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To better bridge research with commercialization, this project sought to develop an improved analytical method for the assay of biosurfactants known as surfactins from *Bacillus subtilis*. We sought to compare levels of production from various strains of *B. subtilis*, including strain 203R, which was isolated from areas prone to oil degraders. The use of Ultra-Performance Liquid Chromatography coupled with High Resolution Mass Spectrometry proved beneficial for the selective identification and quantification of surfactin lipopeptides. Surfactins were eluted in under 10 minutes and quantified using the total area of generated ions. Near baseline separation across the envelope gave an added layer of identification to the ions responsible for surfactins. These lipopeptides, having similar molecular masses to the iturins, nearly coelute complicating the analysis. By coupling retention time with fragmentation pattern, some isoforms could be distinguished. Validation of the method was achieved by obtaining 4 calibration curves on different days and applying linear regression analysis. Strain 203R was shown to be a superior producer of surfactins than the previously reported model strain ATCC 21332.

RAPID AND IMPROVED ASSAY OF SURFACTINS FROM BACILLUS SUBTILIS, 203R VIA UPLC-ESI-MS

by

David M. Wright

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the Faculty of The Graduate School at
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Master of Science

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Approved by

_____
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APPROVAL PAGE

This thesis written by David M. Wright has been approved by the following Committee of the Faculty of the Graduate School at the University of North Carolina at Greensboro.

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Date of Acceptance by Committee

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Date of Final Oral Examination

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CHAPTER I

INTRODUCTION

A wide range of synthetic surfactants have been created with properties of detergency and emulsification that are unparalleled. While these chemicals possess proven performance in many areas, they are unsuitable for use in some pharmaceutical and medical applications. Biosurfactants from microorganisms show continued interest as renewable or green alternatives to petroleum based surfactants. One of the most powerful biosurfactant classes known, the surfactins, have shown use in oil recovery, bioremediation and as emulsifiers in cosmetic formulations (Fracchia et al., 2012).

Their use as excipients in formulations allows alterations in solubility and absorption of pharmaceuticals, and can also provide dissolution and lubricity. Surfactants are also used to alter the flowability of fine particle granular formulations. In the case of bronchodilation, Symbicort® (Astra-Zeneca) utilizes a two component bronchodilator system to give extended efficacy to the patient. For particles to reach the bronchioles, they must be small enough and smooth enough to travel in the air to their target receptors on smooth muscle (Lewis and Copley, 2011). Polyethylene glycol (PEG) 1000 and magnesium stearate are two such preferred surfactants providing the flowability and lubricity necessary for bronchodilator formulations (Astra Zeneca, US 8,461,211 B2). One of the most well documented examples of naturally produced surfactants is the action of pulmonary biosurfactant to effect transfer of oxygen in the alveoli. These

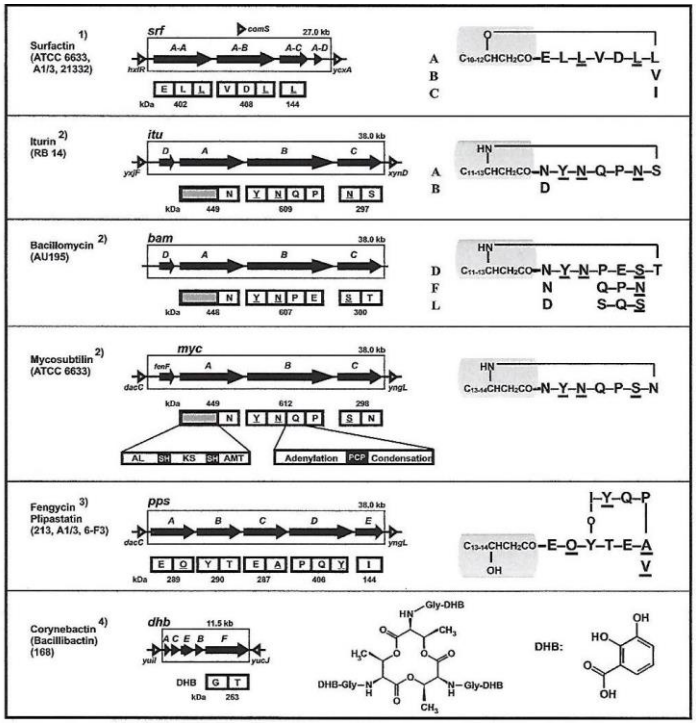
biosurfactants lower the surface tension of the thin aqueous layer in the alveolar lumen to facilitate transfer of oxygen to its eventual heme destination (Silverthorn et al., 2009).

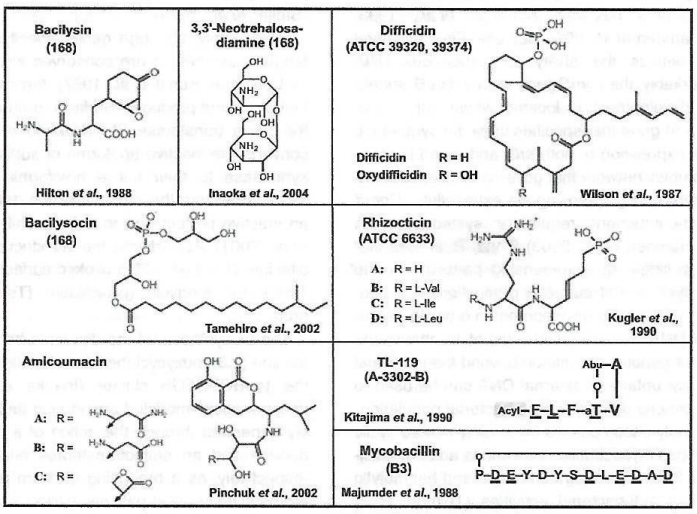
The lipopeptides comprising surfactin are unique among biosurfactants in that they have pronounced ability to lower surface tension in water from 72 to 27 dynes at 20 μmol/L (Chen et al., 2008). They are likely the most prominent antibiotic produced within the *Bacillus* genus and have been much studied for their antibacterial, antimycoplasma (Vollenbroich et al. 1997), antitumor (Lim et al. 2005), insecticidal and even antiviral properties (Das, K. et al. 2006, Vater et al. 2002). The hydrophobic chain moiety of the surfactins is unique since it is incorporated by the non-ribosomal peptide synthetases (NRPS) (Stein et al., 2005). Three genes are required for the biosynthesis of surfactins: *srfA-A*, *srfA-B* and *srfA-C*, which in total comprise the srfA operon. The final step to form the macrolactone occurs via the terminal Te domain of srfA-C (Sonenshein et al., 2002). This biosynthesis of antibiotics is not uncommon to bacteria and fungi.

B. subtilis strains produce a wide spectrum of antibiotics and while no single strain possesses all the genes, the antibiotics can be divided into three main classes: the lantibiotics, lipopeptide antibiotics and small hydrophilic antifungal peptides. The lantibiotics contain crosslinked structures having disulfide bond(s) which can be the site of antibacterial activity via reduction (Stein et al., 2005; Van der Molen et al., 2011). Lipopeptide antibiotics are amphiphilic and often cyclic structures which are membrane active. These compounds comprise the surfactins, iturins, fengycins, mycosubtilin and bacillomycin. Examples of small hydrophilic antifungal peptides are bacilysin and

rhizocticins (Stein et al., 2005, Leenders, 1999). Table 1 below summarizes the structures of all known *B. subtilis* antibiotics and their genetic biosynthesis.

