SOIL WASHING POTENTIAL AND CHARACTERISTICS OF BIOSURFACTANTS FROM *BACILLUS* SP. GY19

Miss Alice Rau

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Environmental Management

(Interdisciplinary Program)

Graduate School

Chulalongkorn University

Academic Year 2012

ศักยภาพในการชะล้างดินและคุณลักษณะของสารลดแรงตึงผิวชีวภาพที่ผลิตโดยใช้จุลินทรีย์ *Bacillus* sp. GY19

นางสาวอลิส รอล์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาการจัดการสิ่งแวดล้อม (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2555

Thesis Title	SOIL WASHING POTENTIAL AND CHARACTERISTICS OF BIOSURFACTANT PRODUCED BY *BACILLUS* SP. GY19
By	Miss Alice Rau
Field of Study	Environmental Management
Thesis Advisor	Assistant Professor Onruthai Pinyakong, Ph.D.
Thesis Co-advisor	Assistant Professor Preecha Phuwapraisirisan, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

…… Dean of the Graduate School

(Associate Professor Amorn Petsom, Ph.D.)

THESIS COMMITTEE

……. Chairman

(Assistant Professor Chantra Tongcumpou, Ph.D.)

……. Thesis Advisor

(Assistant Professor Onruthai Pinyakong, Ph.D.)

……. Thesis Co-advisor

(Assistant Professor Preecha Phuwapraisirisan, Ph.D.)

……. Examiner

(Assistant Professor Ekawan Luepromchai, Ph.D.)

……. External Examiner

(Suwat Soonglerdsongpha, Ph.D.)

อลิส รอล์ : ศักยภาพในการชะล้างดินและคุณลักษณะของสารลดแรงตึงผิวชีวภาพที่ผลิตโดยใช้จุลินทรีย์ *Bacillus* sp. GY19 (Soil washing potential and characteristics of biosurfactant from *Bacillus* sp. GY19) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร.อรฤทัย ภิญญาคง, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.ดร.ปรีชา ภูวไพรศิริศาล, 128 หน้า.

ช่วงทศวรรษที่ผ่านมา สารลดแรงตึงผิวชีวภาพที่ผลิตโดยจุลินทรีย์ชนิดต่างๆ เป็นที่ได้รับความสนใจเป็นอย่างมาก โดยในการศึกษาครั้งนี้ได้มุ่งเน้นการผลิตสารลดแรงตึงผิวชีวภาพโดยแบคทีเรียชนิด *Bacillus* sp. GY19 ซึ่งแยกจากดินที่ใช้ปลูกพืช ซึ่งใช้วัตถุดิบที่สามารถนำกลับมาใช้ใหม่ (กากน้ำตาล ของเสียกลีเซอรอล) และกลีเซอรอลบริสุทธิ์เป็นสารตั้งต้นในการผลิต จากการทดลองพบว่ากากน้ำตาลและกลีเซอรอลบริสุทธิ์สามารถใช้เป็นสารตั้งต้นในการผลิตสารลดแรงตึงผิวชีวภาพได้โดยแบคทีเรียสายพันธุ์นี้ ซึ่งสามารถลดแรงตึงผิวของอาหารเลี้ยงเชื้อได้มากถึง 31% (ค่าแรงตึงผิวต่ำสุด 29.5 มิลลินิวตันต่อเมตร) และ 55% (ค่าแรงตึงผิวต่ำสุด 29.7 มิลลินิวตันต่อเมตร) ตามลำดับ จากการทดสอบองค์ประกอบของสารลดแรงตึงผิวชีวภาพ ได้แก่ ปริมาณไขมัน โปรตีน และคาร์โบไฮเดรตโดยวิธีฟอสโฟ-วานิลิน วิธีเบรดฟอร์ดและวิธีฟีนอล-ซัลฟูริค แสดงเห็นว่าสารลดแรงตึงผิวชีวภาพที่ถูกผลิตขึ้นนั้นเป็นชนิดลิโปเปปไทด์ ซึ่งสามารถยืนยันผลได้โดยใช้เทคนิค NMR นอกจากนี้แบคทีเรีย *Bacillus* sp. GY19 ถูกทดสอบเพื่อตรวจหายีนที่ใช้ในการผลิตสารลดแรงตึงผิวชีวภาพโดยปฏิกิริยาลูกโซ่พอลิเมอเรส พบว่าแบคทีเรียดังกล่าวมียีนของการสร้าง fengycin, plipastatin และ surfactin ร่วมอยู่ด้วย ยิ่งไปกว่านั้นจากการทดสอบการชะล้างน้ำมันดิบออกจากดินสามชนิด พบว่า สารลดแรงตึงผิวที่ผลิตขึ้นมีความเป็นไปได้ในการนำไปใช้เป็นสารชะล้าง ซึ่งศักยภาพในการชะล้างขึ้นอยู่กับชนิดของดิน โดยที่ความเข้มข้นของสารลดแรงตึงผิวชีวภาพ 2 กรัมต่อลิตรที่ผลิตจากการใช้กลีเซอรอลบริสุทธิ์ 3 เปอร์เซ็นต์เป็นสารตั้งต้น สามารถกำจัดน้ำมันดิบออกจากดินร่วนเหนียวปนทรายได้ถึง 91% นอกจากนี้การทดสอบการยับยั้งการงอกของเมล็ดแตงกวา มะเขือเทศ และผักกาดโดยสารสกัดหยาบของสารลดแรงตึงผิวชีวภาพ พบว่าเมล็ดพืชทั้งสามชนิดสามารถงอกได้ ยิ่งไปกว่านั้นยังพบว่าสารลดแรงตึงผิวชีวภาพไม่มีผลในการยับยั้งการเจริญเติบโตของจุลินทรีย์ การศึกษาครั้งนี้จึงสามารถสรุปได้ว่าสารลดแรงตึงผิวชีวภาพที่ผลิตขึ้นโดยใช้กากน้ำตาลและกลีเซอรอลบริสุทธิ์เป็นสารตั้งต้นไม่เป็นพิษต่อสิ่งแวดล้อม ดังนั้นการศึกษานี้ได้แสดงศักยภาพในการใช้ลิโปเปปไทด์จาก *Bacillus* sp. GY19 สำหรับบำบัดพื้นที่ปนเปื้อนน้ำมันดิบ

