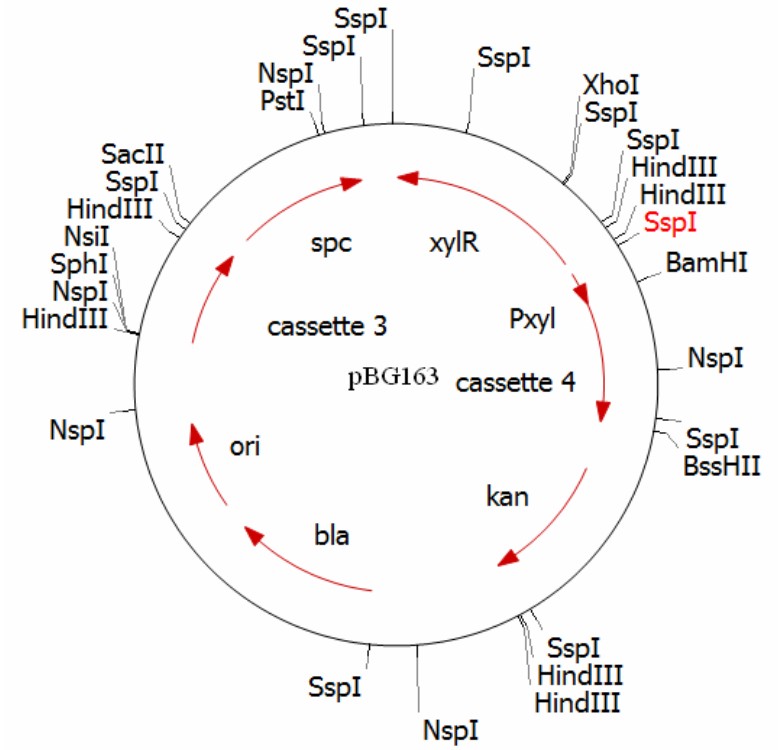

Figure 6.8. Genetic map of pBG163 (7763 bp)

These new plasmids were introduced into JM109 as previously mentioned (§ 2.10.7). To confirm that antibiotic-resistant colonies were true transformants, the colonies first grown on ampicillin 50 µg ml^{-1} were subcultured on LB plates containing the other antibiotics (spectinomycin and kanamycin). After positive growth of these clones, the new plasmids were extracted. They were loaded onto a 0.7% agarose gel to verify the presence of the desired plasmids.

To confirm that the fragments 1 and 3 were inserted in these new plasmids, they were digested with the restriction enzymes *Nsi*I, *Ssp*I and *Sac*II+*Sph*I (Table 6.10, Figure 6.9).

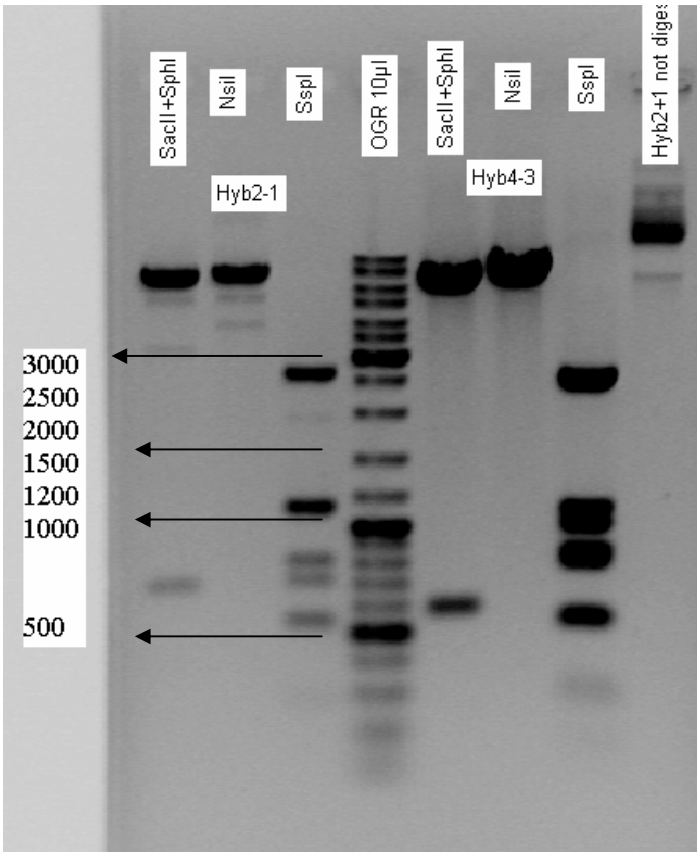

Figure 6.9. Left to right: Lanes 1, 2 and 3: pBG162 digested with *Sac*II+*Sph*I, *Nsi*I and *Ssp*I respectively; lane 4: marker: O'GR; lanes 5, 6 and 7: pBG163 digested with *Sac*II+*Sph*I, *Nsi*I and *Ssp*I, respectively; lane 8: pBG162 not digested, used as control.

Table 6.10. The fragments resulting from digestion of pBG162 and pBG163 with *Sac*II+*Sph*I, *Nsi*I and *Ssp*I (the sizes are in bp)

Plasmid	***Sac*II+*Sph*I**	***Nsi*I**	***Ssp*I**
pBG162	7316, 671	7987	2601, 1287, 1117, 851, 790, 525, 349, 256, 136, 134
pBG163	7466, 580	8046	2601, 1379, 1117, 790, 700, 525, 349, 256, 136, 134

The fragments 1287 and 1379 after digestion of pBG162 and pBG163 with *Ssp*I were not observed. To confirm further, pBG162, pBG163 and pBG214 were digested with *Hin*dIII, *Nsp*I and *Ssp*I (Figure 6.10, Table 6.11).

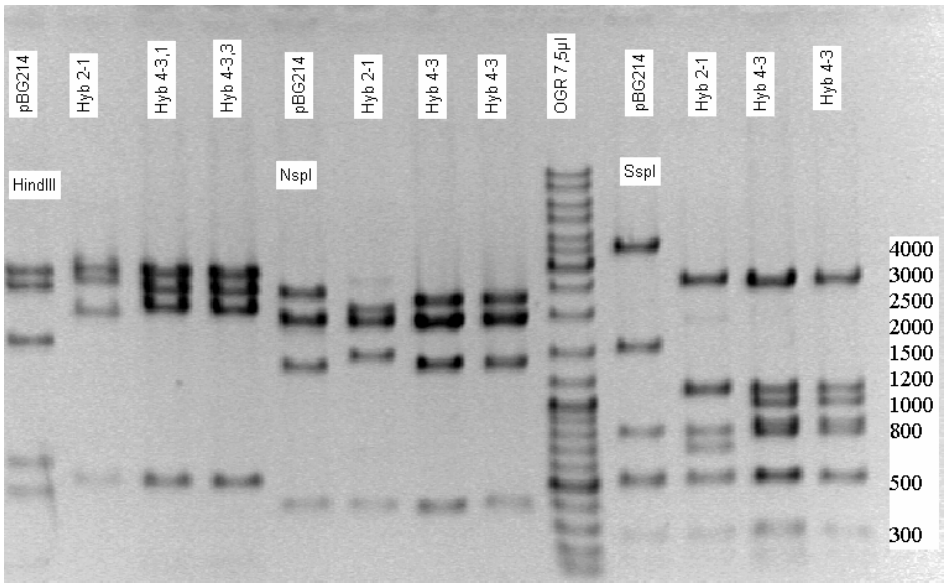

Figure 6.10. Lanes 1, 2, 3 and 4: pBG214, pBG162 (Hyb 2-1), pBG163 (Hyb 4-3,1 and Hyb 4-3,3) digested with *Hin*dIII; lanes 5, 6, 7 and 8: pBG214, pBG162 (Hyb 2-1), pBG163 (Hyb 4-3) digested with *Nsp*I; lane 9: marker, O'GR; lanes 10, 11, 12, 13: pBG214, pBG162 (Hyb 2-1), pBG163 (Hyb 4-3) digested with *Ssp*I.

Table 6.11. The fragments resulting from digestions with *Hin*dIII, *Nsp*I and *Ssp*I (the sizes are in bp)

Plasmid	***Hin*dIII**	***Nsp*I**	***Ssp*I**
pBG214	2777, 2663, 1547, 590, 441, 69, 13	2311, 1917, 1894, 1613, 365	3861, 1543, 790, 525, 506, 349, 256, 136, 134
pBG162	2777, 2723, 2137, 480	2311, 1917, 1613, 365	2601, 1287, 1117, 851, 790, 525, 349, 256, 136, 134
pBG163	2777, 2638, 2137, 485		2601, 1379, 1117, 790, 700, 525, 349, 256, 136, 134

The resulting fragments showed the expected size, except the fragments 1287 and 1379 after digestion of pBG162 and pBG163 with *Ssp*I, respectively.

To confirm further these results, pBG214 and pBG162 were digested with different restriction enzymes *Sac*II+*Xho*I, *Sac*II+*Pst*I, *Sac*II+*Bam*HI and *Bam*HI+*Bss*HII (Table 6.12, Figure 6.11).

The resulting fragments were as expected regarding the restriction map, except for digestion with *Sac*II+*Pst*I in which the fragment 1027 bp was not observed. We supposed a fragment, about 283 bp after *Sac*II region, had been deleted from these plasmids. In consequnce, a fragment of about 800 bp should be observed instead of 1027 bp (Figure 6.10). This finding can explain why the fragments of 1287 bp and 1379 bp were not observed after digestion with *Ssp*I of pBG162 and pBG163, respectively.

Table 6.12. The fragments resulting from pBG214 and pBG162 after digestion with different enzymes (the sizes are in bp)

Plasmid	**Endonucleases**			
	*Sac*II+*Xho*I	*Sac*II+*Pst*I	*Sac*II+*Bam*HI	*Bam*HI+*Bss*HII
pBG214	5854, 2246	7073, 1027	5260, 2840	not digested
pBG162	5741, 2246	6960, 1027	5147, 2840	7420, 567

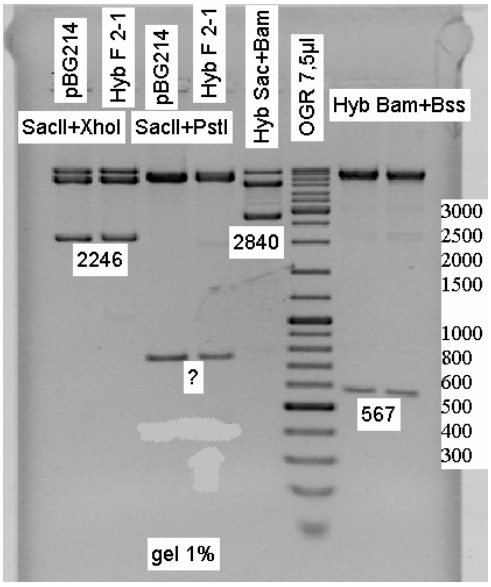

Figure 6.11. Lanes 1 and 2: pBG214 and pBG162 digested with *Sac*II+*Xho*I; lanes 3 and 4: pBG214 and pBG162 digested with *Sac*II+*Pst*I; lane 5: pBG162 digested with *Sac*II+*Bam*HI; lane 6: marker, O'GR; lanes 7 and 8: pBG162 digested with *Bam*HI+*Bss*HII. 1% agarose gel was used.

To confirm this hypothesis, pBG214, pBG162 and pBG163 were digested with *Nru*I and *Pac*I (6863, 6872 bp, respectively) which should be located in this region (after *Sac*II region). The result was negative for all plasmids, showing that this region has been deleted. Comparison of this region in pBG214 sequence and the region sequenced by Cogenics Genome Express in pBG162/pBG163 confirmed the deletion. However, this region was not essential for the constructions, being situated between the cassettes 1/3 and the *spc* gene.

DNA sequencing confirmed the identity of the two fragments inserted into pBG162 and pBG163.