Table 1. Summary of *B. subtilis* Antibiotics.
Stein, T. Molecular Microbiology (2005) 56(4), 849. Reproduced with permission, John Wiley and Sons and Copyright Clearance Center, April 26, 2018.





Traditionally, the ATCC 21332 strain of *Bacillus subtilis* has been used as the model producer of surfactins (Mohammadipour et al., 2009). The discovery of another strain, 203R described herein, has shown to yield increased amounts of lipopeptides. These lipopeptides and other antibiotics having surface activity can be enriched from batch fermentation systems through a process known as foam fractionation, acid precipitation or a combination of both (Chen, et al., 2008). To bridge research with commercialization, we sought to develop an improved analytical method for the assay of surfactins using Ultra Performance Liquid Chromatography (UPLC) coupled to Electrospray Ionization Mass Spectrometry (ESI-MS) with a LTQ Mass Spectrometer utilizing Orbitrap Technology. The goal of this work was to develop and validate the new method of surfactin analysis, and to apply it to compare quantity of surfactin produced by several strains of *B. subtilis*.

CHAPTER II

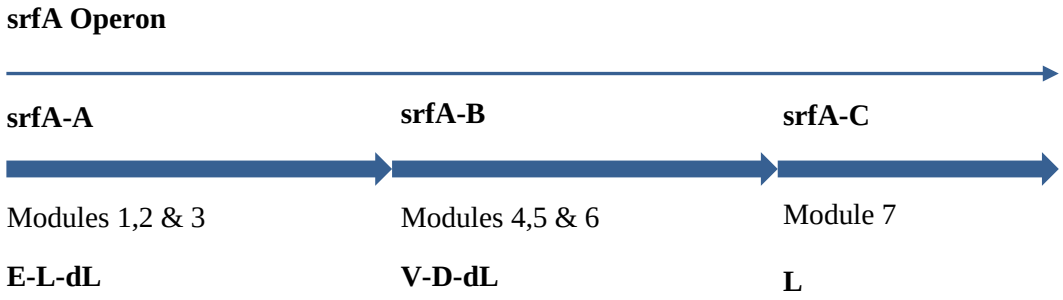
BIOSYNTHESIS

NRPS Assembly

Secondary metabolite production in fungi, bacteria and plants utilizes a different pathway for the incorporation of amino acids. In these instances, the central dogma gives way to processes largely independent of mRNA. These secondary metabolites are built using large multienzyme complexes such as the polyketide synthases (PKS) or nonribosomal peptide synthetase (NRPS) systems. The range of nonproteinogenic amino acids used extends well beyond the canonical essential and nonessential ones. For this reason, they are often referred to as monomers for the NRPS modules. The primary structure of these peptidyl regions is short, from two to about fifty monomers, rarely linear and can contain branchings and cycles (Grünewald and Marahiel, 2013).

Contained within this NRPS complex are repeating domains of condensation, adenylation, thiolation, epimerization, termination and cyclization. Repeating domains which give one complete elongation cycle can be grouped and give rise to modules (Mootz, et al., 2002). For the production of surfactins, the srfA operon can be broken down into the genes srfA-A, srfA-B and srfA-C to make the groups of modules as shown below in figure 1. The srfA-A gene contains the nucleotides to make the first three enzyme modules and so forth (Lee, et al., 2007).

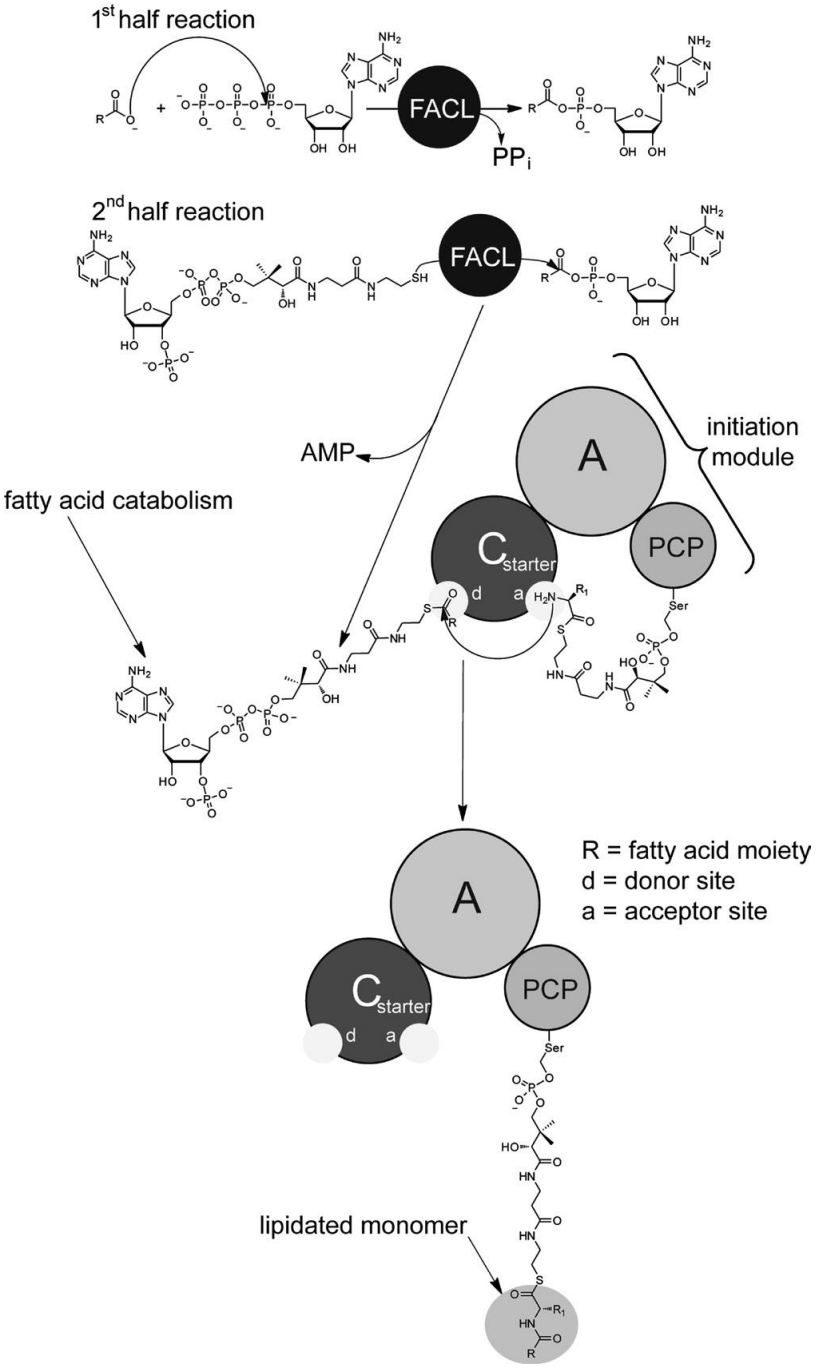
Figure 1. The srfA Operon and Genes for Seven Modules.