สาขาวิชา: การจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต .....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5487615120 : MAJOR ENVIRONMENTAL MANAGEMENT

KEYWORDS : Biosurfactant / Soil washing / *Bacillus* sp. GY19 / Crude oil / Ecotoxicity

ALICE RAU : SOIL WASHING POTENTIAL AND CHARACTERISTICS OF BIOSURFACTANTS FROM *BACILLUS* SP. GY19. ADVISOR : ASST. PROF. ONRUTHAI PINYAKONG Ph.D., CO-ADVISOR : ASST. PROF. PREECHA PHUWAPRAISIRISAN, Ph.D., 128 pp.

Biosurfactants, produced by various microorganisms, gained several interests during the past decades. This study aims to take a closer look at the biosurfactant production by *Bacillus* sp. GY19, isolated from planted soil using renewable substrates (molasses, bottom glycerol) and pure glycerol. Molasses and glycerol can be effectively used as substrates for biosurfactant production by this strain, as they reduce surface tensions of culture media up to 31% (29.5 mN/m) and 55% (29.7 mN/m) compared to the control, respectively. Colorimetric tests to quantify the content of lipid, protein and carbohydrates (sulpho-phospho-vanillin-test, Bradford-test and phenol-sulfuric acid test, respectively) revealed the produced biosurfactant as one of lipopeptide type, which could be confirmed by NMR. *Bacillus* sp. GY19 has been tested for biosurfactant producing genes by Polymerase Chain Reaction and found to contain genes responsible for fengycin, plipastatin and surfactin production. Furthermore, soil washing batch experiments, using three types of soil spiked with crude oil, showed potential of an application of this biosurfactant as a soil washing agent, depending on the type of soil. Solutions containing 2 g/l biosurfactant using 3% pure glycerol as substrate could remove 91% of crude oil from a sandy clay loam soil. Additionally, germination tests with cucumber, tomato and lettuce seeds were conducted with aqueous solutions of the crude biosurfactant extract, showing no inhibition of germination to all tested plants. Furthermore, no microbial inhibition or bactericidal effects could be determined of the biosurfactant solutions, leading to the conclusion that the extracted crude biosurfactant solutions, using molasses and pure glycerol as substrates, are not toxic to the environment. Hence, this study showed potential utilization of lipopeptide from *Bacillus* sp. GY19 for remediation of crude oil contaminated sites.

Field of Study : Environmental Management Student's Signature .....

Academic Year : 2012 ..... Advisor's Signature .....

Co-advisor's Signature .....

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and greatest appreciation to my advisors, Assistant Professor Dr. Onruthai Pinyakong and Assistant Professor Dr. Preecha Phuwapraisirisan, for their helpful advices, guidance, support and encouragement throughout this research work.

I wish to express my thanks to the Environmental and Hazardous Waste Management, International Programs (EHWM) for providing me a full scholarship, research funding and supporting facilities to complete this work and the PTT Research and Technology Institute for supporting me with providing waste glycerol and crude oil samples.

Acknowledgement of my gratitude should also be expressed to Dr. Ekawan Luepromchai, Dr. Mark Nanny, Dr. David Sabatini and Dr. Rudolf Müller who gave valuable directions to my work.

I would like to thank the laboratory staff and students in the Department of Microbiology, Chulalongkorn University and the National Research Center for Environmental and Hazardous Waste Management (NRC-EHWM) who supported me with administrative issues and lab guidance.