6.4.5. *B. licheniformis* ATCC 14580 transformation

Different transformation methods (§ 2.10.10) were performed to transform *B. licheniformis* ATCC 14580. The selection methods used in this study include hemolysis, antibiotic selection and spreading on semi-solid media (Leclere *et al*. 2006), including the swarming property.

If the desired plasmids are transferred into *B. licheniformis* ATCC 14580, colonies should be observed on different media according to Table 6.13 and it is expected to obtain two kinds of mutants resulting from homologous recombination. If one crossing-over happens, transformed colonies containing pBG162 or pBG163 are able to grow on LB plates containing spectinomycin or kanamycin, while they lyse erythrocytes only in the presence of xylose, since the latter is

Table 6.13. Patterns of growth and hemolysis of *B. licheniformis* ATCC 14580 on different media after transformation

Medium	**Spontaneous mutant**	**Mutant with one crossing-over**	**Mutant with 2 crossing-over**
LB+Spc	++	++	++
LB+Kan	-	++	-
LB+blood (hemolysis)	+/-	-	-
LB+blood+xylose (hemolysis)	+/-	++	++

the inducer of the xyl promoter now controlling lichenysin production. If two crossing-over happen, mutants grow on LB plates containing only spectinomycin but not on LB containing kanamycin. Moreover, hemolysis will occur only in the presence of xylose.

B. licheniformis ATCC 14580 was transformed three times by electroporation and three times by natural competence, but every time many spontaneous colonies were observed, which were resistant to spectinomycin (we had to increase concentration of spectinomycin up to 400 μg ml^{-1}). Therefore, it was difficult to find the desired colonies among hundreds of colonies.
Three of these colonies (resistant to spectinomycin), which showed both hemolytic (in the presence of xylose) and swarming property (cultivated on B medium), were selected for production, ST determination and PCR experiments (primers were mentioned in Table 6.8). HPLC results demonstrated that one of them produced a biomolecule which is eluted in the same conditions than surfactin, with a 10-fold higher yield than the wild type (data not shown). In addition, the culture supernatant showed ST reduced to 35.6 mN m^{-1} (standard deviation ± 0.1). However, the absence of a positive specific PCR amplification showed that they were spontaneous mutants.

In conclusion, it seems that the antibiotic pressure chosen in this study was insufficient and inadequate to differentiate true mutants from spontaneous ones.

6.5. Discussion

B. licheniformis is closely related to the well-studied model organism *B. subtilis* (Xu, 2003). The availability of a complete genome sequence for *B. licheniformis* ATCC 14580 facilitates the design and construction of improved industrial strains and allows for comparative genomics.
Regions shared between the genomes of *B. licheniformis* ATCC 14580 and *B. subtilis* 168 are approximately 84.6% identical at the nucleotide level and show extensive organizational similarity.
As noted by Lapidus *et al*. (2002), there are broad regions of colinearity between the genomes of *B. licheniformis* and *B. subtilis*. These observations clearly support previous hypotheses that *B. subtilis* and *B. licheniformis* are phylogenetically and evolutionarily close. Despite the broad colinearity of *B. licheniformis* and *B. subtilis* genomes, there are local regions that are individually unique. These include chromosome segments that comprise DNA restriction-modification systems, antibiotic

synthases, and a number of extracellular enzymes and metabolic activities that are not present in *B. subtilis*.

B. licheniformis ATCC 14580 possesses the operon coding for the synthesis of lichenysin and also a potentially functional *sfp* gene (that is frame-shifted in *B. subtilis* 168, rendering this strain unable to produce surfactin). The modular organization found in the lichenysin synthetase LchA to -C is completely identical to the synthetase SrfA-A to -C. In addition, *B. licheniformis* genome encodes all of the late competence functions described in *B. subtilis* (such as *comC*, *comEFG* operons, *comK*, *mecA*), but it lacks an obvious *comS* gene. Moreover, *comP* gene (necessary to stimulate surfactin production in *B. subtilis* via phosphorylation of *comA* gene product) in this strain is punctuated by an insertion sequence element (Randy, 2004). Therefore, *B. licheniformis* ATCC 14580 cannot produce large amounts of lichenysin. This justify the strategy chosen here, i.e. the replacement of the *lchA* promoter.

In this study, the comparisons were performed with each ORF of different *Bacillus* species against a database of the second one: *B. licheniformis* ATCC 14580/ *B. subtilis* 168, *B. licheniformis* ATCC 14580/ *B. amyloliquefaciens* FZB42 and *B. amyloliquefaciens* FZB42/ *B. subtilis* 168. Bioinformatic analyses confirmed higher similarities between *B. amyloliquefaciens* FZB42/ *B. subtilis* 168 than between *B. licheniformis* ATCC 14580 and the two other strains.

Analyses performed by NRPS Predictor (§ 6.2) showed that surfactin produced by *B. subtilis* 168 and *B. amyloliquefaciens* FZB42 have the same amino acid composition, but lichenysin produced by *B. licheniformis* ATCC 14580 has differences in the first and seventh amino acids in which Glu and Leu are substituted by Gln and Ile, respectively (Table 6.5).

Konz *et al*. 1999 reported cloning and overexpression of the adenylation domain from the first module of the lichenysin synthetase LicA in *B. licheniformis* ATCC 10716 and showed that it specifically activates L-glutamine.

For domains that activate amino acids with polar sidechains, one or two universally conserved polar residues can be identified that presumably interact with the substrate sidechain through hydrogen bonding and electrostatic interactions. The remaining residues in the specificity pocket are hydrophobic and vary among domains that activate the same amino acid. In contrast, for substrates with hydrophobic sidechains, the residues lining the specificity pockets are all hydrophobic, except for proline. In fact, many of these domains have lower substrate selectivity

than those activating polar amino acids. Although some of these residues appear to be highly conserved, variation in other residues seems to be tolerated (Challis *et al*. 2000).
However, a more detailed analysis is required to identify important residues for substrate recognition.

Regarding bioinformatic analyses in this study, molecular techniques were used to bioengineer *B. licheniformis* ATCC 14580. The aim of this research was modification of native promoter of *lchA* operon from *B. licheniformis* ATCC 14580 (which was not capable of producing large amounts of lichenysin) to tailor the strain for a high-yield lichenysin production. The studies presented here include: 1) getting information of promoter of *lchA* operon; 2) designing primers; 3) construction of plasmids; 4) using different transformation techniques to replace the native promoter P_{lchA} by the strong and tightly regulated promoter P_{xylA} from the *Bacillus megaterium* xylose isomerase, which was shown previously to be strong and inducible by xylose in *B. subtilis* ATCC 6633 (Fickers *et al*. 2009) after insertion by homologous recombination.
In this research, several transformation methods were performed to generate *B. licheniformis* ATCC 14580 transformants. The colonies appearing on selective media (LB supplemented by spectinomycin and blood at the presence of xylose) were examined on B medium to observe swarming phenomenon, and then analyzed by PCR for presence of transformants, but they lacked the expected sequences.
On one side, the strain is not easily amenable to genetic manipulations by techniques used in this study; on the other side, due to the presence of strongly dominant spontaneous mutants, and despite being an effective selection procedures to get transformants in previous experiments (Leclere *et al*. 2006 and Coutte *et al*. 2008: not published/in press), we could not get the desired colonies.
It seems that the antibiotic pressure (spectinomycin used in this study) was not adequate to select desired transformants, therefore future attempts to transform the strain could be focused on the construction of plasmids containing resistance genes to other antibiotics.

The improvement of many bacterial strains has been hampered by lack or low efficiency of genetic transformation systems. In general, transformation efficiencies obtained by electroporation of gram-positive bacteria are relatively low, compared to those of gram-negative species (Trevors *et al*. 1992). In addition, *B. licheniformis* does not appear to be a strain naturally competent in contrast to

some *B. subtilis* strains. The synthesis of a glutamyl polypeptide capsule has also been identified as a potential barrier to transformation of *B. licheniformis* strains (Thorne, 1966).

Furthermore, the transformation frequency of protoplasts generated from the strain used here was found low in comparison with *B. subtilis* (Maghnouj *et al*. 2000).

Although current knowledge on the expression of peptide synthetases indicate that it should be possible to achieve much higher levels of synthesis of lichenysin A by genetic optimization, most attempts to genetically manipulate *B. licheniformis* strains with this purpose have so far been unsuccessful.

7. Conclusion and perspectives

7. Conclusion and perspectives

Biosurfactants have gained importance in the various fields of food, cosmetic, pesticide, detergent, pharmaceutical industries, enhanced oil recovery, transportation of heavy crude oil and bioremediation (Georgiou, 1992; Desai, 1997), owing to their unique properties such as lower toxicity, higher biodegradability (Zajic, 1997; Woo, 2004), higher foaming and better environmental compatibility (Georgiou, 1992; Banat, 1995).

This study was on the basis of a multidisciplinary approach, which includes:

- genetic manipulation of microbial cells to increase biosurfactant production,
- production and purification of biosurfactants,
- characterization of biosurfactants by different analytical methods,
- study of conditioned substrata by these biosurfactants by goniometry and XPS analyses to demonstrate their ability to modify surfaces properties,
- study of *B. cereus* 98/4 spores attachment on these different conditioned substrata.

The members of three families of lipopeptidic compounds produced by *B. subtilis* strains including surfactin S1, iturin A and mycosubtilin (two members of the iturin family), and fengycin, as well rhamnolipids (glycolipids) produced by *P. aeruginosa* PTCC 1637, were investigated.
Purified forms of some of them were provided by Sigma or kindly supplied by Dr Magali Deleu from Gembloux Agro-Bio Tech (surfactin S1, iturin A and fengycin) and the other lipopeptides (iturin A, mycosubtilin and fengycin) were produced, purified and characterized by several analytical methods (TLC, HPLC, LC/MS and MALDI-TOF/MS). The optimal conditions were considered on the basis of previous studies for production of lipopeptides in Landy medium.
Efficiency of purification in best conditions was 45% for fengycin and much lesser for other lipopeptides. Regarding low efficiency of purification of final product, extraction methods should be improved.

Rhamnolipids from *P. aeruginosa* PTCC 1637 were also produced in Lindhardt and 3M medium. Lindhardt medium with 6% corn oil as carbon source was the best medium for rhamnolipid production, which gave a yield about 20 g l^{-1}. Rhamnolipids were characterized by different

analytical methods (TLC, HPLC, HPLC/MS and FTIR). The analyses showed that they are a mixture from mono- and di-rhamnolipids.

The different solutions of biosurfactants used in this study contained several homologous compounds from the same family. As the critical micelle concentration is slightly different for each homologous compound (Deleu *et al*. 2003), differences were observed between ST and CMC of biosurfactants used here and those of the literature.

The compounds were then examined for their ability to modify the surface hydrophobicity of the two substrata stainless steel (SS) and Teflon.

These modifications were evaluated by water contact angle measurements and XPS analyses. Conditioning of stainless steel with the various biosurfactants resulted in more or less marked increase of the hydrophobic character, but the changes varied among them. The effects depend on the biomolecule, the concentration, and the substratum (Table 7.1).

Table 7.1. Effect of different lipopeptides on SS and Teflon: comparison of goniometry and XPS results

Lipopeptide	**Goniometry**	**XPS**	**XPS**
	Increase in hydrophobicity on SS	**Increase in C signal at highest concentration, on SS**	**Increase in N signal at highest concentration, on SS**
Surfactin	+++	++	++
Iturin A /Mycosubtilin	_	+	++
Fengycin	++ up to CMC, then decrease	+ up to CMC, then decrease	++
	Decrease of hydrophobicity on Teflon	**Increase in C signal at highest concentration, on Teflon**	**Increase in N signal at highest concentration, on Teflon**
Surfactin	+	+++	++
Iturin A /Mycosubtilin	+++	+	+
Fengycin	+/-	+	+

Two families of lipopeptides increased the hydrophobicity of stainless steel, *viz* surfactin and fengycin. Iturin A and mycosubtilin had no effect. The difference between the families can be mainly attributed to the variability in the primary structure of the peptide cycle, which is responsible for the 3D structure at the interface (Deleu *et al*. 1999). The 3D structure of peptide combined with the presence of the lipidic chain could be a crucial parameter in changing surface properties.