Lipoinitiation and Adenylation

Two theories abound for the lipidation of monomers both of which involve fatty acyl-CoA ligase (FACL) (Baltz et al., 2005). One proposed scheme involves adenylation of the fatty acid from primary metabolism followed by conversion into the energy-rich CoA thioester. This CoA thioester is then recruited to the donor site of the starter condensation domain where it is attacked by the nucleophilic nitrogen of the tethered amino acid as shown below in figure 2. Amino acids are not incorporated directly into the growing peptide chain. They must first be adenylated in the adenylation domain to impart reactivity toward the terminal sulfhydryl of 4'-phosphopantetheine (4'-PP) (Kleinkauf, 1995, Stachelhaus, et al., 1998).

Figure 2. Lipoinitiation and Adenylation. In this proposed mechanism, fatty acids are first adenylated and then recruited to the donor site as acyl CoA donor substrates. Nascent monomers attached to the 4'-PP arm can then enter the acceptor site where they can condense with the thioester to form the lipidated monomer. Grünewald and Marahiel, Handbook of Biologically Active Peptides, 2013.

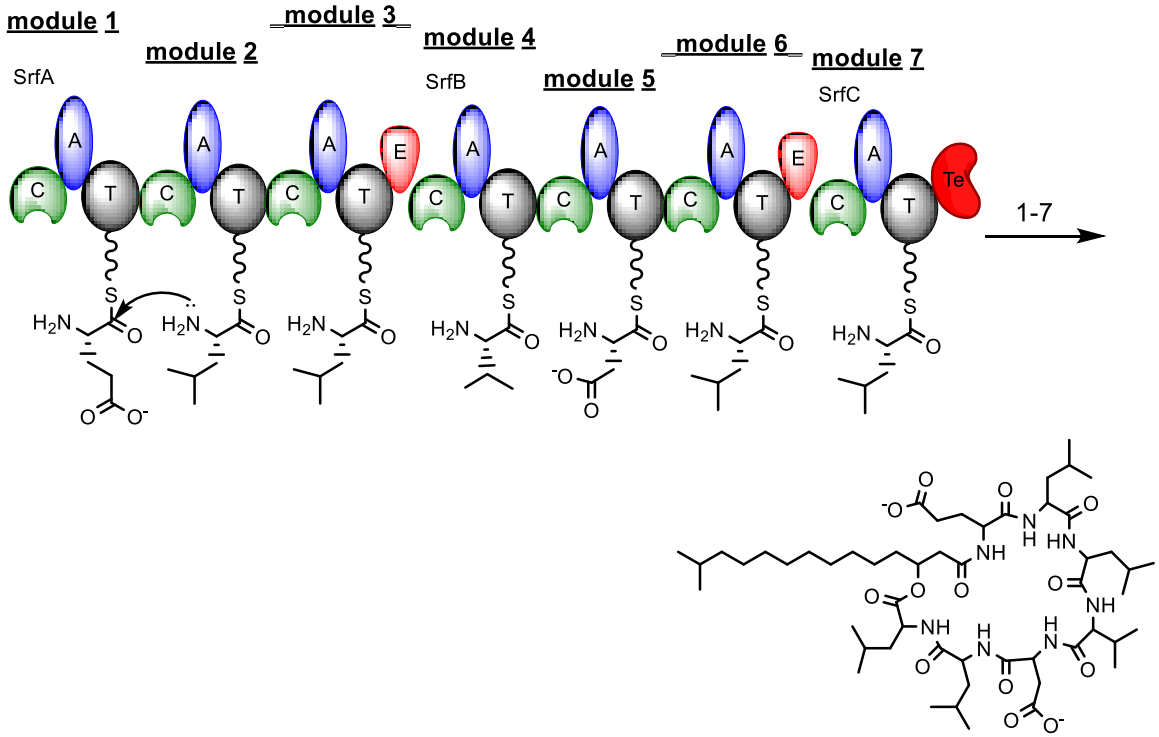


Peptide Elongation

The importance of the peptidyl carrier protein (PCP) or thiolation domain of the multi-enzyme complex has led to the "PCP-centered view" of lipopeptide biosynthesis. Nascent amino acids become tethered to the peptidyl carrier protein via the highly flexible 4'-PP cofactor. The transfer of the 4'-PP cofactor to the peptidyl carrier protein occurs due to the presence of the sfp gene which encodes for the enzyme phosphopantetheinyl transferase (Lee, et al., 2007, Galli, et al. 1994). This cofactor is posttranslationally attached to the PCP via a highly conserved serine residue to give rise to the thiolation domain (Grünewald 2013). In this way, monomers are added to each module in an almost assembly-line fashion as shown in figure 3 below.

In addition to condensation, adenylation and thiolation; epimerization, N-methylation and heterocyclization can occur (Kleinkauf, 1990). In the case of epimerization, it has been recognized that formation of d building blocks impart resistance to proteases which are l specific (Grünewald, 2013). The incorporation of a d stereoisomer directly has been known to occur but most often proceeds through epimerization (Silverman, 2002).

Figure 3. Peptide Elongation. Each reactive monomer is tethered to the condensation domain via the 4'-PP (wavy line).



Termination and Cyclization

According to the Norine database, macrolactonization and macrolactamization account for 64% of all nonribosomally produced peptides. This final step serves to rigidify the lipopeptide and constrain it into a biologically active form. Research performed by Hoefler and colleagues suggests that the cyclized form of surfactin is indeed necessary for activity against the bacterial pathogen *Streptomyces* sp. Mg1 (Hoefler et al., 2012). Similarly, the cyclized form of fengycins was shown to be absolutely necessary for antifungal activity (Tosco et al., 2015). In order for cyclization to occur, the thioester of the most downstream PCP domain is attacked by the hydroxyl of

serine in a catalytic triad of histidine and aspartate. The resulting peptidyl-O-TE oxoester can then undergo attack by an amine or hydroxyl group to complete cyclization and release from the thioesterase domain (Grünewald, 2013).

The exact mechanism by which surfactins are excreted is unknown. It is believed that excretion occurs via diffusion since a transporter has yet to be identified. Gram positive lantibiotic producers such as *Bacillus subtilis* have ways to obviate the action of their own products. For the lantibiotics, this is accomplished through ATP binding cassette (ABC) transporters which move the lantibiotic to the extracellular space. For surfactins, it is believed this is accomplished via the YerP gene. This gene is an example of a RND (resistance, nodulation and cell division) family of multidrug efflux pumps in Gram positive bacteria (Stein et al., 2005).