Very special thanks and deep appreciation to all my laboratory friends (no particular order): Ms. Nichakorn Khondee, Ms. Witchaya Kaewtip Ms. Wanwasan Wongwongsee, Ms. Chanokporn Muangchinda, Ms. Natthariga Laothamteep, Ms. Nanthorn Paorach, Ms. Suthasinee Jittimanee, Ms. Thipupsorn Rungthaweemanuschai, Ms. Piyamart Khongkhaem, Ms. Chutima Ploychankul and Mr. Sitti Thatong for their guidance and untiring help in all matters. Without you, this work wouldn’t have been successful, not even possible. Not to forget all the remaining members of lab room 406 and 462, but to mention all of you would exceed the limited space I have.

Finally, I am very thankful to my family, for their love, support and, most importantly, never ending belief in the success of their daughter and sister. I am very pleased to thank all my friends in Bangkok, Germany and other parts of the world, who supported me in showing how beautiful life is outside of lab. This work would not have been possible without their moral support.

CONTENTS

Page

ABSTRACT IN THAI....IV

ABSTRACT IN ENGLISH....V

ACKNOWLEDGEMENTS....VI

CONTENTS....VII

LIST OF TABLES....XI

LIST OF FIGURES....XII

LIST OF ABBREVIATIONS....XV

CHAPTER I INTRODUCTION....1

1. Statement of the problem....1
2. Objectives....2
3. Research Hypothesis....3
4. Scope of the work....3

CHAPTER II LITERATURE REVIEW....6

1. Surface active compounds....6
   - 1.1 Critical Micelle Concentration....7
2. Biosurfactants....9
   - 2.1 Classification and properties....10
     - 2.1.1 Glycolipids....15
     - 2.1.2 Lipoproteins....15
     - 2.1.3 Polymeric biosurfactants....17
3. Biosynthetic pathways and molecular genetics of biosurfactant production....18
4. Substrates for biosurfactant production....21
   - 4.1 Molasses....22
   - 4.2 Glycerol and waste/bottom glycerol....22

Page

5. Characterization of biosurfactants....23
6. Applications of biosurfactants....25
7. Soil washing....26
8. Behavior in the environment and toxicity....27
9. *Bacillus* sp. strain GY19....28
CHAPTER III METHODOLOGY....29
1. Materials....29
1.1 Chemicals....29
1.2 Culture media and solutions....29
2. Methods....29
2.1 Biosurfactant production....29
2.1.1 Inoculum preparation....29
2.1.2 Production step....29
2.1.3 Surface tension measurement....30
2.1.4 Foamate extraction....30
2.1.5 Extraction of crude biosurfactant....31
2.2 Soil sorption and soil washing....31
2.2.1 Soil sorption....31
2.2.2 Soil washing....31
2.2.2.a Spiking of soil with crude oil....31
2.2.2.b Washing experiments....31
2.3 Ecotoxicity....32
2.3.1 Seed germination and root elongation tests....32
2.3.2 MIC and MBC....32
2.4 Detection of biosurfactant producing genes....33
2.4.1 DNA extraction....34

Page

2.4.2 PCR 34
2.4.3 Cloning and sequencing 35
2.4.3.a Ligation 35
2.4.3.b Transfer into competent cell 35
2.4.3.c Plasmid extraction and digestion 35
2.5 Chemical characterization of crude biosurfactant 36
2.5.1 Sulpho-phospho-vanillin test 36
2.5.2 Bradford assay 36
2.5.3 Sulphuric acid test 37
2.5.4 TLC autographic method for detection of amino acids 37
2.5.5 Analytics of fatty acid components in lipids 37
2.5.5.a Preparation of FAME 37
2.5.5.b Gas chromatography of FAME 38
2.5.6 Nuclear Magnetic Resonance Spectroscopy 38
2.6 Statistical analysis 38
CHAPTER IV RESULTS 39
1. Biosurfactant production by different renewable substrates 39
1.1 Screening of renewable substrates 39
1.2 Critical Micelle Concentrations 40
2. Soil sorption and soil washing 41
2.1 Soil sorption 42
2.2 Soil washing 44
2.2.1 Sandy clay loam 1 44
2.2.2 Sandy clay loam 2 46
2.2.3 Sandy loam 3 48

Page

2.2.4 Summary of soil washing results 50
3. Ecotoxicity 51
3.1 Seed germination 51
3.2 Root elongation 54
3.3 MIC and MBC 55
1. Detection of biosurfactant producing genes 56
2. Chemical characterization of the crude biosurfactant 58
2.1 Colorimetric tests 58
2.2 TLC with Ninhydrin staining 59
2.3 Analysis of FAME 60
2.4 NMR 62
CHAPTER V DISCUSSION AND CONCLUSIONS 64
1. Discussion 64
1.1 Renewable substrates 64
1.2 Applications in soil washing and soil sorption 65
1.3 Ecotoxicity 67
1.4 Characterization 68
2. Conclusions 70
3. Recommendations 70
REFERENCES 72
APPENDICES 85
APPENDIX A 86
APPENDIX B 88
APPENDIX C 100
APPENDIX D 108
VITAE 113

LIST OF TABLES

Table 1 Overview of the groups and types of biosurfactants, its producing microorganisms and possible applications (modified from Pacwa-Płociniczak et al., 2011 and Soberon-Chavez and Maier, 2011) 11