Treatment of stainless steel with different concentrations of surfactin S1 showed an increase of the hydrophobicity between 1 and 100 mg l^{-1}. The surfactin peptide cycle is more hydrophobic than that of iturin A and fengycin (Deleu *et al*. 1999), which can explain the higher contact angle observed with the former.

The presence of the carboxylic groups of both glutamate and aspartate in surfactin was found to be important for surface activity (Bonmatin *et al*. 2003; Youssef *et al*. 2005). The negative charges of Glu and Asp are important for the adhesion of surfactin to the stainless steel surface. Indeed, the molecule could no longer increase stainless steel hydrophobicity when these charges were neutralized by 0.1 M $NaHCO_3$. According to previous studies (Deleu *et al*. 1999), it is supposed surfactin orients perpendicular to the stainless steel, with the hydrophilic head groups in contact with the stainless steel (Figure 7.1).

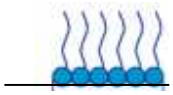

Figure 7.1. Monolayer of surfactin on SS.

XPS analyses of surfaces treated by lipopeptides confirmed the presence of the different biomolecules. Following the presence of surfactin on stainless steel, typical elements of substrate (Ni, Cr, Fe and Mo) clearly decreased and the N and C signals increased at its highest concentration.

On the contrary, fatty acid chains can interact with the hydrophobic surfaces and the polar peptidic moieties would be exposed to the environment (Figure 7.2). In these conditions, a decrease in the hydrophobicity of the surface could be observed.

Teflon conditioning resulted in a more or less marked reduction of the contact angle in all lipopeptides.

Conditioning of Teflon with surfactin resulted in a slight but significant decrease (*p*-values < 0.0001) of the water contact angle. According to the XPS results, when surfactin adhered to Teflon it

caused an increase of the N, O and C signals (these signals do not exist in Teflon) that were more marked at 100 mg l^{-1} compared to 10 mg l^{-1}.

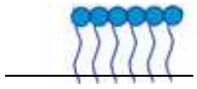

Figure 7.2. Monolayer of surfactin on Teflon.

This could be also correlated to the results obtained by goniometry for surfactin with a reduction of hydrophobicity on Teflon. In addition, surfactin has surface area greater than fengycin, and can extend on the surface better than the latter which is in accordance with XPS results.

Fengycin increased hydrophobicity on stainless steel up to its CMC (6.25 mg l^{-1}). However, at concentrations higher than CMC, different results were observed for fengycin and a decrease in the contact angle occurred. Under these conditions, there are three hypotheses to justify the behaviour of the molecule:

1) a decreased number of molecules on stainless steel (The molecules would be organised in micelles in solution and this configuration would be thermodynamically more favourable than adsorption to the surfaces);

2) adhesion on stainless steel with the formation of micelles;

3) adhesion on stainless steel with the formation of a fully interdigitated bilayer, where each hydrocarbon tail spans the entire hydrocarbon width of the bilayer, and interacts laterally, through hydrophobic forces, with the hydrocarbon tail of fengycin molecules from the opposing lamellar leaflet.

Such a hypothesis was suggested for the organization of iturin in solution (Grau *et al*. 2001; Yu *et al*. 2002).

The XPS results demonstrated that the carbon signal after adsorption of fengycin on stainless steel increased up to the concentration 6.25 mg l^{-1} (CMC of molecule) and then decreased at 25 mg l^{-1} (its highest concentration), but the nitrogen signal increased progressively. Other typical elements of stainless steel decreased following more adsorption of fengycin.

The XPS results confirmed the changes of hydrophobicity measured by goniometer. Considering XPS results, the first hypothesis is denied. However, further studies are required to discriminate micelle formation from bilayer one.

Conditioning of Teflon with fengycin resulted in a slight but significant decrease (*p*-values < 0.0001) of the water contact angle, while Teflon remained highly hydrophobic even at 100 mg l^{-1}. XPS results for fengycin on Teflon exhibited that N, O and C signals increased at higher concentrations of this lipopeptide but in less extent compared to surfactin. This result does not correspond to those obtained by goniometry for fengycin in which at higher concentrations of fengycin, hydrophobicity does not change. As it was mentioned previously, fengycin forms micelles or bilayers at higher concentrations, and could no longer change hydrophobicity on Teflon at these concentrations, whereas according to the XPS results, these supramolecular structures resulted in increased amounts of the respective elements on Teflon.

The slight modifications induced on stainless steel by conditioning with iturin A and mycosubtilin were not significant (*p*-value = 0.3022). The absence of charged amino acid in the peptidic moiety of iturin A and mycosubtilin could explain the lack of effect of such compounds on stainless steel.
XPS analyses of stainless steel teated with iturin A demonstrated that N and C signals increased at higher concentrations. This result does not correspond to the results obtained by goniometry in which at higher concentrations no modification of hydrophobicity was observed.
It is supposed iturin molecules form a bilayer at higher concentrations on stainless steel, and would be unable to change hydrophobicity under these conditions.
All lipopeptides decreased the hydrophobicity on Teflon (Figure 7.1). Indeed, the change in the hydrophobicity depends on the whole hydrophobic character of the lipopeptide film adsorbed onto the surface, which was due to both lipid and peptide parts. With iturin A and mycosubtilin, a remarkable decrease (*p*-value < 0.0001) in the contact angles was observed at concentrations over 1 mg l^{-1}, and the lowest value was at 100 mg l^{-1}. At this concentration, conditioned Teflon was hydrophilic.
In this regard, the length of the fatty acid chain could be determinant. This hypothesis was confirmed by the results obtained with mycosubtilin. Mycosubtilin differs from iturin A by the sequence inversion of two adjacent Ser6-Asn7 residues. In addition, it has a longer carbon atom chain (16-17) in comparison with iturin A (14-15) which led to an enhanced effect on Teflon.
XPS analyses of Teflon teated with iturin A demonstrated that the N, O and C signals increased at higher concentrations, but in less extent compared to surfactin. This result does not correspond to the results obtained by goniometry in which at higher concentrations hydrophobicity increased noticeably.

Regarding surface area of iturin A (300 $Å^2$) that is clearly greater than the two others (262 and 181 $Å^2$ for surfactin and fengycin, respectively), it seems that small amounts of molecule can introduce significant modifications in surface properties.

The consequences of spores adhesion of *B. cereus* 98/4 were then investigated on both substrata previously conditioned by biosurfactants.
Interestingly, despite great differences in the effects of biosurfactants on hydrophobicity of stainless steel and Teflon, the adhesion profiles of spores of *B. cereus* 98/4 on theses substrata were close to those of the water angle.
Results exhibited that there are promising correlations between hydrophobicity modifications of surfaces and the attachment of *B. cereus* 98/4 spores to these surfaces. Enhancement in hydrophobicity of surfaces increases the number of adhering spores to them and vice versa.

As previously mentioned (§ 2.3), iturin A was produced in our laboratory too. In surface property experiments, iturin A obtained from Belgium was used. The results showed that contact angles of mycosubtilin were significantly lower than iturin A (Figure 4.8). Regarding the length of fatty acid chain which is longer in mycosubtilin, it led to a better effect on Teflon.
In adhesion experiments, iturin A purified in the Lab was used. It showed more noticeable effects than mycosubtilin (6.5-fold more spores against 2.5-fold) on Teflon. The MS analysis for both samples showed that homologous C15 exists in higher amounts than C14 in the Lab sample (Figure 3.7).
It seems that the correlation between fatty acid chain length, hydrophobicity modification and spore adhesion is more complex. An optimal mixture of fatty acid length could lead to the best modification of surface properties. This hypothesis needs more experiments to be confirmed.
In addition, the purity of the different samples would also be determinant. Little contaminants could also influence surface properties.

Rhamnolipids also varied surface hydrophobicity on stainless steel possibly in the same way as the other lip peptides. As the substrate is a hydrophilic surface, it is supposed rhamnolipids interact with it by their rhamnose moiety.

In other respects, a LC/MS analysis of Sigma sample was also performed in our Lab. Surfactin usually contains homologues C13, C14 and C15 with high abundance in the two latter. Regarding

our results, homologue C13 was not present and only homologue C15 (ion mass 1058) of surfactin was observed, while homologues C15/16 (ion mass 1072) and C16/17 (ion mass 1086) were also detected with high intensity. Therefore, we make the hypothesis that the observed compound should be pumilacidin, a lipopeptide which contains Ile (C15 and C16) or Val (C16 and C17) in position 7 instead of Leu in surfactin (Table 7.2).

Table 7.2. Ion masses of of surfactin and pumilacidin homologues.

Lipopetide	**1058 $[M+Na]^+$**	**1072 $[M+Na]^+$**	**1086 $[M+Na]^+$**
Surfactin	C15	-	-
Pumilacidin	C14, Ile	C15, Ile	C16, Ile
Pumilacidin	C15, Val	C16, Val	C17, Val

Lichenysin A is a cyclic lipoheptapeptide produced by *B. licheniformis* strains ATCC 10716, BAS50 and BNP29. Our strategy during this research concentrated on effects of biosurfactants in modification of surface properties.

Since lichenysin have many similarities with surfactin and in addition, it has potent biosurfactant properties (§ 1.1.5.2.2), *B. licheniformis* ATCC 14580 whose genome is completely sequenced was selected to produce it.

The strain has the capacity of lichenysin synthesis regarding to respective gene sequences (obtained from bioinformatic analyses) and produces the low amount of product. A strategy was designed to overproduce this less-studied lipopeptide. Genetic manipulations were performed on the basis of replacement of the native promoter of lichenysin synthetase P_{lchA} by P_{xylA} a strong promoter inducible by xylose in *B. subtilis* ATCC 6633 (Fickers *et al*. 2009), using homologous recombination.

Those attemps were unsuccesfull, mainly because of the selection of spontaneous resistants.

It seems that the antibiotic pressure (spectinomycin used in this study) should be improved to help us in the selection of transformants; therefore future attempts to transform the strain could be focused on construction of plasmids containing resistance genes to the other antibiotics.

Regarding results achieved in this research, the following perspectives are proposed:

1. Large-scale production of these biosurfactants has not still been achieved because of low yields in production processes and high purification costs. In respect to the importance of biosurfactants in various fields such as enhanced oil recovery, environmental bioremediation, food processing and pharmaceuticals owing to their unique properties, large-scale production of these molecules should be considered.

Development of strategies overcoming these difficulties by using cheaper substrates, optimising the process to improve yields, and integrating the process to reduce downstream processing steps seems necessary.

Unlike chemical surfactants, which are mostly derived from petroleum feedstock, some of these molecules (rhamnolipids) can be produced by microbial fermentation processes using cheaper agro-industrial substrates and waste materials, with special emphasis on the development and use of mutant and recombinant hyperproducers of biosurfactants, and indication of direction towards their commercial production that lead to reduced pollution caused by those wastes.

2. A more detailed study of monolayers of biosurfactants by XPS coupled with beam angle change.

3. The comprehensive study of the mechanism of hydrophobicity modification by the fatty acid moiety or peptide moiety of lipopeptides on the different surfaces by other techniques, in order to determine and predict correlation between the structure-function.

4. Adhesion of microorganisms to food processing equipment surfaces and the problems it causes, are a matter of concern to the food industry. Moreover, the conditioning film affects the hygienic status of various materials (Herrera *et al*. 2007). Therefore, using new treatments in food industry are of importance to prevent microorganism adhesion.

5. The improvement of genetic transformation methods in *B. licheniformis* ATCC 14580.

6. The modification of genes involved in amino acid recognition at synthetase level in *B. subtilis* 168 to make it to produce lichenysin regarding this strain is transformed easier than *B. licheniformis* ATCC 14580.