CHAPTER III

LIPOPEPTIDE FUNCTION

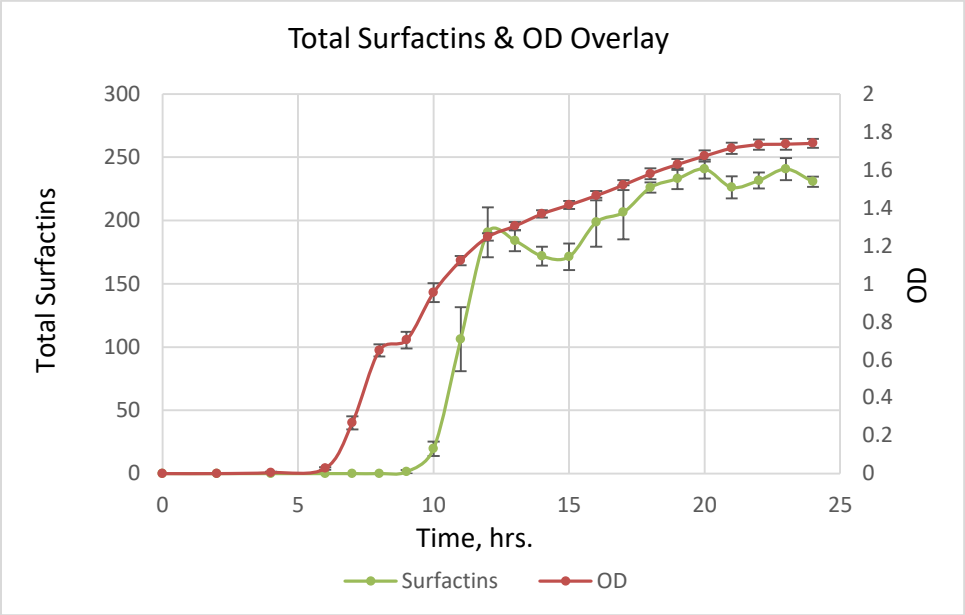
Regulation of Surfactin Production

The regulation of surfactin biosynthesis is coordinated through signals such as starvation, sporulation, genetic competence development and production of degradative enzymes (Losick et al., 1986; Marahiel et al., 1993). The depletion of one or more nutrients such as glucose can induce starvation. When this occurs some cells can begin sporulation which releases DNA into the extracellular matrix. This uptake of exogenous DNA is termed genetic competence. Mutational analysis has shown that a gene termed comS is embedded within the srfA-B gene and is dependent upon the srfA promoter for expression. This comS gene is required for genetic competence (Solomon, et al., 1996). Competence is also dependent upon the buildup of an extracellular peptide encoded by the comX gene (D'Souza et al., 1994). In this way, srfA expression is controlled through a quorum sensing mechanism. ComX binds at the cellular membrane to a membrane bound histidine kinase comP and the response regulator comA. ComA autophosphorylates and then transfers its phosphate to comA which initiates transcription of the srfA operon (Sullivan, 1998; Magnuson, 1994).

Quorum Response

Surfactin production is initiated when cell density reaches sufficient quantities to produce the small signal peptide from comX. The two component regulatory response which follows the binding of the comX peptide is a common theme for Gram positive bacteria. Experiments to monitor surfactin production in the 203R strain showed that lipopeptide formation was slightly delayed but followed exponential phase of growth starting at an optical density of 0.7 as shown in figure 4 below.

Figure 4. Surfactin Production over 24 Hours.



For Gram negative bacteria, quorum sensing induces a N-acylhomoserine lactone (an autoinducer) to bind to a transcriptional activator to control expression of genes. Quorum sensing is common to bacteria and can control the production of virulence factors,

biosurfactant production, secondary metabolites, transfer of genetic material as well as swarming (Sullivan, 1998).

Swarming and Motility

Motility in bacteria can be used to avoid antimicrobial substances as well as gain access to nutrients. There are three recognized forms of movement for *Bacillus subtilis*: sliding, swimming and swarming. Hyperflagellation of vegetative cells is the most recognized stage of differentiation for *B. subtilis* and is necessary for swarming. In the planktonic state, *B. subtilis* is able to swim as single cells through aqueous medium. If the medium is sufficiently viscous to support *B. subtilis*, hyperflagellated cells align closely along their long axis and join to form rafts and move across the surface via swarming (Fraser, et al., 1999; Liu, et al., 2018). Experiments by Kinsinger have shown the necessity of surfactin and K^+ ion in motility of *B. subtilis*. In this case, cells from the leading edge of dendritic growth did not show the presence of flagella when Ryu stained (Heimbrook, et al., 1989) and the mechanism of movement was determined as sliding instead of swarming (Kinsinger, et al., 2003).

Other Functions

As previously mentioned, the antiviral, antitumor, hemolytic and antibacterial properties of surfactin are believed to stem from its ability to perturb lipid membranes and viral envelopes. Particularly in Gram positive organisms, where the cell wall is simpler, this is perceived as a defense mechanism. The disruption of lipid membranes, as determined via POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine)

liposomes, was found to begin at a surfactin concentration of 2 μM (Heerklotz and Seelig, 2007).

Furthermore, the very nature of lipopeptides gives them an inherent ability to increase the surface area of hydrophobic water-insoluble growth substrates. In this way the bioavailability of nutrients is increased. Finally, the connection between attachment and detachment from surfaces and production of surfactin cannot be underestimated (Rosenberg and Ron, 1999). *B. subtilis* biofilm formation is dependent on the early sporulation gene product SpoOA and transcription factors σ^H and AbrB (Stein, 2005; Marahiel, 1993; Hamon and Lazazzera, 2001). In 2004 it was shown that colonization around plant roots and the subsequent biofilm formation were connected with surfactin production (Bais et al., 2004)

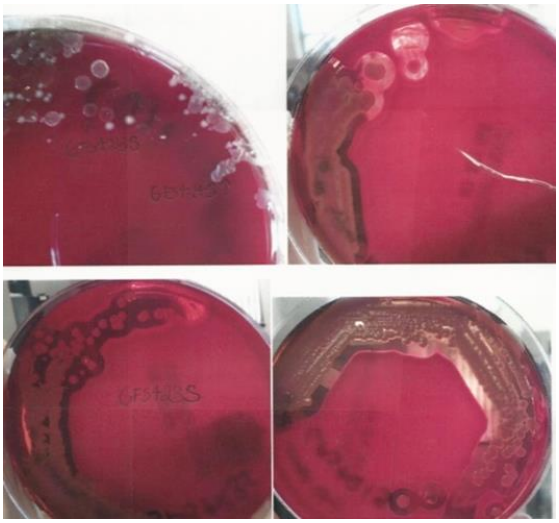
CHAPTER IV

METHODS

Discovery of the Organism

In order to find superior surfactin producing strains without using genetic modification, soil samples were taken from areas prone to oil degraders (Vater et al., 2002). Samples were heat treated in a sand bath at 80°C in order to kill vegetative cells and leave only the spore forming Bacilli. Gram sized aliquots were taken and cultured in media enriched with yeast extract and optimized for growth of *Bacillus subtilis* via the Taguchi method (Wei et al., 2007). Aliquots of seed culture were transferred to fresh broth to reduce the soil content for spread plating. Upon regrowth, samples were serial diluted on agar containing 5% sheeps blood. Surfactin producing strains were determined by measuring radii of β-hemolysis (Mulligan et al., 1989) as the ones shown in figure 5 below.