Table 2 Comparison of bio- and synthetic surfactants in terms of critical micelle concentrations. 41

Table 3 Selected Soil Characteristics – OM: Organic Matter; Avai P: Available Phosphorous; Avai K: Available Potassium; OC: Organic Carbon; N: Nitrogen; CEC: Cation Exchange Capacity. 42

Table 4 R^2 and K_d values of 3 different sorption models 42

Table 5 Concentration of BSF calculated in times of CMC (conc. of BSF divided through CMC from Table 2). 51

Table 6 Means (M) of root length in cm and standard deviation (SD) of three species of plant seeds with different test solutions. 55

Table 7 Results of soil bacteria toxicity assay of four test solutions; N.D. – not detectable. 56

Table 8 PCR products obtained from different primer pairs specific for BSF producing genes. 56

Table 9 Loading scheme of agarose gel presented in Figure 25 57

Table 10 Similarity of PCR products to biosurfactant encoding genes ([1]shown is the name of the biosurfactant encoded by the gene, which could be detected by the corresponding primer pair; [2]accession number of the sequenced gene compared with the database using blastx program) Numeration of bands referred to bands numbered in Figure 23. 58

Table 11 Summary of colorimetric tests: lipid, sugar and protein contents in % of two studied crude BSF. 59

Table 12 Retention times of standard FAME mixture of GC-FID. 61

Table 13 Retention times of FAME of crude biosurfactant of *Bacillus* sp. GY19 using 3% pure glycerol as substrate of GC-FID. 62

LIST OF FIGURES

Figure 1 The global surfactant market (Royal Society of Chemistry 2003). 2

Figure 2 Accumulation of biosurfactants at the water-air interface (Pacwa-Płociniczak et al., 2011). 6

Figure 3 Wilhelmy plate method to measure the surface tension (Kibron Inc., 2012). 7

Figure 4 Different types of aggregates formed by biosurfactants (Maier, 2003). ...8

Figure 5 Relationship between surface tension and concentration of the biosurfactant (Pacwa-Płociniczak et al., 2011). 9

Figure 6 Lactonicsophorolipid (Smyth et al., 2010a). 15

Figure 7 Structure of the cyclic lipopeptide surfactin from Bacillus sp. (Smyth et al., 2010b). 16

Figure 8 Structure of a biopolymer emulsan from Acinetobacter RAG-1 (Smyth et al., 2010b) 17

Figure 9 Potential biosynthetic pathways of Biosurfactant production in microorganisms. (Satpute et al., 2010b) based on (Syldatk and Wagner, 1987) 19

Figure 10 Rhamnolipid synthesis in Pseudomonas spp. By two quorum sensing systems: QSS is present at different regions of the chromosome. Thick black bold arrows: genes on chromosome of Pseudomonas; black arrows: protein synthesis from gene; dotted oval indicates inactive regulatory protein; continuous oval: active complex of regulatory protein and autoinducer (Satpute et al., 2010a). 20

Figure 11 Experimental set up for foamate extraction. 30

Figure 12 Surface tension reduction of supernatant after cultivating *Bacillus* sp. GY19 with three different substrates at different concentrations added to the production media (media with substrate = control) 39

Figure 13 Yields after extraction of the crude biosurfactant from the supernatant produced by Bacillus sp. GY19 using different substrates and concentrations. 40

Figure 14 Surface tensions [mN/m] at different concentrations of the crude biosurfactant using 3% pure glycerol and 3% molasses as substrate, respectively. Results are represented as the means +/- standard deviation (n=3).....41
Figure 15 Concentration of BSF (3% PG) in adsorbed to soil (Cs) plotted versus concentration of BSF (3% PG) in aqueous solution (Cw).....43
Figure 16 Concentration of BSF (3% molasses) in adsorbed to soil (Cs) plotted versus concentration of BSF (3% molasses) in aqueous solution (Cw) according to Langmuir sorption model.....43
Figure 17 Removal of crude oil [in % to control] of sandy clay loam 1 (OC=3.74%, CEC=9.2 cmol/kg) by different washing solutions (BSF = crude biosurfactant, M = molasses, PG = pure glycerol).....45
Figure 18 Removal of crude oil [in % to control] of sandy clay loam 2 (OC=0.84%, CEC=22.9 cmol/kg) by different washing solutions (BSF = crude biosurfactant, M = molasses, PG = pure glycerol).....47
Figure 19 Removal of crude oil [in % to control] of sandy loam 3(OC=2.55%, CEC=4.6 cmol/kg) by different washing solutions (BSF = crude biosurfactant, M = molasses, PG = pure glycerol). 49
Figure 20 Germination in % after 5 days of three different plant species and different test solutions, concentration = 1 g/l; (BSF [3% PG] – crude biosurfactant extract with 3% pure glycerol as substrate; BSF [3% M] – crude biosurfactant with 3% molasses as substrate). 52
Figure 21 Germination of tomato seeds over investigated time with different test solutions, concentration = 1 g/l; (BSF [3% PG] – crude biosurfactant extract with 3% pure glycerol as substrate; BSF [3% M] – crude biosurfactant with 3% molasses as substrate). 53
Figure 22 Germination of lettuce seeds over investigated time with different test solutions, concentration = 1 g/l; (BSF [3% PG] – crude biosurfactant extract with 3% pure glycerol as substrate; BSF [3% M] – crude biosurfactant with 3% molasses as substrate).....53
Figure 23 Germination of cucumber seeds over investigated time with different test solutions, concentration = 1 g/l; (BSF [3% PG] – crude

biosurfactant extract with 3% pure glycerol as substrate; BSF [3% M] – crude biosurfactant with 3% molasses as substrate). 54