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Effect of different *Bacillus subtilis* lipopeptides on surface hydrophobicity and adhesion of *Bacillus cereus* 98/4 spores to stainless steel and Teflon

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Various lipopeptides produced by *Bacillus subtilis* were examined for their ability to modify the surface hydrophobicity of two substrata, stainless steel (SS) and Teflon. These modifications were evaluated by water contact angle measurements. The effects depended on the lipopeptide, its concentration, and the tested substratum. Treatment of SS with different concentrations of surfactin S1 showed an increase of the hydrophobicity between 1 and 100 mg l^{-1}. On the same substratum, fengycin increased hydrophobicity up to its critical micelle concentration (6.25 mg l^{-1}). With higher concentrations of fengycin, hydrophobicity decreased. Surfactin, mycosubtilin, and iturin A decreased hydrophobicity on Teflon. The different effects of these three families of lipopeptides were related to their structural differences. A good correlation was shown between hydrophobicity modifications of surfaces and the attachment of *B. cereus* 98/4 spores. Enhancement in the hydrophobicity of the surfaces increased the number of adhering spores.

Keywords: biosurfactant; lipopeptide; surface properties; adhesion; *Bacillus cereus*; spore

Introduction

Biosurfactants are a structurally diverse group of surface-active molecules mainly synthesized by microorganisms (Desai and Banat 1997). Interest in microbial surfactants has been steadily increasing in recent years, as they have numerous advantages compared with chemical surfactants, including a lower toxicity, higher biodegradability, higher foaming, better environmental compatibility, and higher specific activity at extreme temperature, pH levels, and salinity (Georgiou et al. 1992; Banat 1995). They have applications in a wide variety of industrial fields such as the food, cosmetic, pesticide, detergent, and pharmaceutical industries, and in enhanced oil recovery, transportation of heavy crude oil, and bioremediation (Georgiou et al. 1992; Desai and Banat 1997). Lipopeptides are an interesting class of microbial surfactants because of their many attractive properties such as detergency, emulsification, foaming, dispersion (Razafindralambo et al. 1996; Desai and Banat 1997), antifungal and antimicrobial activities (Yoo et al. 2005), and induction of systemic resistance in plants (Ongena et al. 2005, Ongena and Jacques 2008). These biomolecules are synthesized by large multienzymatic proteins called Non-Ribosomal Peptide Synthetases (NRPS). They are produced as mixtures of components varying in their peptidic and/or lipidic structure including homologous and isoform series, which differ in the length and branching of the fatty acid side chains and the amino acid substitutions in the peptide rings, respectively (Ongena and Jacques 2008). Three lipopeptide families are excreted by *Bacillus subtilis* strains including surfactin, iturin, and fengycin (Table 1). These molecules are cyclic peptides composed of seven (surfactins and iturins) or 10 (fengycins) amino acid residues linked to one unique β-amino (iturins) or β-hydroxy (surfactins and fengycins) fatty acid. The length of this fatty acid chain may vary from C_{13} to C_{16} for surfactins, from C_{14} to C_{17} for iturins, and from C_{14} to C_{18} in the case of fengycins giving different homologous compounds and isomers (*n, iso, anteiso*) for each lipopeptide (Ongena and Jacques 2008). This diversity of structure leads to different biological and physicochemical properties. Surfactins can interfere with biological membrane integrity in a dose-dependent manner. They display hemolytic, antiviral, antimycoplasma, and antibacterial activities and reduce the surface tension (ST) of water from 72 mN m^{-1} to values in the range of 25–30 mN m^{-1} (Bonmatin et al. 2003). These properties are mainly related to their amphiphilic character due to the presence of the long chain fatty acid and some lipophilic amino acids and a hydrophilic part composed by two acidic amino acid

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Table 1. Structural characteristics of the different lipopeptides used in this study.

Name	Primary structure of the peptide moiety	Main fatty acid chains
Fengycin family	Decapeptide with a lactone ring between OH group of Tyr3 and carboxy-terminal group of Ile_{10}	β-OH fatty acids
Fengycin A	L-Glu-D-Orn-D-Tyr-D-aThr-L-Glu-D-Ala-L-Pro-L-Gln-L-Tyr-L-Ile	*ai*-C_{15}, *i*-C_{16}, *n*-C_{16}
Fengycin B	L-Glu-D-Orn-D-Tyr-D-aThr-L-Glu-D-Val-L-Pro-L-Gln-L-Tyr-L-Ile	*ai*-C_{15}, *i*-C_{16}, *n*-C_{16}, *ai*-C_{17}
Iturin family	Heptapeptide closed by a lactam ring with the β-NH_2 group of the fatty acid chain	β-NH_2 fatty acids
Iturin A	L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	*n*-C_{14}, *i*-C_{15}, *ai*-C_{15}
Mycosubtilin	L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Ser-L-Asn	*n*-C_{16}, *i*-C_{16}, *ai*-C_{17}
Surfactin family	Heptapeptide closed by a lactone ring with the β-OH group of the fatty acid chain	β-OH fatty acids
Surfactin	L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu	*i*-C_{14}, *n*-C_{14}, *i*-C_{15}, *ai*-C_{15}

residues (Deleu et al. 2003). Surfactin has a critical micellar concentration (CMC) of 9.4 μM in 0.1 M $NaHCO_3$ at pH 8.7 (Ishigami et al. 1995) and forms rod-like micelles with an aggregation number of ~170 (Heerklotz and Seelig 2001). Iturins are widely known for their antifungal activity. Though they are also strongly hemolytic, their biological activity is different from that of surfactins: they display a strong *in vitro* antifungal action against a large variety of yeast and fungi but only limited antibacterial and no antiviral activities (Ongena and Jacques 2008). Iturin A forms ion-conducting pores in lipid bilayers and not membrane disruption or solubilization as caused by surfactins (Grau et al. 2001). This neutral lipopeptide has a CMC of *ca.* 40 μM. It has been shown that, in addition to the monomer or micellar organizations, at higher concentrations, another type of aggregate different from the micelle is preferred, probably a lamellar vesicle, whose proportion increases as the concentration of iturin is raised. For iturin A micelles, an aggregation number of 7 has been proposed (Grau et al. 2001). One member of the iturin family is mycosubtilin, which shows two different structural traits from iturin A: an inversion of two amino acids (Ser_6-Asn_7 instead of Asn_6-Ser_7) and a higher fatty acid chain length (mainly C_{16} and C_{17}) than iturin A (mainly C_{14} and C_{15}). Fengycins are less hemolytic than iturins and surfactins but retain a strong fungitoxic activity more specifically against filamentous fungi. Its peptidic moiety contains three charged amino acids (two glutamic acids and one ornithine). Mechanistically, the action of fengycins is less well-known compared with other lipopeptides, but they also readily interact with lipid layers and to some extent retain the potential to alter cell membrane structure (packing) and permeability in a dose-dependent way (Deleu et al. 2005). However, in general the available literature related to fengycin micelle formation is far from complete.

B. cereus strains are widespread in environments such as soil, air, and foodstuffs and have often been implicated in foodborne gastroenteritis. Their spores are often isolated from surfaces due to their ability to firmly adhere to a wide variety of materials. In addition, the spores are highly resistant to most of the thermal and chemical treatments used in the food industry. One of the commonly considered factors involved in adhesion of bacteria (vegetative cells and spores) to substrata is bacterial cell surface properties, especially hydrophobicity (Faille et al. 2002). In addition, the surface properties of materials have been shown to change by the formation of a conditioning layer (Mettler and Carpentier 1998; Storgards et al. 1999; Verran and Whitehead, 2006). This surface contamination comes from the organic and inorganic compounds present in the bulk fluid carried toward the surface either by diffusion or turbulent flow, which quickly accumulate at the solid–liquid interface, giving rise to a greatly modified surface status (Barnes et al. 1999; O'Sullivan et al. 2000; Herrera et al. 2007; Jullien et al. 2008). For example, detergents (Boulangé-Petermann 1996; Cloete and Jacobs 2001) and surfactants (Nitschke and Costa 2007) have been shown to condition surfaces and further affect their hygienic properties.

In the present study, the influence on the hydrophobicity and hygienic status of materials conditioned with lipopeptides was investigated. Three lipopeptides (surfactin S1, iturin A, and fengycin) were tested for their conditioning properties on two substrata, *viz* stainless steel (SS) and Teflon. The hygienic status of the unconditioned and conditioned materials was estimated by the number of *B. cereus* spores able to adhere under defined conditions. Some hypotheses are suggested to explain the different effects of the families of lipopeptides.

Materials and methods

Lipopeptide purification

Surfactin S1 (~98% purity, C_{14}–C_{15}.) was purchased from Sigma (St Louis, MO). Iturin A (90% purity, C_{13}–C_{16}) and fengycin (95% purity, C_{13}–C_{18}) were

kindly provided by Dr Magali Deleu (Agricultural University of Gembloux, Belgium). Mycosubtilin (C_{16}–C_{17}) was produced by *B. subtilis* BBG100 (Leclère et al. 2005; Guez et al. 2007) in Landy medium for 72 h, at 30°C. The culture was centrifuged at 13,000*g* for 30 min at 4°C and the supernatant was loaded onto C_{18} Maxi-Clean cartridges (Extract-Clean SPE 500 mg, Altech). Lipopeptides were eluted with pure methanol (high-performance liquid chromatography (HPLC) grade; Acros Organics, Geel, Belgium) and then purified by thin layer chromatography (TLC) (silica gel plates: F_{256}) or silica gel column (Razafindralambo et al. 1997). Purity and identification of the mycosubtilin were controlled by HPLC and mass spectrometry (Leclère et al. 2005, data not shown).

Determination of lipopeptide concentrations

Lipopeptide solutions were analyzed by HPLC to confirm or determine their precise concentrations (Guez et al. 2007) using a C_{18} column (5 μm; 250 by 4.6 mm; VYDAC 218 TP;VYDAC, Hesperia, CA) with the acetonitrile–water–trifluoroacetic acid solvent system (40:60:0.1, v/v/v for iturin and mycosubtilin, 80:20:0.1, v/v/v for surfactin and gradient from 45:55:0.1, v/v/v to 55:45:0.1, v/v/v in 30 min for fengycin) and a flow rate of 1 ml min^{-1}. The retention time and second derivatives of UV–visible spectra (Waters PDA 996 photodiode array detector; Millennium Software) of each peak were used to identify the eluted molecules.

CMC determination

The ST of lipopeptide solutions used for substratum conditioning was measured by the ring method using a Du Nouy tensiometer TD1 (Lauda, Königshofen, Germany). CMC was determined by plotting the ST as a function of the lipopeptide concentration. The average of three independent measurements was taken.

Substratum conditioning and contact angle measurement

SS (304L with a 2R finish, hydrophilic) and Teflon (hydrophobic) coupons were provided in the form of 45 × 15 mm^2. Before each experiment, coupons were subjected to the cleaning and disinfection protocol previously described (Faille et al. 2002). A goniometer (Digidrop, GBX model ASE, www.gbxonline.com) was used for contact angle measurement. To determine the influence of lipopeptides on the hydrophilic/hydrophobic characteristic of surfaces, different concentrations of lipopeptides were prepared, ie 1, 10, 25, 50, and 100 mg l^{-1} in 10% methanol (v/v) for surfactin, iturin, and mycosubtilin and 0.25, 2.5, 6.25, 12.5, and 25 mg l^{-1} for fengycin.

SS and Teflon coupons were covered by the lipopeptide solutions at different concentrations for 1 h at room temperature and after removing the solutions, they were dried. Four water droplets (5 μl) were applied on each coupon at 20°C, and water contact angles were measured. Each analysis was performed at least three times. Coupons subjected to 10% methanol were used as controls (it was determined that diluted 10% methanol did not influence surface properties). Ultra high purity MilliQ water was used throughout the experimental procedures.