Figure 5. Isolates from Various Locations in Buck Swamp, Goldsboro, NC.



Promising colonies were subjected to 16S-rRNA sequencing for identification (Pyoung Il et al., 2010).

Solvent Systems for UPLC and Sample Preparation

The dissolution of the cyclic heptapeptide surfactin presented many difficulties. In order to minimize sample matrix effects, the optimal sample solvent is one that is identical to the mobile phase, however this solvent fails to dissolve surfactin efficiently. A solvent system was designed that would dissolve purified surfactin and yet mimic the culture broth matrix. This system was found to be 95% ethanol containing 5% culture matrix containing only the sodium and potassium phosphates diluted to 25% original concentration. For the method validation, a 500 ppm stock solution of surfactin standard (Sigma-Aldrich) was made and diluted arithmetically in half to a concentration of 0.9735 ppb. Samples were ran from lowest to highest concentration with triple injections and a

wash between each concentration to prevent sample carryover. A total of 4 calibration curves were performed on different days and averaged to a final curve. Mass tolerance for the selected ion chromatograms of each parent ion was set to ±5 ppm. The best chromatography was found utilizing an Accucore C30 column, 150 X 2.1 mm, 2.6µm particles held at 30ºC. Solvent conditions for the UPLC are given below in Table 2.

Table 2. Reversed Phase UPLC Ramp Program.

A: H_2O with 0.1% Formic Acid			
B: ACN with 0.1% Formic Acid			
Time	**Flow**	**%A**	**%B**
0.0	0.3	40	60
0.5	0.3	40	60
2.0	0.3	20	80
10.0	0.3	11	89
10.5	0.3	0	100
10.75	0.3	0	100
11.0	0.3	40	60

The preparation of samples was accomplished by dilution in ethanol and spin filtered to an anticipated concentration within the linear dynamic range.

Electrospray Ionization Conditions

High Resolution Mass spectrometry was accomplished via a Thermo LTQ Mass Spectrometer utilizing Orbitrap technology. Mass calibration was achieved using a positive ion calibration mix containing caffeine, the small peptide MRFA and Ultramark 1621 at a B ring distance from the capillary transfer tube. Acidic conditions were chosen in our reversed phase method to facilitate retention within the column. The sheath gas

was heated for introduction of ions from the source at a distance C from the capillary transfer tube. Values for the tune file are listed in Table 3 below.

Table 3. Tune File Conditions

Capillary Temp.	350°C
Sheath Gas Flow	35
Aux Gas Flow	30
Source Voltage	3.70 kV
Capillary Voltage	44.00 V
Tube Lens	135.00 V

Surfactin Production in Various Strains

In order to further establish the 203R strain as a prominent producer of surfactins, multiple strains of *Bacillus subtilis*: 21332, NRRL B-558, NRRL NRS-1270, NRRL B-3383, 168 and 6633, and one *Pseudomonas aeruginosa* strain 17934 were cultured in the same way. These cultures were grown from cryostock in identical media, temperature and aeration. Samples were cultured for 24 hours and frozen until evaluated for lipopeptide content.

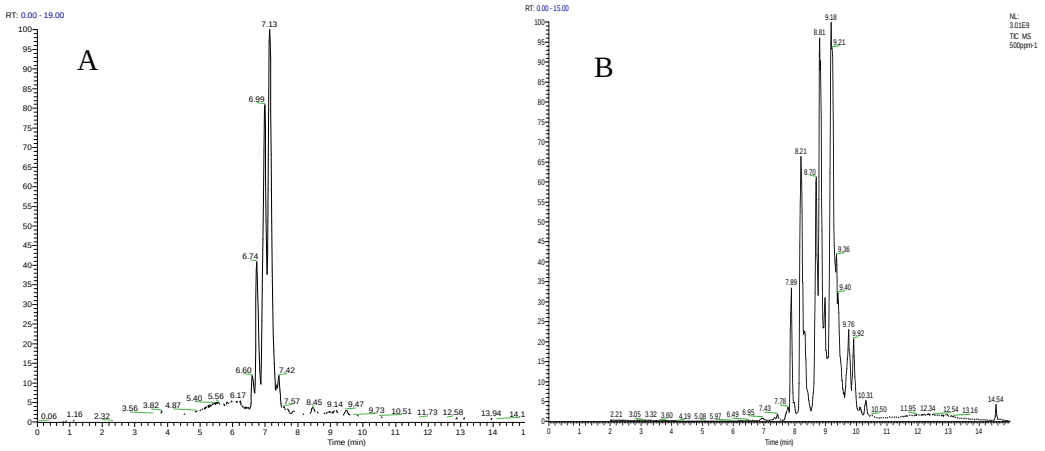
CHAPTER V

RESULTS AND DISCUSSION

UPLC Separation

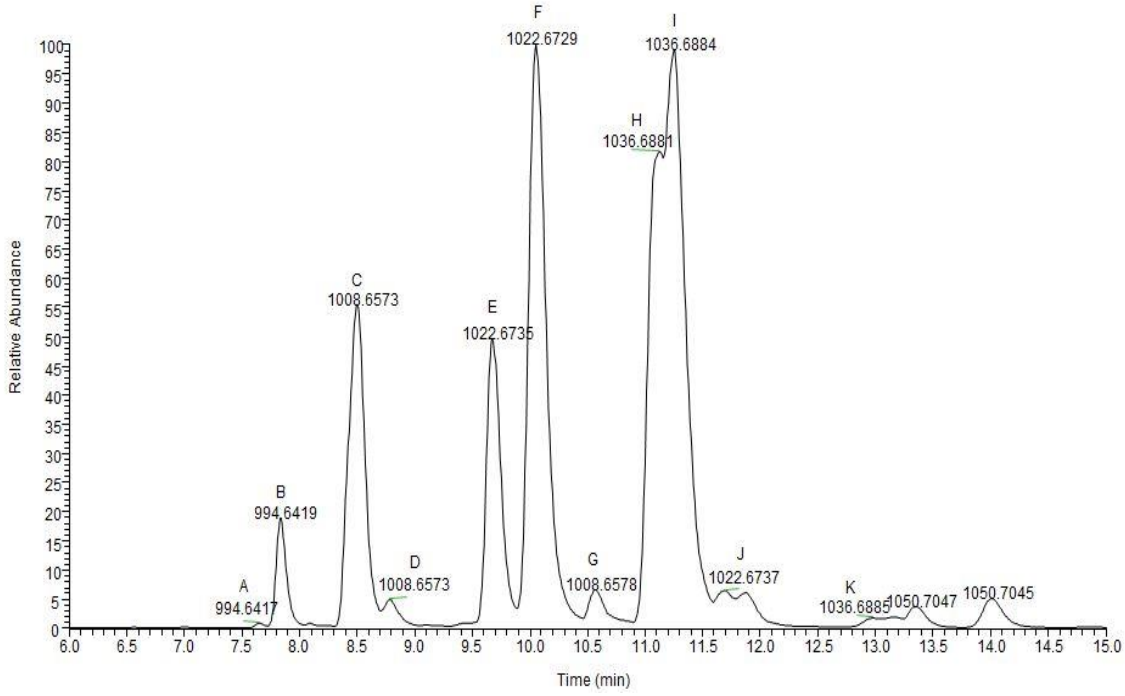
Several UPLC columns were used to improve the resolution of surfactin homologs and isoforms: Waters Acquity UPLC BEH Amide, 130Å, 1.7 µm, 2.1 X 150 mm (data not shown), Agilent Zorbax Bonus RP C_{18} Amide, 2.1 X 150 mm, 1.8 µm and Waters Acquity BEH C_{18}, 2.1 X 150 mm, 1.7 µm. Although the amide columns had higher anticipation to separate the cyclic lipopeptides of interest, the presence of the lipopeptide chain appeared to dominate the separation mechanism and thus a reverse phase separation mode was used. Methanol failed to elute the surfactins from the Zorbax Bonus RP Amide phase and thus ethanol was chosen for the separation. A second reason for the use of ethanol was its' lower viscosity than isopropyl alcohol and thus reduced back pressure. Likewise, analytes were separated under acidic conditions and acetonitrile was found to yield a superior separation over methanol and ethanol as shown in figure 6 below.