Figure 24 Presented are the means of root length of germinated seeds (Cucumber, Tomato, and Lettuce) with different test solutions, concentration = 1 g/l. 55

Figure 25 Agarose gel after running gel electrophoresis with PCR products using specific primer pairs for biosurfactant producing genes; loading scheme is presented in Table 9 57

Figure 26 Silica-TLC sheet with spotted sample of hydrolyzed crude BSF (3% M as substrate shown as M, 3% PG as substrate shown as PG), control: unhydrolyzed sample (shown as C-M and C-PG, respectively); solvent system: 4:1:1 ButOH – CH3COOH – H2O.....60

Figure 27 ^{13}C-NMR of crude biosurfactant extract obtained with 3% pure glycerol as substrate. 63

Figure 28 Relationship between organic carbon content and K_d values of crude biosurfactant extracts. 65

LIST OF ABBREVIATIONS

BSF	Biosurfactant
CEC	Cation Exchange Capacity
CMC	Critical Micelle Concentration
DNA	Deoxyribonucleic acid
FID	Flame ionization detector
FAME	Fatty acid methyl ester
g	Gram
GC	Gas chromatography
l	Liter
LB	Luria Bertani
M	Molasses
MALDI TOF MS	Matrix Assisted Laser Desorption Time of Flight Mass Spectrometry
MBC	Minimal bactericidal concentration
MIC	Minimal inhibitory concentration
m	Meter
mg	Milligram
min	Minute
mN	Milli Newton
µl	Microliter
NB	Nutrient Broth
NMR	Nuclear magnetic resonance spectroscopy
OC	Organic Carbon
OM	Organic Matter
PCR	Polymerase Chain Reaction
PG	Pure Glycerol
QSS	Quorum sensing system
RT	Room temperature
SDS	Sodiumdodecylsulfate

CHAPTER I

INTRODUCTION

1. Statement of the problem

With industrialization and exploration of fossil fuels and its energy potential for industry and daily life, environmental pollution came as a harmful side effect. Leaks and accidental spills of petroleum based products and crude oil are common in the modern world due to the broad usage of petrochemical fabrications (Marinescu et al., 2012). It is estimated that 53% of crude oil entering the environment comes from human activities (Kvenvolden and Cooper, 2003). Crude oil and its refined products, are the main reason for soil and water pollution (Holliger et al., 1997). Furthermore, due to the accumulation of pollutants in animals and plant tissues they cause severe damage and may cause death or mutations (Alvarez and Vogel, 1991). Hence remediation technologies are needed to reduce and if possible remove contaminants from the environment. Having a closer look on soil remediation, several technologies of physical, chemical or biological nature are used, either *ex situ* or *in situ*. Soil flushing and soil washing could be combined with washing solutions containing surfactants (Mulligan et al., 2001), where it comes to biosurfactants as promising substitutes to chemical (synthetic) produced surfactants. Biosurfactants can be produced by plants, animals and microorganisms; the highest research interest is within the microbial production of biosurfactants. The produced surfactants play different roles in the organisms' life cycle (Ron and Rosenberg, 2001) and show important biological activities e.g. antibiotic, antifungal, insecticidal, antiviral, antitumor activities etc., hence biosurfactants have a wide interest in special applications and industries. Details in broad applications are mentioned in point 6.

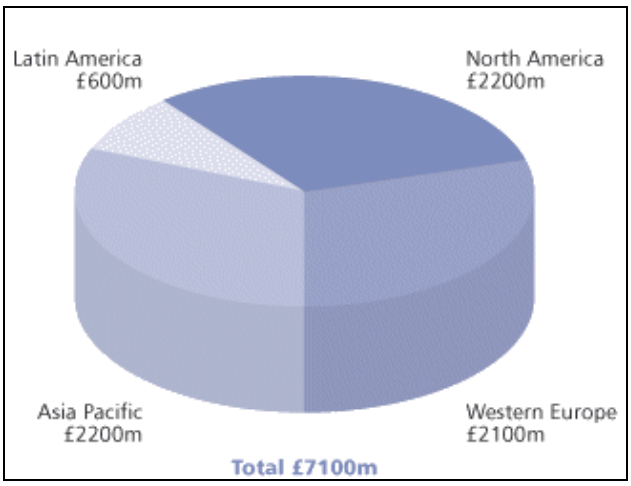


Figure 1 The global surfactant market (Royal Society of Chemistry 2003).