Similar experiments were performed with surfactin dissolved in 10% methanol or in 10% methanol/0.1 M $NaHCO_3$ solution (10/90, v/v) to investigate the role of the lipopeptide charge in substratum conditioning.

Adhesion tests of B. cereus *98/4*

B. cereus CUETM (Collection Unité EcoToxicologie Microbienne,Villeneuve dAscq, France) 98/4, isolated from a dairy line, was chosen for adhesion tests to coupons because of its high adherence to various materials (Faille et al. 1999). Spores were produced (Faille et al. 1997) and kept for up to 3 months in distilled water (10^9 spores ml^{-1}) at 4°C. SS and Teflon coupons, conditioned as indicated before, were vertically immersed in spore suspensions in sterile MilliQ water containing $\sim 10^6$ spores ml^{-1}, for 4 h at room temperature. The coupons were rinsed by dipping into a beaker containing distilled water to remove non-adhering spores. They were then placed into tubes containing 10 ml of 2% Tween 80 and 10 mg l^{-1} of peptone water (Biokar, Diagnostic, Beauvais, France). Adherent cells were detached from the surfaces using an ultrasonic bath (Deltasonic Meaux, France, 40 kHz) for 2 min and 30 s followed by 20 s vortexing with quick speed and then an ultrasonic bath again for 2 min and 30 s. Detached spores were enumerated in duplicate using the serial dilution technique on nutrient agar after 24–48 h incubation at 30°C. Each experiment was performed at least three times.

Statistical analysis

Data were analyzed by general linear model procedures using SAS V8.0 software (SAS Institute, Cary, NC). For each surfactant, variance analyses were performed to determine the influence of the concentration of the conditioning solution on surface hydrophobicity and further on the ability of *B. cereus* 98/4 spores to adhere, taking into account the variability between trials. This was followed by a multiple comparison procedure by Tukey's test (α level = 0.05).

Results

Critical micelle concentration of lipopeptides

The different solutions of lipopeptides used in this study contain several homologous compounds from the same family. The CMC is slightly different for each homologous compound (Deleu et al. 2003). In addition, the presence of 10% methanol could slightly modify CMC values. The CMC of the different mixtures used in this study was thus determined by using the ring method. The results are shown in Table 2 and compared with the values shown in literature.

Influence of conditioning with lipopeptides on substratum hydrophobicity

Water contact angle measurements on SS are presented in Figure 1. Treating SS with 10% methanol gave contact angle values in the range of 30–35°, close to the values obtained for untreated surfaces (data not shown), showing that methanol in contact with surfaces does not affect their hydrophobic/hydrophilic character. Conditioning with the various lipopeptides resulted mostly in a more or less marked increase in hydrophobicity, but the changes varied among the lipopeptides. When SS was conditioned with fengycin, the contact angles were significantly higher (p value <0.0001) for concentrations ranging from 2.5 to 12.5 mg l^{-1} (intermediate concentrations) and the maximal value of 61.73° was obtained at a concentration of 6.25 mg l^{-1}, which corresponds to the CMC of the mixture of compounds used (Figure 1A). For higher concentrations, the contact angle decreased. The slight modifications (Figure 1B) induced by conditioning with iturin A were not significant (p value $= 0.3022$). Conversely, when surfactin S1 was used, a significant increase (p value < 0.0001) in the contact angles was observed following conditioning with concentrations >1 mg l^{-1} (Figure 1C). This effect increased further with increasing lipopeptide concentration (even for concentrations over the CMC) and reached a value of 81.1° at 100 mg l^{-1}, the highest value obtained among the other tested lipopeptides.

Table 2. ST and CMC of lipopeptides.

	Results from the literature[a]		Results from this study	
Lipopeptide	ST (mN m^{-1})	CMC (μM)	ST (mN m^{-1})	CMC (μM)
Surfactin S1	31	9	31 ± 0.05[b]	10
Iturin A	54	43	45 ± 0.35[b]	40
Fengycin	42	4.6	43 ± 0.05[b]	6.25

[a]Thimon et al. 1992, Deleu 2000; [b]Standard deviation.

In the presence of 10% methanol, Teflon exhibited very high hydrophobicity (water has a contact angle around 115°), close to the value of 120° obtained without any conditioning step. Whatever the lipopeptide used, conditioned Teflon resulted in a reduction in the contact angle (Figure 1). With iturin A, a noticeable decrease (p value < 0.0001) in the contact angle was observed at concentrations > 1 mg l^{-1}, and the lowest value was obtained at 100 mg l^{-1}. At this

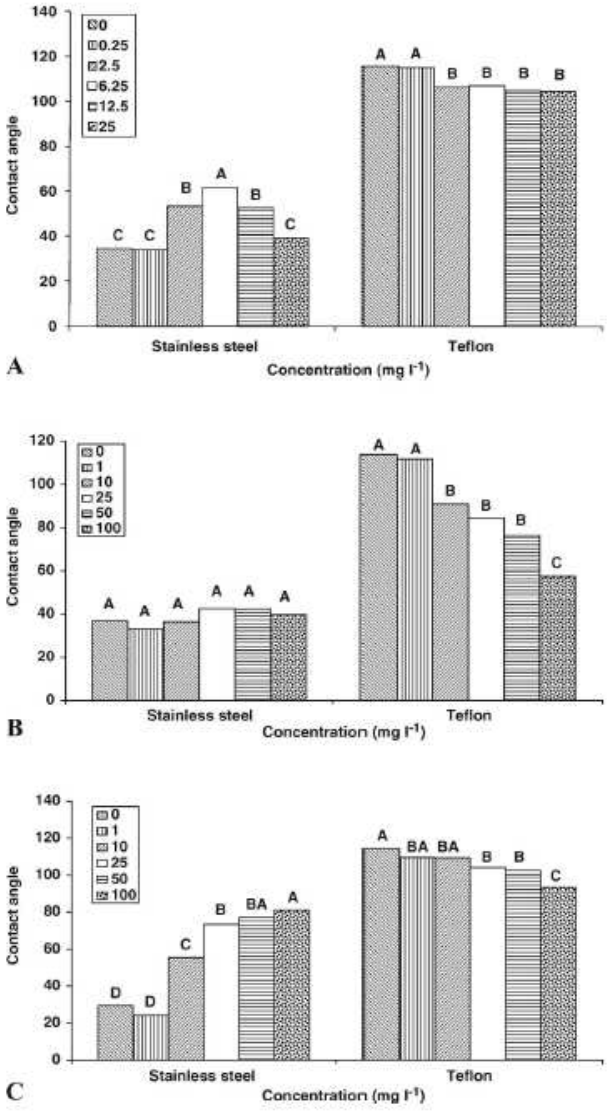

Figure 1. Effect of conditioning on SS and Teflon with fengycin (A), iturin A (B), and surfactin (C). Hydrophobicity was estimated by the water contact angle values. Coupons treated with 10% methanol solution were used as control. The results were analyzed by a multiple comparison procedure by Tukey's test (α level $= 0.05$). Bars marked with the same letter are not statistically different from each other.

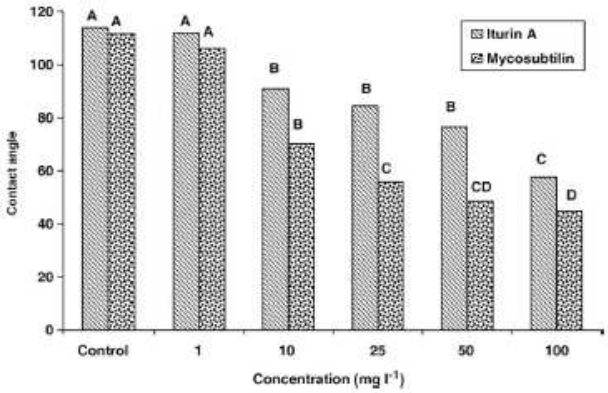

Figure 2. Effect of conditioning with iturin A and mycosubtilin on Teflon. Hydrophobicity was estimated by the water contact angle values. Coupons treated with 10% methanol solution were used as control. The results were analyzed by a multiple comparison procedure by Tukey's test (α level = 0.05). Bars marked with the same letter are not statistically different from each other.

concentration, conditioned Teflon was rather hydrophilic, with a water contact angle around 58°. On the contrary, conditioning of Teflon with fengycin or surfactin only resulted in a slight but significant (p values < 0.0001) decrease in the water contact angle, and Teflon remained highly hydrophobic (angle = 100°) even at 100 mg l^{-1}.

Effect of carbon chain length of lipopeptides belonging to the iturin family on hydrophobicity

Similar trends (Figure 2) were observed when iturin A or mycosubtilin were used to condition Teflon, but the decrease was more marked with mycosubtilin. At intermediate concentrations, eg 10–25 mg l^{-1}, the contact angle was significantly lower with mycosubtilin than with iturin A. Conversely, at higher concentrations (100 mg l^{-1}), a similar water contact angle was obtained for both lipopeptides.

Influence of conditioning with lipopeptides on the ability of B. cereus *spores to adhere*

The consequences of conditioning both substrata with lipopeptides on adhesion of the hydrophobic spores of *B. cereus* were investigated. The results are summarized in Figure 3. Despite great differences between the effects of lipopeptides on the hydrophobicity of SS, the adhesion profiles obtained were close to the water angle profiles presented in Figure 1. Conditioning with fengycin significantly affected spore adhesion (p value = 0.0047). The maximal number of spores (1.9×10^4) was found at the intermediate fengycin concentrations of 2.5 and 6.25 mg l^{-1}. For surfactin, considerable differences

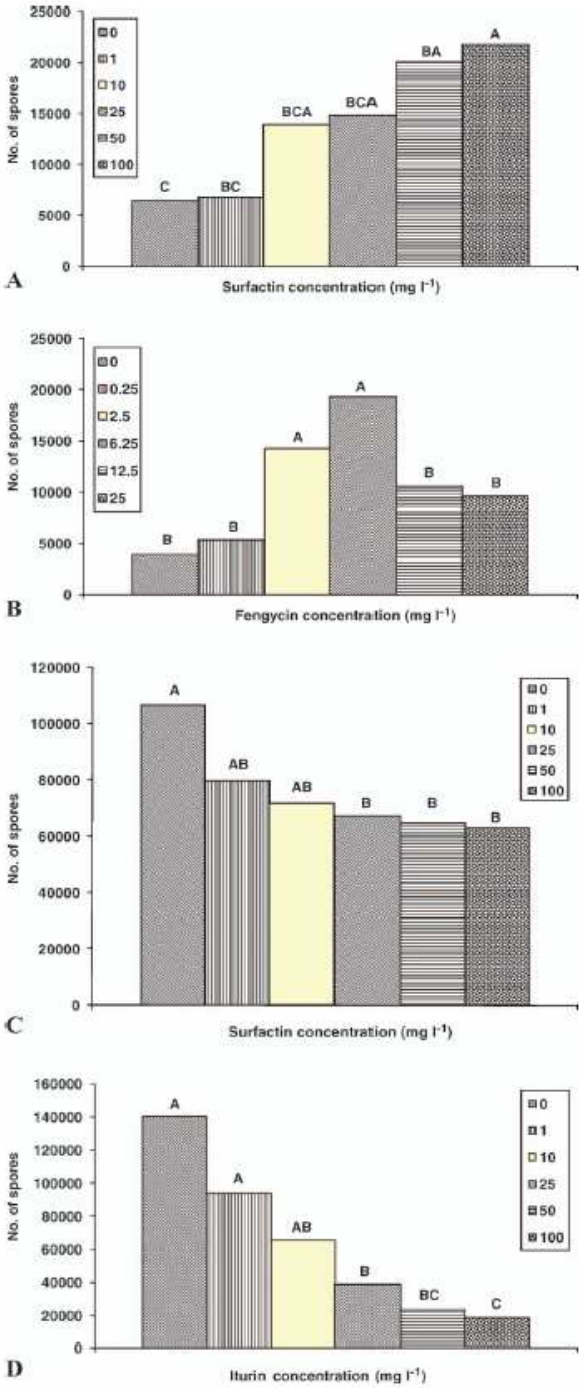

Figure 3. Effect of conditioning with surfactin (A) and fengycin (B) on *B. cereus* spore attachment to SS coupons and surfactin (C) and iturin A (D) on *B. cereus* spore attachment to Teflon coupons. Coupons treated with 10% methanol solution were used as control. The results were analyzed by a multiple comparison procedure by Tukey's test (α level = 0.05). Bars marked with the same letter are not statistically different from each other.

were also observed between concentrations, with the number of adherent spores varying from around 5×10^3 at 0 and 1 mg l^{-1} to around 2×10^4 at 50 and 100 mg l^{-1} (p value = 0.0017). However, iturin A did not significantly affect spore adhesion (data not shown).