Figure 6. UPLC Column Optimization. A: Chromatogram obtained using Zorbax Bonus RP, C_{18} amide and ethanol solvent ramp. B: Chromatogram obtained using Waters Acquity BEH C_{18} phase and acetonitrile solvent ramp.



Distinguishing between the A and C isoforms of surfactins presented a challenge. Since these two isoforms vary only at the seventh amino acid residues, which are leucine and isoleucine, they are isobaric. Surfactin B varies with a valine residue at the seventh position and is more easily separated on the basis of its hydrophobic chain length owing to the reverse phase conditions. We were able to obtain near baseline resolution for the A and C isoforms through C14. Figure 7 below shows the total ion chromatogram.

Figure 7. Base Peak of Total Ion Chromatogram. TIC obtained using Accucore C_{30} phase and acidic water/acetonitrile solvent ramp: Peak A: C_{12}-Isoform A, B: C_{12}-Isoform C: C_{13}-Isoform A, D: C_{13}-Isoform C, E: C_{14}-Isoform A, F: C_{14}-Isoform C, G: C_{14}-Isoform B, H: C_{15}-Isoform A, I: C_{15}-Isoform C, J: C_{15}-Isoform B, K: C_{16}-Isoform B.



Masses and Fragmentation

The use of sodium and potassium phosphates in the culture matrix created both adducts in the source and must be taken into consideration for the quantification of total surfactins. Table 4 below lists the ions present and upon closer examination shows a repeating pattern of masses for the valine isoform with the addition of a methylene group for the next higher homolog.

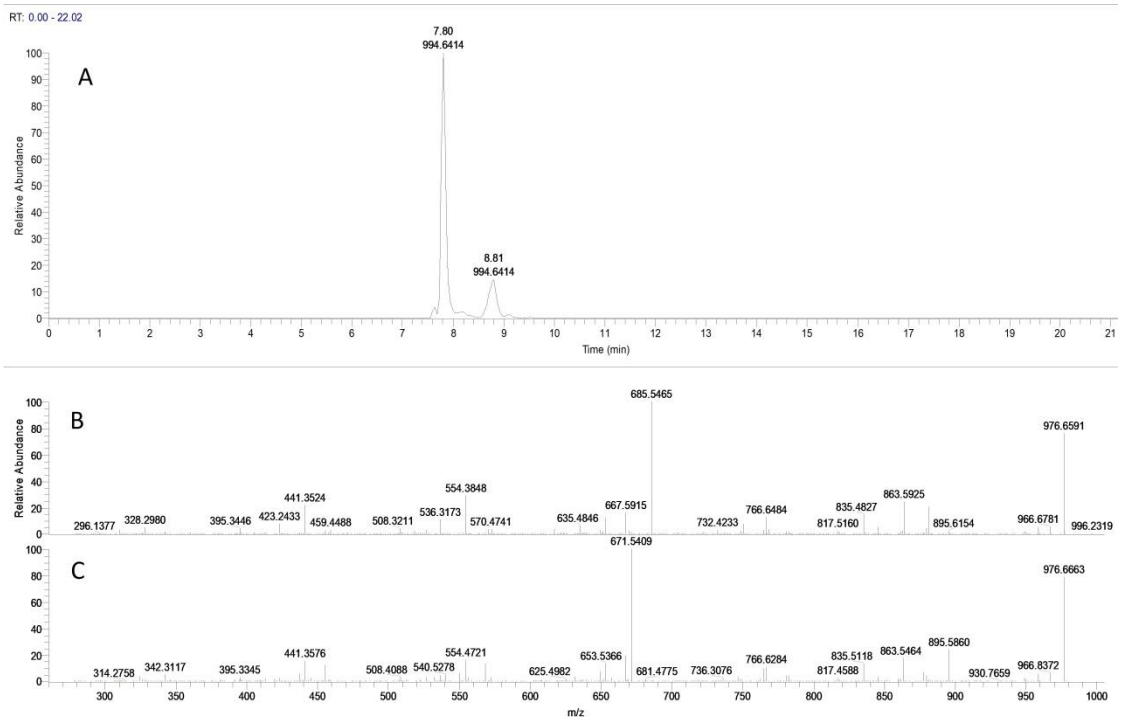
Table 4. Ions used in Quantification of Total Surfactins.