Furthermore, the surfactant industry is a huge business (Figure 1) serving broad markets (Royal Society of Chemistry, 2003). Trends towards sustainable so called "green" products are increasing, where biosurfactants become even more interesting (IHS Chemical, 2010).

There is still a part of raw materials in surfactant industry derived from fossil resources. Using renewable substrates for biosurfactant production by microorganisms could help to conserve our natural fossil resources. To decrease surfactant production with fossil resources is one aim under the European Commission's European Climate Change Program (Patel, 2003).

2. Objectives

The main objectives of this study are to get a better understanding of the biosurfactants produced by *Bacillus* sp. GY19 from genes to applications. The objectives, in detail, are listed as follows:

1. Determine the soil washing potential, soil sorption and ecotoxicology of the biosurfactant by using renewable resources as substrate for biosurfactant production.
2. Detecting biosurfactant producing genes in *Bacillus* sp. GY19 and study the properties and chemical characteristics of the obtained crude biosurfactant.

3. Research Hypothesis

Biosurfactants obtained from *Bacillus* sp. GY19 can be used to wash crude oil from soil and is less toxic to the environment compared to the synthetic surfactants SDS and Tween 80®. Biosurfactant producing genes can be detected in *Bacillus* sp. GY19. Biosurfactant produced by *Bacillus* sp. GY19 is of a lipopeptide type but chemical characteristics and performance of the biosurfactants differ depending on the substrate used.

4. Scope of the work

This work consists of two main parts: A) Environmental applications of biosurfactants produced by *Bacillus* sp. GY19 using renewable substrates and B) Chemical characterization of the biosurfactant and detection of biosurfactant producing genes in *Bacillus* sp. GY19. Furthermore part A was divided into several subparts explained below.

1. Screening of renewable substrates for biosurfactant production

Three different substrates were screened as carbon sources to determine the most effective biosurfactant for further study (Bottom Glycerol, Pure Glycerol, and Molasses). Therefore, *Bacillus* sp. GY19 was incubated in a medium supplemented with the mentioned carbon sources in different concentrations (3%, 7%, 10%) for five days at ambient temperature, 230 rpm. Cell-free media was obtained to measure the surface tension and was further extracted to receive the yield of each batch. The criteria to choose was the reduction of surface tension compared to the uncultured media and yield [g/l]. Selected substrate was used for further production of biosurfactant to study the chemical properties, soil washing, soil sorption and ecotoxicology.

2. Production of biosurfactant in a large scale

The selected substrate was used to cultivate *Bacillus* GY19 and to finally obtain a crude extract of biosurfactants after a liquid-liquid solvent extraction.

3. Chemical properties of the crude extract

The following properties were determined to compare the crude extracts according to the used substrate:

a) Protein content by Bradford Assay (Coomassie blue method).
b) Sugar content by phenol-sulphuric acid method.
c) Lipid content by sulfo-phospho-Vanillin method.

Furthermore, critical micelle concentration was analyzed.

4. Soil washing tests

Soil spiked with crude oil was used to determine the ability of a biosurfactant in different forms (solution of a crude extract, cultured broth, foamate) to wash crude oil from soil. Synthetic surfactants (SDS and Tween 80®) were used to compare the performance.

5. Soil sorption

Three different soils (details are indicated in Material and Methods part) were used to study the sorption behavior of aqueous solutions of the produced biosurfactant to those soil types.

6. Ecotoxicology

In this study, ecotoxicological information was obtained by carrying out germination tests and the determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of aqueous solutions of the biosurfactant and synthetic surfactants (SDS and Tween 80®).

Part B can be separated into two parts as described below:

1. Detection of biosurfactant-producing genes

Bacterial DNA of *Bacillus* sp. GY19 was tested for specific biosurfactant producing genes by Polymerase Chain Reaction, using specific primers as described in Tapi et al. (2010).

2. Chemical characterization of the biosurfactant

A rough characterization of the obtained biosurfactant cultured with pure glycerol as substrate was done to confirm the proposed lipopeptide type. Therefore, colorimetric tests such as sulfo-phospho-vanillin test, phenol-sulfuric acid test and Bradford assay to estimate the amount of lipids, sugars and proteins, respectively, were carried out. Further structural information was achieved by Thin Layer Chromatography and Nuclear Magnetic Resonance spectroskopy as far as possible and fatty acid analysis by gas chromatography.

CHAPTER II

LITERATURE REVIEW

Biosurfactants are amphiphile compounds produced by microorganisms such as fungi, yeasts and bacteria. This structurally diverse group of substances shows surface-active properties with at least one hydrophobic and one hydrophilic moiety (Desai and Banat, 1997).

The hydrophobic part of the molecule is often a fatty acid chain, due to its Surface-active nature, and varies in the chain length. The amphiphile compounds tend to accumulate at interfaces (e.g. water-air, Figure 2) and decrease the repulsive forces between the two phases, which results in a better ability for the two phases to mix, thus helps to solubilize hydrophobic substances (Desai and Banat, 1997). More details of the theoretical background of biosurfactants are summarized in the following sections.