On Teflon, the adhesion profiles were clearly related to the profiles obtained for water contact angles, and a more or less marked decrease in the number of adherent spores was observed following every conditioning with lipopeptides. However, the effects with iturin A were more remarkable and the number of adherent spores decreased 6.5 fold at 100 mg l^{-1}, and this was highly significant (p values < 0.0001). Conversely, conditioning of Teflon with any concentration of fengycin (data not shown) or surfactin had little effect on *B. cereus* spore adhesion, and the number of adherent spores decreased < 2-fold. However, statistical analysis confirmed the influence of surfactin on *B. cereus* spore adhesion (p value = 0.0083).

Relationship between surface hydrophobicity and spore adhesion

To investigate the relationship between substratum hydrophobicity and *B. cereus* spore adhesion, the numbers of adherent spores were plotted against water contact angles for each conditioning of both substrata (SS and Teflon). Linear regressions were performed for the whole data set, and for each conditioning, including 10% methanol (Table 3). The results indicate that, following conditioning with lipopeptides, changes in hydrophobicity would explain most of the variation in the ability of *B. cereus* spores to adhere. When each conditioning procedure was analyzed separately, fengycin did not behave like the others; despite its significant influence on substratum hydrophobicity. Indeed, the number of adherent spores was poorly affected by substratum hydrophobicity (slope = 0.0062) compared with the other lipopeptides (slopes ranging from 0.0132 to 0.0162).

Discussion

Lipopeptides have an important role in adhesion to various surfaces by hydrophobic interactions. After contact with a surface, lipopeptides can form a conditioning film, thereby varying surface properties like hydrophobicity (Rosenberg and Ron 1999). This effect on surface hydrophobicity arises from the amphiphilic structure of these molecules by orientation of peptide cycle and fatty acid chains in relation to the hydrophobic/hydrophilic character of the surface. One explanation is that on the hydrophilic surface, lipopeptide molecules are probably oriented in such a way that the peptide cycle is adsorbed onto the surface and the hydrocarbon chains are exposed to the surrounding medium. Therefore, the surface becomes more hydrophobic (Ahimou et al. 2000). The spatial organization of the lipopeptide molecules may be influenced by their concentration and the surface environment too (Ahimou et al. 2000). Manne et al. (1994) proposed an adsorption model for ionic surfactant molecules at the graphite-aqueous solution interface in which at low concentration (almost 10% of CMC), molecules are adsorbed with alkane chains extended on the substrate plane, and this chain is gradually desorbed with an increase in the concentration. At concentrations near CMC, the surfactant molecules were oriented perpendicular to the plane of the substratum, with the hydrophilic head groups in contact with the aqueous phase (Ahimou et al. 2000). On the contrary, fatty acid chains can interact with hydrophobic surfaces and polar peptidic moieties can be exposed to the environment. In these conditions, a decrease in the hydrophobicity of the surface can be observed.

In this study, the influence of structurally different types of lipopeptide was studied on modification of the surface properties of two substrata, SS and Teflon, using contact angle measurements. The water contact angle provides a suitable evaluation of surface hydrophobicity (Ahimou et al. 2001).

Two families increased the hydrophobicity of SS, *viz* surfactin and fengycin. Iturin A had no effect. The difference between the families can be mainly attributed to the variability in the primary structure of the peptide cycle which is responsible for the 3D structure at the interface (Deleu et al. 1999). The 3D structure of peptide combined with the presence of the lipidic chain could be a crucial parameter in changing surface properties. The study of the 3D structure of surfactin, that is the structural basis of its important surface activities, showed that the carboxylic groups of both glutamate and aspartate form a minor hydrophilic domain and the nonpolar residues in position 4, and to a lesser extent, positions 2 and 7 from the peptidic part, along with the lipid tail, form major hydrophobic domains. The presence of these two domains was found to be important for surface activity (Youssef et al. 2005). The importance of the negative charges of Glu and Asp in adhesion of surfactin to the SS surface

Table 3. Relationship between substratum hydrophobicity and spore adhesion for the various conditionings.

Conditioning	Slope of the linear regression	R^2
Methanol 10%	0.0139	0.8807
Fengycin	0.0062	0.6205
Iturin A	0.0159	0.9485
Mycosubtilin	0.0162	0.7873
Surfactin	0.0132	0.9755
All data	0.0107	0.6384

was proved when these charges were neutralized by 0.1 M $NaHCO_3$. In this case, as shown in Figure 4, it was observed that surfactin could no longer change the surface hydrophobicity of SS.

The spatial organization of fengycin is not known but this compound also contains charged amino acid residues in the peptidic moiety. These charged residues could interact with charges present on SS. The absence of charged amino acid in the peptidic moiety of iturin could explain the absence of an effect of such compounds on SS. The surfactin peptide cycle is more hydrophobic than that of iturin A and fengycin (Deleu et al. 1999), which can explain the higher contact angle observed with the former. Indeed, the change in the hydrophobicity depends on the whole hydrophobic character of the lipopeptide film adsorbed onto the surface, which is due to both the lipid and peptide parts. The presence of five hydrophobic amino acids in the peptide moiety of surfactin increases its whole hydrophobic character. However, at concentrations higher than CMC, different results were observed for fengycin. A decrease in the contact angle occurred. At concentrations higher than CMC, fengycins might form a fully interdigitated bilayer, where each hydrocarbon tail spans the entire hydrocarbon width of the bilayer, and interacts laterally, through hydrophobic forces, with the hydrocarbon tail of fengycin molecules from the opposing lamellar leaflet. Such a hypothesis was suggested for the organization of iturin in solution (Grau et al. 2001).

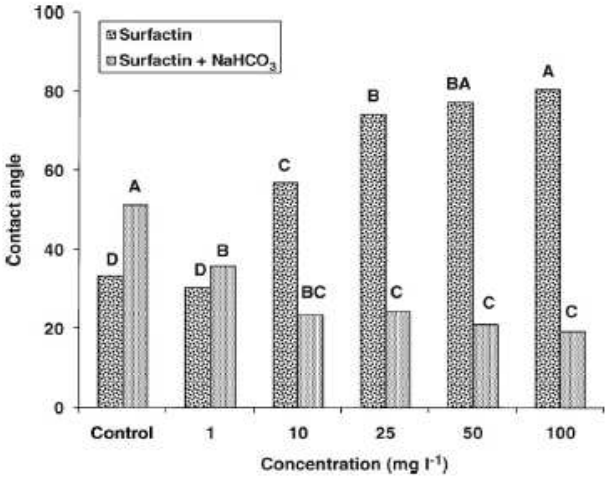

Figure 4. Effect of surfactin and neutralized surfactin treatment on SS hydrophobicity. Hydrophobicity was evaluated by goniometry. Coupons treated with 10% methanol or methanol/0.1 M $NaHCO_3$ solution (10/90, v/v) were used as control. The results were analyzed by a multiple comparison procedure by Tukey's test (α level = 0.05). Bars marked with the same letter are not statistically different from each other.

All lipopeptides decreased the hydrophobicity on Teflon. The higher hydrophobicity decrease was observed for iturin A compared with surfactin and fengycin. As previously mentioned, the iturin A peptide moiety has a lower hydrophobicity than the surfactin peptide chain (on one side, the presence of 4 Leu and 1 Val in surfactin and, on the other side, the presence of polar groups on the hydroxyl amino acid residues, Ser and Tyr, in iturin A) and it could be one of the reasons why differences were observed between these two families of lipopeptides. However, the length of the fatty acid chain should also be a determinant, which was confirmed by the results obtained with another group of biosurfactants, *viz* mycosubtilins. Mycosubtilin differs from iturin A by the sequence inversion of two adjacent Ser6-Asn7 residues. In addition, it has a longer carbon atom chain (16–17) in comparison with iturin A (14–15) in the alkyl chain and as shown in Figure 2, this longer fatty acid chain leads to an enhanced effect on Teflon.

It was then determined which biosurfactants would modify the adhesion of *B. cereus* 98/4 spores to the conditioned surfaces. According to previous studies, pre-treatment of SS by surface-active compounds produced by *P. fluorescens* 495 gave rise to a substantial reduction in the number of adherent cells of *Listeria monocytogenes* LO28 (Meylheuc et al. 2001). Similarly, pre-treatment of silicone rubber with *S. thermophilus* surfactant inhibited the adhesion of *Candida albicans* by 85% (Busscher et al. 1997) and a biosurfactant of *Lactococcus lactis* 53 inhibited adhesion of four bacterial and two yeast strains to silicon rubber (Rodrigues et al. 2004).

Pre-treatment of substrata with some of the biosurfactants caused a change (a reduction or increase depending on the lipopeptide type and the substrata) in the number of adherent spores on SS and Teflon. These modifications perfectly fit with the hydrophobicity changes measured by contact angle, especially for experiments with surfactin and iturin A. As *B. cereus* 98/4 spores are so hydrophobic, they adhere to hydrophobic surfaces with a higher affinity. Therefore, according to the results, more hydrophobic surfaces attract a greater number of spores. The lower correlation observed with mycosubtilin could be due to the degree of purity (85%) of the samples used, which was a little lower than that of iturin (90%). The effect of fengycin was completely different from the other lipopeptides, especially on SS, as an increase in the hydrophobicity with the fengycin concentration was followed by a decrease. Such behavior, which could be linked to the formation of a bilayer as previously suggested, could explain the less good correlation coefficient observed with this lipopeptide.

Conclusions

There is a correlation between the modification of surface properties measured by goniometry and the modification of *B. cereus* spores adsorption on the surfaces. Water contact angle measurement is thus a suitable indicator to identify the behavior of modified substrata. Lipopeptides produced by *B. subtilis* are able to modify the surface hydrophobicity of some substrata and consequently, influence microbial adhesion to these surfaces. These effects depend on the lipopeptide type, the concentration, and the substratum. The bioconditioning of surfaces using microbial surfactants has been suggested as a new strategy for reducing adhesion of bacteria to the surfaces.