SURFACTIN A, Isoform N→C, ELLVDLL				SURFACTIN B, Isoform N→C, ELLVDLV				SURFACTIN C, Isoform N→C, ELLVDLI			
Homologs	Ion	Formula*	Mass	Homologs	Ion	Formula*	Mass	Homologs	Ion	Formula*	Mass
C-12	H^+	$C_{50}H_{88}O_{13}N_7$	994.64	C-12	H^+	$C_{49}H_{86}O_{13}N_7$	980.63	C-12	H^+	$C_{50}H_{88}O_{13}N_7$	994.64
	Na^+	$C_{50}H_{87}O_{13}N_7Na$	1016.63		Na^+	$C_{49}H_{85}O_{13}N_7Na$	1002.61		Na^+	$C_{50}H_{87}O_{13}N_7Na$	1016.63
	K^+	$C_{50}H_{87}O_{13}N_7K$	1032.60		K^+	$C_{49}H_{85}O_{13}N_7K$	1018.58		K^+	$C_{50}H_{87}O_{13}N_7K$	1032.60
C-13	H^+	$C_{51}H_{90}O_{13}N_7$	1008.66	C-13	H^+	$C_{50}H_{88}O_{13}N_7$	994.64	C-13	H^+	$C_{51}H_{90}O_{13}N_7$	1008.66
	Na^+	$C_{51}H_{89}O_{13}N_7Na$	1030.64		Na^+	$C_{50}H_{87}O_{13}N_7Na$	1016.65		Na^+	$C_{51}H_{89}O_{13}N_7Na$	1030.64
	K^+	$C_{51}H_{89}O_{13}N_7K$	1046.62		K^+	$C_{50}H_{87}O_{13}N_7K$	1032.60		K^+	$C_{51}H_{89}O_{13}N_7K$	1046.62
C-14	H^+	$C_{52}H_{92}O_{13}N_7$	1022.68	C-14	H^+	$C_{51}H_{90}O_{13}N_7$	1008.66	C-14	H^+	$C_{52}H_{92}O_{13}N_7$	1022.68
	Na^+	$C_{52}H_{91}O_{13}N_7Na$	1044.66		Na^+	$C_{51}H_{89}O_{13}N_7Na$	1030.64		Na^+	$C_{52}H_{91}O_{13}N_7Na$	1044.66
	K^+	$C_{52}H_{91}O_{13}N_7K$	1060.63		K^+	$C_{51}H_{89}O_{13}N_7K$	1046.62		K^+	$C_{52}H_{91}O_{13}N_7K$	1060.63
C-15	H^+	$C_{53}H_{94}O_{13}N_7$	1036.69	C-15	H^+	$C_{52}H_{92}O_{13}N_7$	1022.68	C-15	H^+	$C_{53}H_{94}O_{13}N_7$	1036.69
	Na^+	$C_{53}H_{93}O_{13}N_7Na$	1058.67		Na^+	$C_{52}H_{91}O_{13}N_7Na$	1044.66		Na^+	$C_{53}H_{93}O_{13}N_7Na$	1058.67
	K^+	$C_{53}H_{93}O_{13}N_7K$	1074.65		K^+	$C_{52}H_{91}O_{13}N_7K$	1060.63		K^+	$C_{53}H_{93}O_{13}N_7K$	1074.65
C-16	H^+	$C_{54}H_{96}O_{13}N_7$	1050.71	C-16	H^+	$C_{53}H_{94}O_{13}N_7$	1036.69	C-16	H^+	$C_{54}H_{96}O_{13}N_7$	1050.71
	Na^+	$C_{54}H_{95}O_{13}N_7Na$	1072.69		Na^+	$C_{53}H_{93}O_{13}N_7Na$	1058.67		Na^+	$C_{54}H_{95}O_{13}N_7Na$	1072.69
	K^+	$C_{54}H_{95}O_{13}N_7K$	1088.66		K^+	$C_{53}H_{93}O_{13}N_7K$	1074.65		K^+	$C_{54}H_{95}O_{13}N_7K$	1088.66

* Formulas and Masses include protonation

To distinguish the valine isoform from the surfactins A and C, fragmentation of the precursor masses was performed. The difference of a single methylene unit in valine created a fragment with *m/z* 671.86 versus cleavage at the same positions for the surfactin A and C isoforms which yielded *m/z* 685.88 (Pecci et al., 2010). Figure 8 shows the 994.6414 precursor ions and their resulting MS-MS spectra for each retention time.

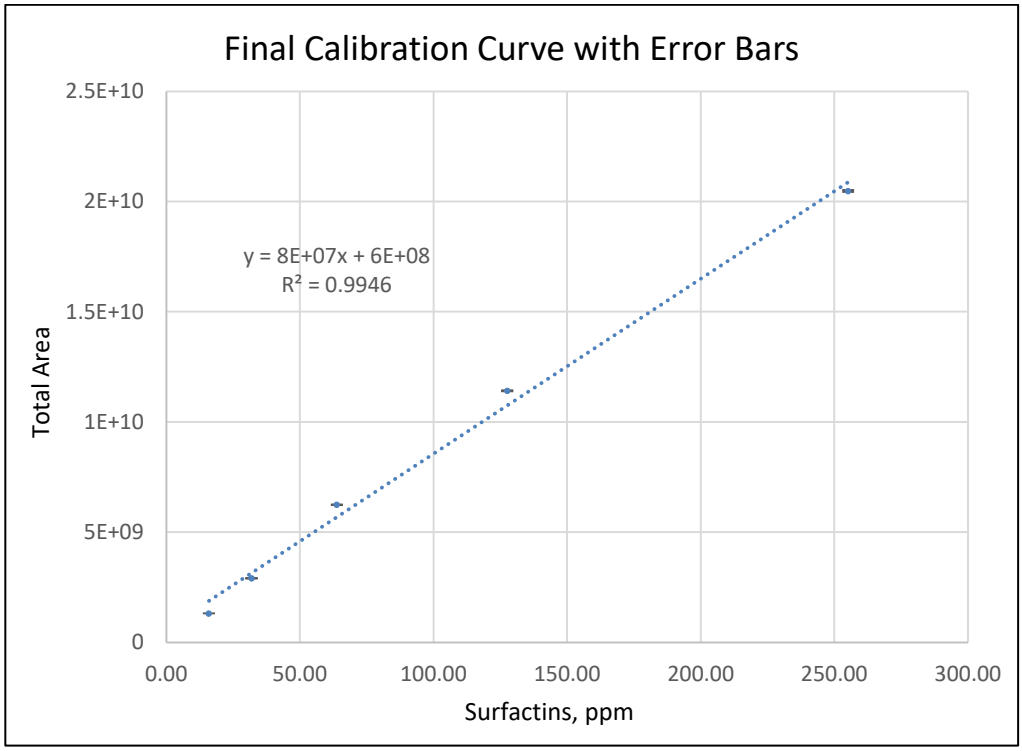
Figure 8. Extracted Ion Chromatogram. The 994.6414 precursor ion and resulting fragmentation. A: All ions of *m/z* 994.6414 eluting over the given time range. B: Fragmentation pattern of the ions at retension time 7.80 minutes. C: Fragmentation pattern of the ions at retension time 8.81 minutes.



Method Validation for Surfactin Quantification

An analysis of the log-log curves for each calibration curve revealed that linearity deviated below 0.1246 ppm and above 250 ppm. The final averaged calibration curve is shown in Figure 9.

Figure 9. Average of Four Calibration Curves.



Applying linear regression analysis gave a correlation coefficient of 0.995 as shown in Table 5 below.

Table 5. Linear Regression Parameters.

Validation Parameter	Result
Correlation coefficient	0.995
Standard error	6.58×10^{8}
y-intercept	$6.20 \times 10^{8} \pm 4.5 \times 10^{8}$
Slope of regression line	$7.94 \times 10^{7} \pm 3.4 \times 10^{6}$
Number of data points	5
Range	15.9 - 255 ppm
Limit of Detection (LOD)[a]	8.0 ppm
Limit of Quantification (LOQ)[b]	16 ppm

a. LOD defined as the lowest concentration which gave a total ion signal three times greater than the noise.
b. LOQ defined as the lowest concentration which gave a residual of 15% or less.

When determining residuals, the low concentrations extended from 15 to 2100%. All concentrations below 15% residuals were truncated (Junio, et al., 2013) yielding a calibration curve with a range through one order of magnitude. This small linear dynamic range is likely due to further saturation at the upper limit and poor repeatability for the ion areas at low concentrations. Table 6 shows the accuracy and precision for the validated method.

Table 6. Accuracy and Precision.