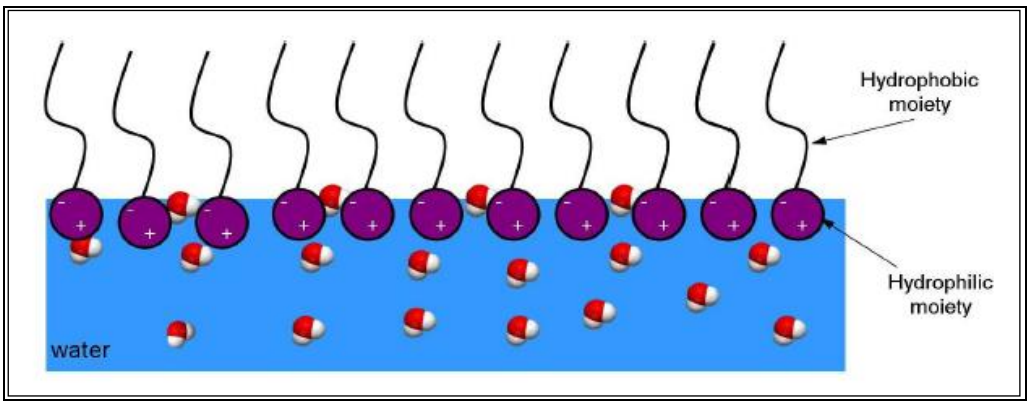


Figure 2 Accumulation of biosurfactants at the water-air interface (Pacwa-Płociniczak et al., 2011).

1. Surface active compounds

A comprehensive start into the terminology of surface and interfaces is given by Rosen and Kunjappu (2012)“A surfactant (a contraction of the term surface-active-agent) is a substance that, when present at low concentration in a system, has the

property of adsorbing onto the surfaces or interfaces of the system and of altering to a marked degree the surface or interfacial free energies of those surfaces (or interfaces). The term interface indicates a boundary between any two immiscible phases; the term surface denotes an interface where one phase is a gas, usually air" (Rosen and Kunjappu, 2012).

Furthermore, the minimum amount of work required to create an interface is called the interfacial free energy, which is measured per area and determined as interfacial or surface tension.

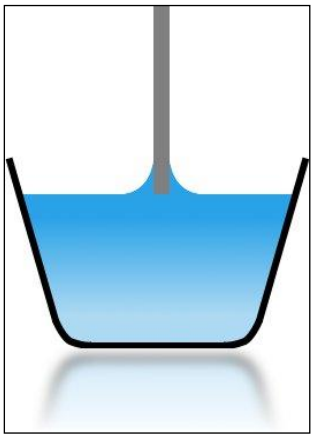


Figure 3 Wilhelmy plate method to measure the surface tension (Kibron Inc., 2012).

One possible method to measure the surface tension can be carried out with a Wilhelmy plate (Figure 3), where a thin plate with known perimeter is lowered to the surface of a liquid and the downward force to the plate is measured.

1.1 Critical Micelle Concentration

The critical micelle concentration is a parameter to determine the effectiveness of a (bio) surfactant. It is considered the concentration where surfactant molecules (monomers) are forming aggregates such as micelles (Figure 4). Meaning that above the CMC no further surface tension reduction can be achieved (Figure 5). This is due to a variety of weak chemical interactions between the non-polar and polar moieties of the molecules, which means, the CMC strongly depends on the structure of the surfactant molecules. (Maier, 2003; Soberon-Chavez and Maier, 2011).

The CMC is therefore commonly used to measure the efficiency of the biosurfactant, thus, the lower the CMC, the less biosurfactant is needed to reduce the surface tension. Remarkably low CMCs have been reported of biosurfactants e.g. <1 mM to 10 mM for rhamnolipid mixtures, depending on the ionic strength of the solution (Lebron-Paler, 2008). With increasing pH, CMC is increasing, due to deprotonation of the rhamnolipid.