Acknowledgements

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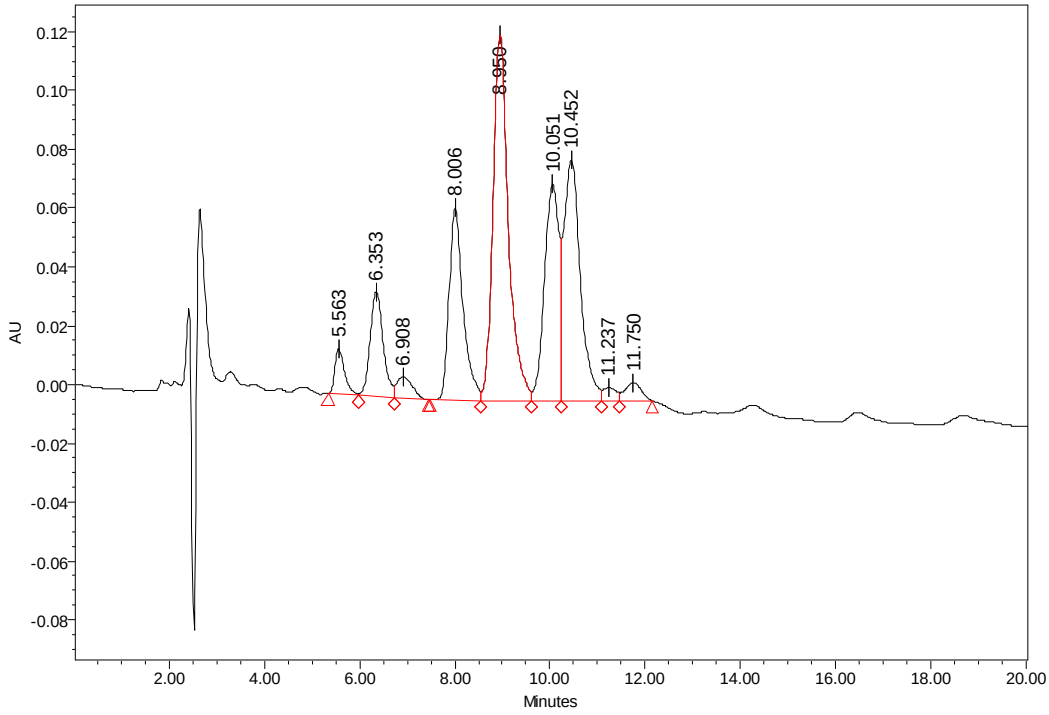
Index II

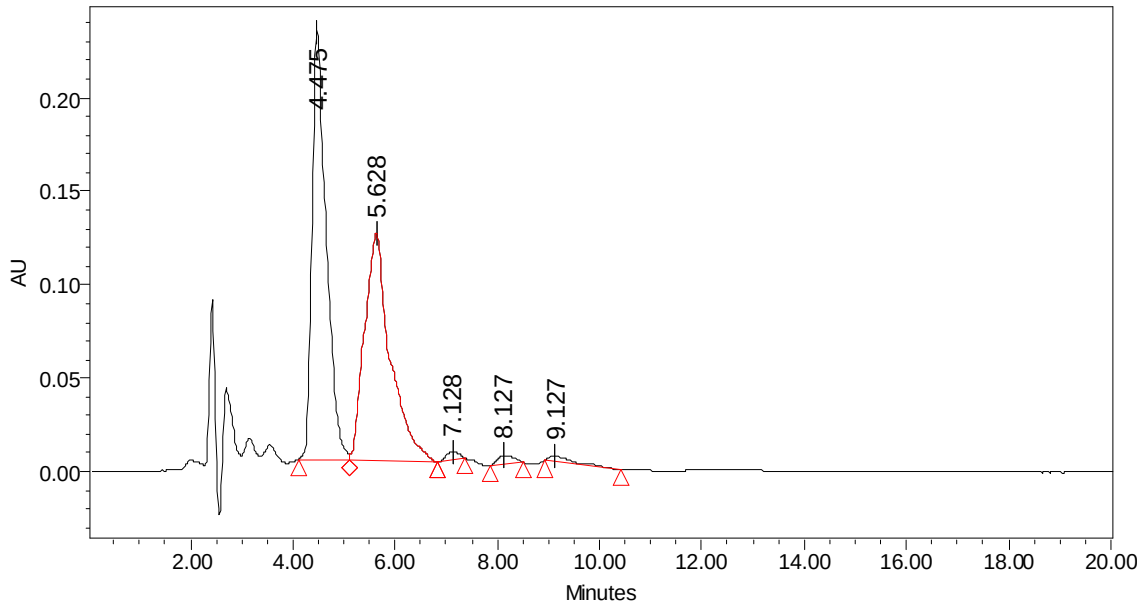
Chromatograms and standard peaks of lipopeptides: surfactin, fengycin and iturin A

1- Surfactin standard spectrum



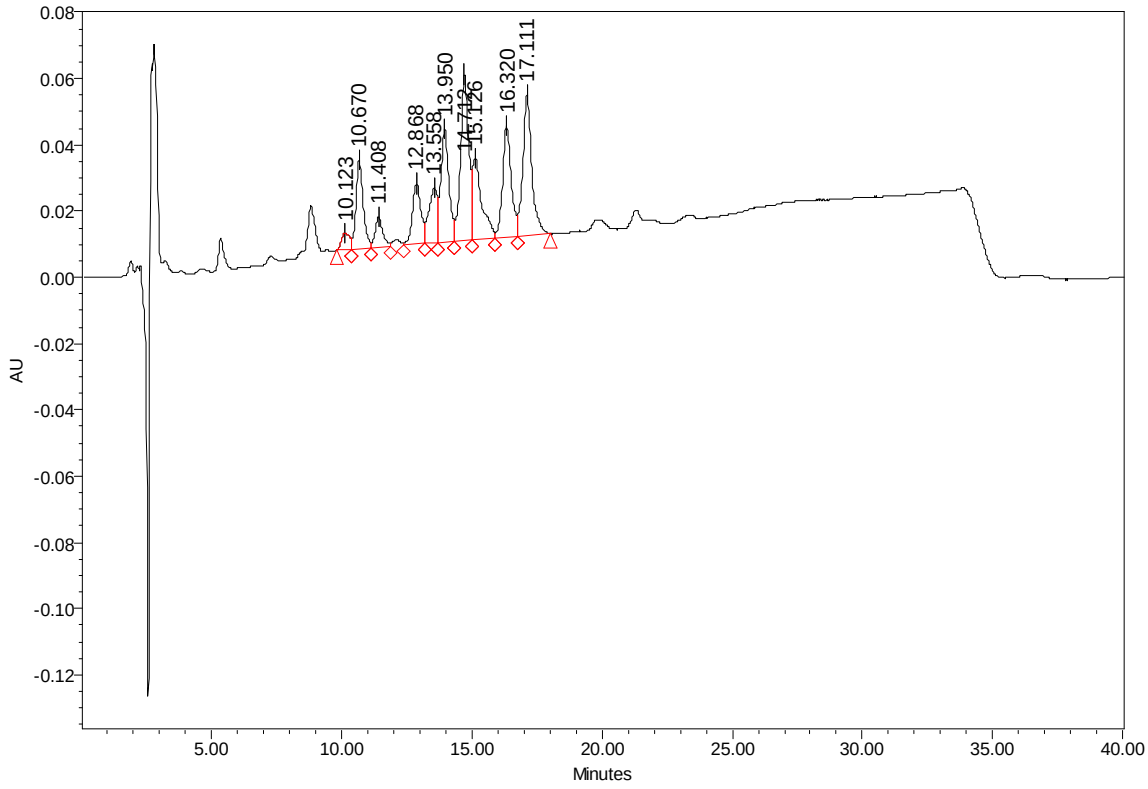
AU : Arbitrary Unit

2- Iturin A standard spectrum



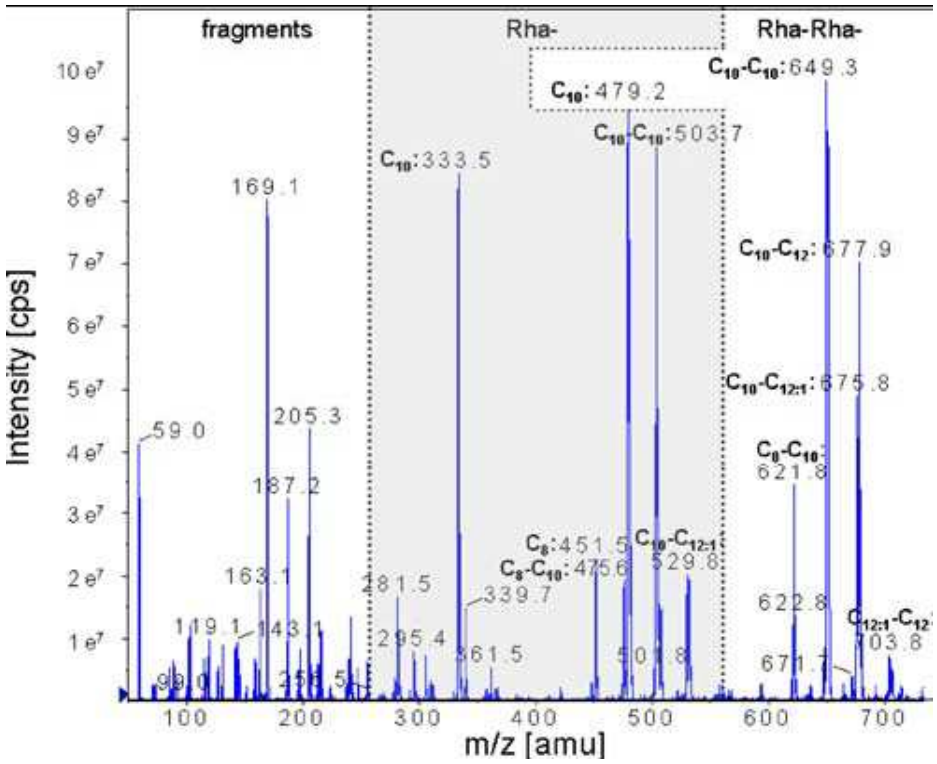
AU : Arbitrary Unit

3- Fengycin standard spectrum

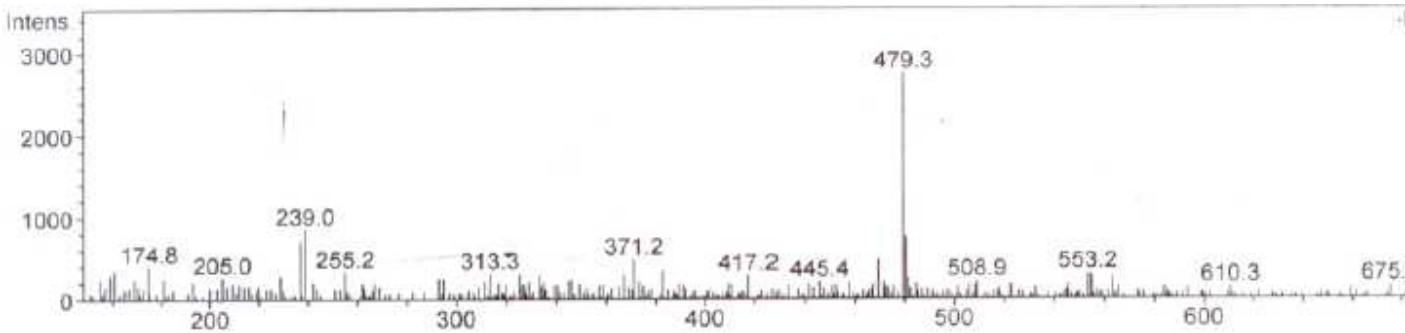


AU : Arbitrary Unit

Index III



MS spectra of a crude rhamnolipid extract divided into a mass range for monorhamnolipids and dirhamnolipids with different fatty acid chain lengths and some of their fragments. Rh is rhamnose.



MS spectrum of rhamnolipid extract (LH7) produced in Lindhardt medium for 7 days.