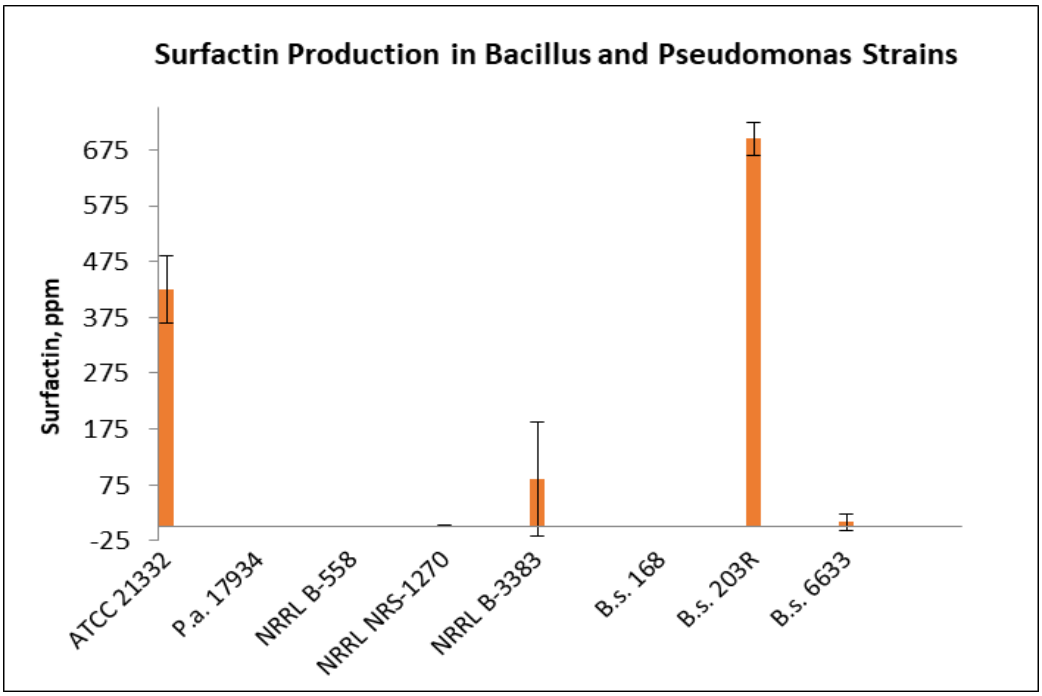
Theoretical Conc. (ppm)	Measured Conc. (ppm)	Residuals[a] (%)	Repeatability[b] (%)	Intermediate Precision[c] (%)
15.95	13.49	-15	5.7	2.9
31.9	22.32	3.4	12	3.1
63.8	73.80	16	10	2.0
127.6	137.1	7.5	0.23	3.2
255.2	248.1	-2.8	4.4	0.84

a. Residuals were calculated as follows: (measured concentration-theoretical concentration)/ theoretical concentration x 100.
b. Repeatability is expressed as the % relative standard deviation for back calculated surfactin concentrations determined by triplicate analyses conducted on a single day.
c. Intermediate precision is expressed as the % relative standard deviation of the four back-calculated surfactin concentrations (each an average of triplicate measurements determined on four separate days).

Production of Surfactins in Various Strains

Many strains of *B. subtilis* and other species contain the genes for antibiotic production. We sought to further investigate multiple *B. subtilis* strains from the USDA as well as one *P. aeruginosa* strain for surfactin production. *B. subtilis* 168 was used as a potential negative control because of its years of laboratory cultivation and use as a model *Bacillus* strain. This original Marburg 168 strain was reportedly exposed to X-rays in the 1940s and does not produce lipopeptides or polyketides (Burkholder and Giles, 1947; Stein, T. 2005; Kinsinger, R. 2003). It has also been documented that a frameshift mutation in the sfp gene of strain 168 resulted in an inactive form of PPan transferase and thus the 4'-PP arm cannot be transferred to the thiolation domain (Mootz et al, 2001). Results for the various strains are shown below in figure 10.

Figure 10. Surfactin Production across Multiple Strains of *B. subtilis*.



Although *Pseudomonas aeruginosa* 17934, NRRL B-558 and *B. subtilis* 168 did not produce surfactin above the limit of quantification (16 ppm), ions corresponding to the masses and retension times of surfactin were detected in the culture media for these organisms. Their total area sums remained below the limit of detection, as defined by the method validation, and even several orders of magnitude below the y-intercept value for the calibration curve. Consistent with published literature, the previous model strain ATCC 21332, produced detectable surfactins of 425 ppm (Mohammadipour et al., 2009).

CHAPTER VI

CONCLUSION

The solubility of purified surfactin is very limited when compared to being synthesized by the organism and excreted in situ into the culture broth matrix. This limited solubility makes it impossible to dissolve the analytical standard, which was purified by acid precipitation, in the starting mobile phase or culture matrix. To ameliorate this, a suitable solvent system was created to mimic the ions found in the actual culture matrix. Since actual samples from a batch process would contain a disproportionate amount of sodium and potassium ions compared to the calibration standards, all possible ions were summed in the calibration and culture samples to give an equal weighting to both.

Prior literature suggested using reversed phase chromatography utilizing C_{18} phases for separation of surfactins. Our method involving an Accucore C_{30} column improved resolution of the surfactin envelope particularly throughout the C_{14} chain lengths. When coupling the UPLC retention times with the fragmentation pattern of the precursor ions, it was possible to discriminate the A and C isoforms from the surfactin B isoform. This gave an added layer of identification to surfactin peaks and helped prevent misidentification if other identical masses are present.

Four calibration curves collected on different days with each concentration in triplicate gave good repeatability, within the linear dynamic range, with a correlation

coefficient of 0.995. Although breakdown of linearity was clearly identified from the log-log transformations, other factors obligated the linear dynamic range to be further truncated. Upon analysis of the % residuals it became apparent that at concentrations less than 16 ppm quantification was not possible. Even though surfactins could easily be detected at concentrations of 2 ppm and less, our chosen definition for LOD of signal to noise ratio of 3:1 meant a LOD of 8 ppm was set.

The 203R strain of *Bacillus subtilis* was originally screened for surfactin production based on clearing zones of β-hemolysis. Using mass spectrometry enabled a quantitative way of further establishing this strain as a superior producer of surfactins as compared to other *B. subtilis* strains evaluated. Although UPLC coupled to HRMS is a far superior method for quantifying surfactins than gravimetric or surface tension methods, the inherent similarity between different classes of biosurfactants can produce situations of coelution. In our case, the iturins also contain the same range of fatty acyl chains and a seven-membered cyclic heptapeptide but are joined through lactam cyclization. Since the mode of UPLC separation was reversed phase, the iturins coeluted within the surfactin envelope. The iturin class of lipopeptides produced several ions of similar mass but not within the 5ppm mass tolerance set for the surfactin quantification. For this reason, the HRMS application coupled with an improved separation before the MS interface allowed differentiation between surfactins and iturins as well as the surfactin valine isoform from the A and C forms.

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APPENDIX A

SUPPLEMENTARY DATA

Figure 11. Glucose Depletion throughout Culture.