Intens: intensity

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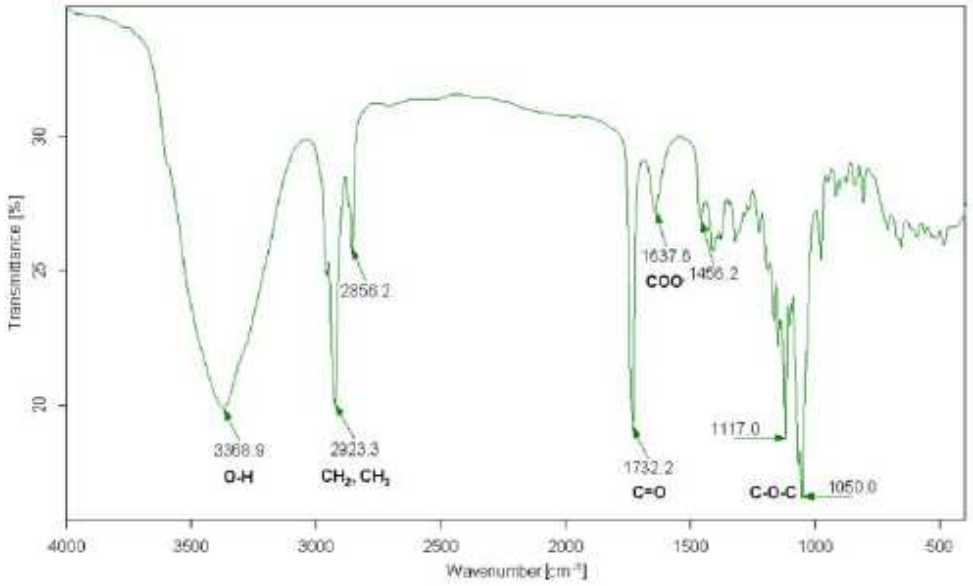

Fourier transform infrared (FTIR) transmittance spectrum of a potassium bromide pellet of rhamnolipid Rha-Rha-C10-C10 from *Pseudomonas aeruginosa* measured with the IFS 66 FTIR spectrometer from Bruker

Frequencies and band assignments for FTIR spectra.

Wavenumber (cm^{-1})	Assignment[a] to frequency group	Molecular group
~1054	ν_s CO–O–C	Polysaccharides
~1178	ν_{as} CO–O–C	Ester
~1247	CNH / PO	Amide III
~1305	O–P–O	Phospholipids
~1386	δ_s CH_3	Methyl
~1544	CNH	Amide II
~1653	ν C=O	Amide I
~1738	νC=O	Esters
~2877	ν_s CH_2 /CH_3	Methyl.
~2939	ν_{as} CH_2	Methylene
~2995	ν_{as} CH_3	Methyl group
~3304	ν NH	Amides

[a] abbreviation used in the table: ν-stretching; δ-bending; s-symmetric; as-asymmetric

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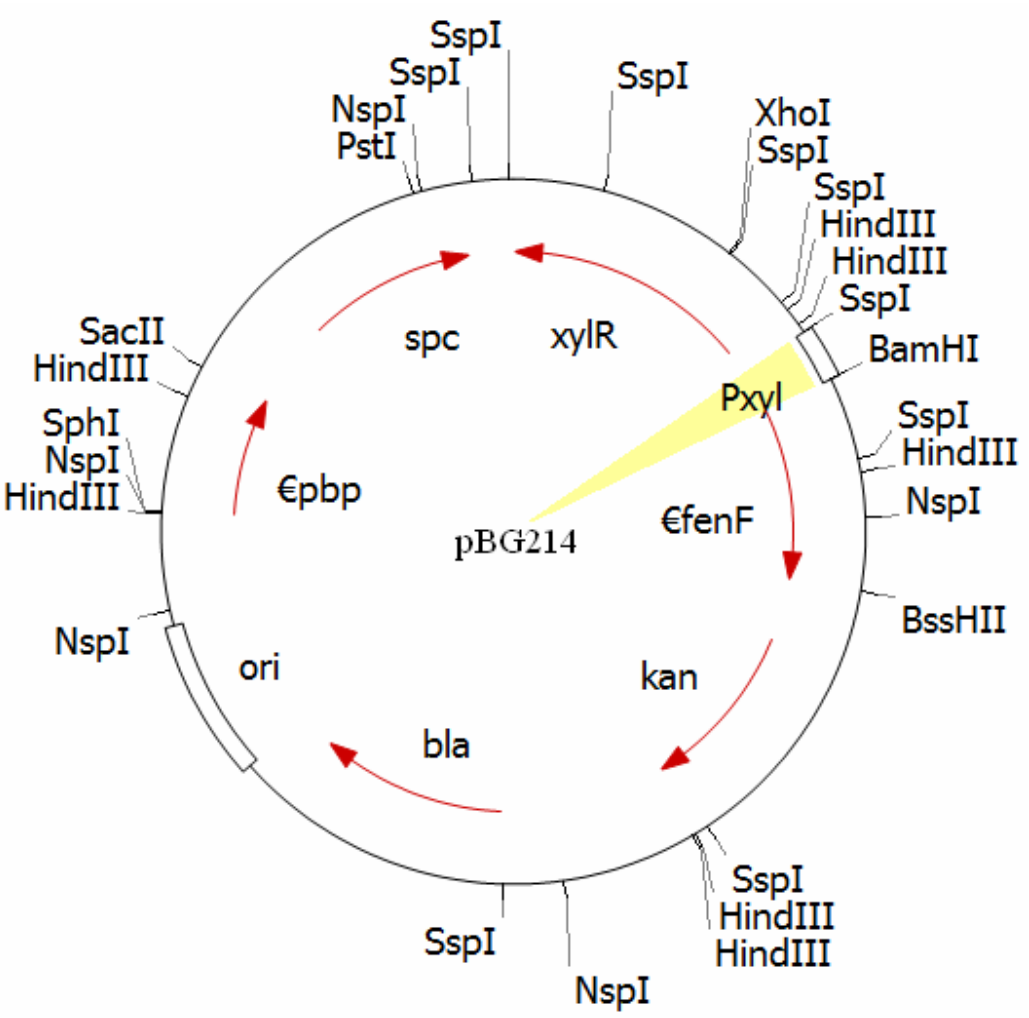
Genetic map of pBG214 (8100 bp)



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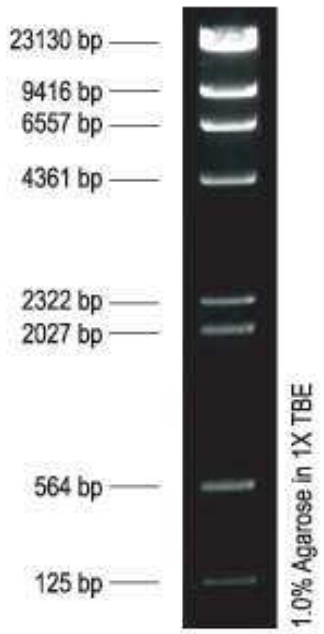
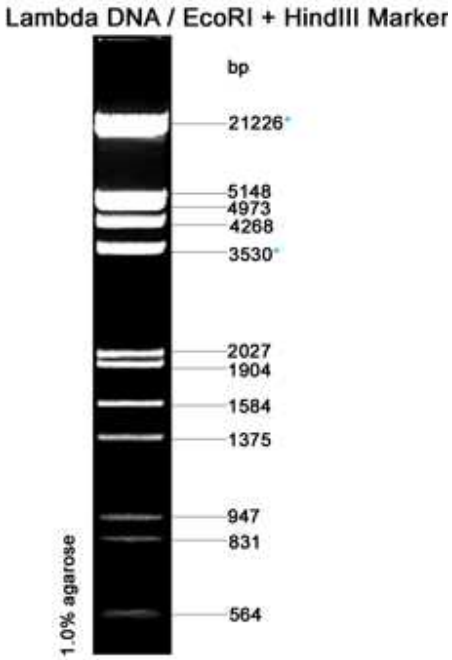
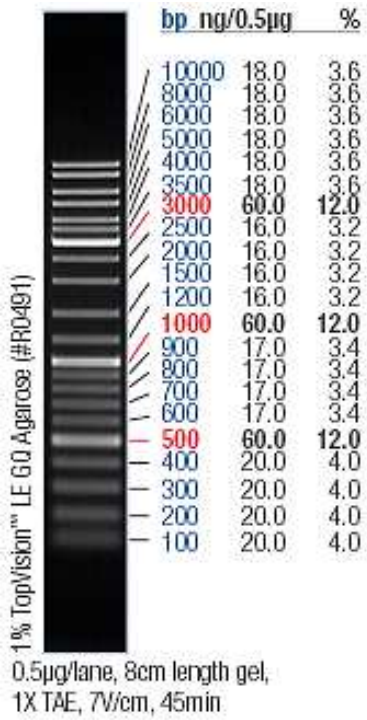
Discription of different markers size



Lambda+*Hin*dIII



Lambda+*Eco*RI+*Hin*dIII



O 'GeneRuler

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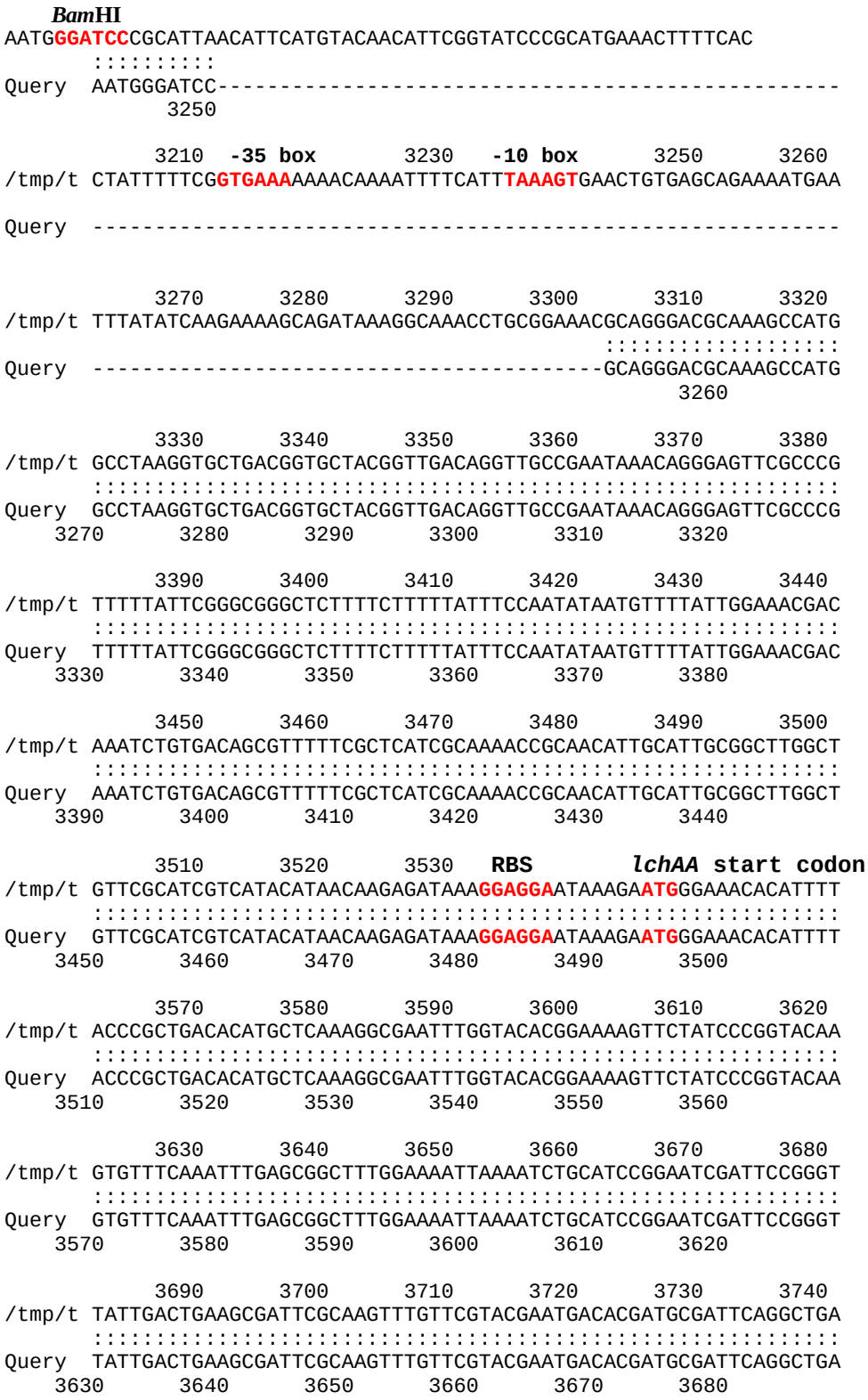
Comparison of pBG163 and pBG162 sequences (partially)



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