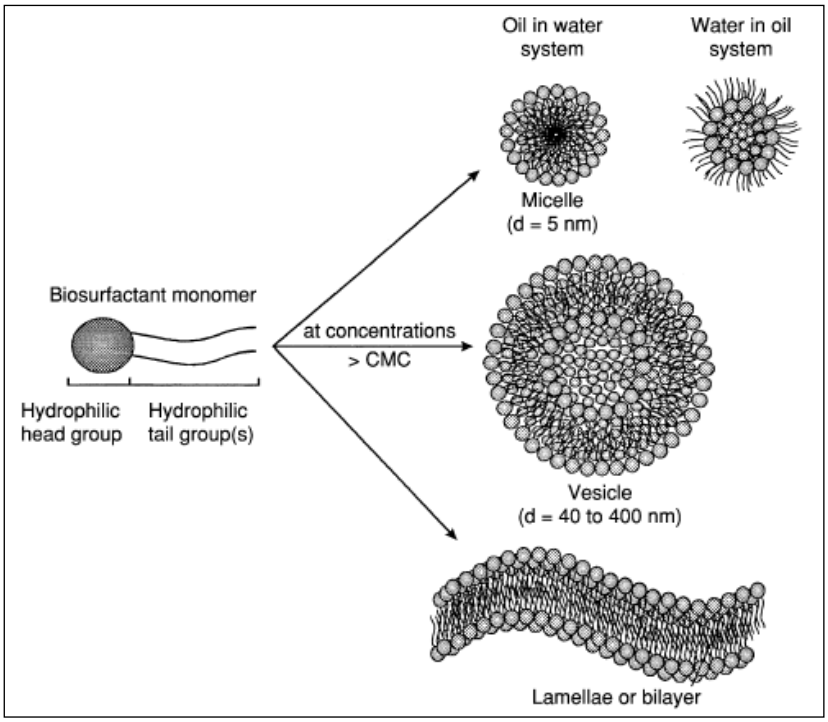


Figure 4 Different types of aggregates formed by biosurfactants (Maier, 2003).

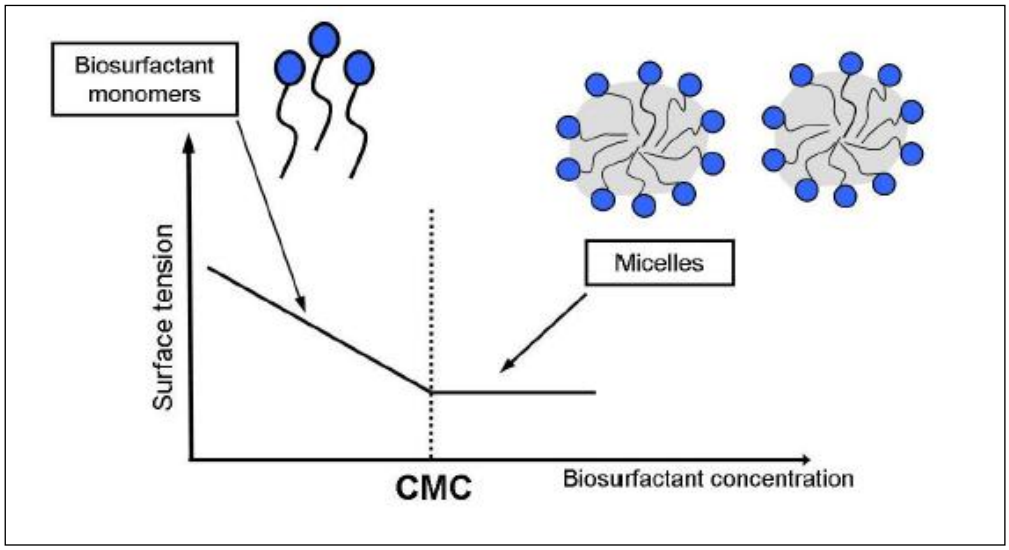


Figure 5 Relationship between surface tension and concentration of the biosurfactant (Pacwa-Płociniczak et al., 2011).

2. Biosurfactants

Biosurfactants can be produced by plants, animals and microorganisms; the highest research interest is within the microbial production of biosurfactants. The produced surfactants play different roles in the organisms' life cycle (Ron and Rosenberg, 2001) and show important biological activities e.g. antibiotic, antifungal, insecticidal, antiviral, antitumor activities etc., hence biosurfactants have a wide interest in special applications and industries (See point 6 for more details).

Biosurfactants can enhance hydrocarbon bioremediation due to their surface tension lowering properties. Thus, they increase the microbial availability of water insoluble compounds while the surface area of those compounds is increased leading to a higher mixing meaning greater mobilization, solubilization or emulsification (Bai et al., 1997; Nguyen et al., 2008). Different roles of biosurfactants referring to the cell hydrophobicity of microorganisms while contacting hydrocarbons, are proposed by Franzetti et al. (2010).

The two best studied biosurfactants are rhamnolipids produced by *Pseudomonas aeruginosa* and surfactin, synthesized by *Bacillus subtilis*. They can for example, reduce the surface tension from 73 mN/m to 25 mN/m. Rhamnolipids and surfactins

were first mentioned in literature in 1949 (Jarvis and Johnson, 1949) and 1968 (Arima et al., 1968), respectively.

2.1 Classification and properties

Biosurfactants are usually classified according to their structural properties and the producing organism within the main groups of glycolipids, lipoproteins (or lipopeptides), phospholipids and polymeric biosurfactants. They are produced in considerable mixtures rather than in pure state for example as described for a mixture of mono- and dirhamnolipids from a clinical *P. aeruginosa* strain (Rendell et al., 1990).

Soberon-Chavez and Maier (2011) (adopted by Pacwa-Płociniczak et al., 2011) gave an overview of the main groups of biosurfactants, its applications, producing organisms and structure (Table 1).

Table 1 Overview of the groups and types of biosurfactants, its producing microorganisms and possible applications (modified from Pacwa-Płociniczak et al., 2011 and Soberon-Chavez and Maier, 2011)

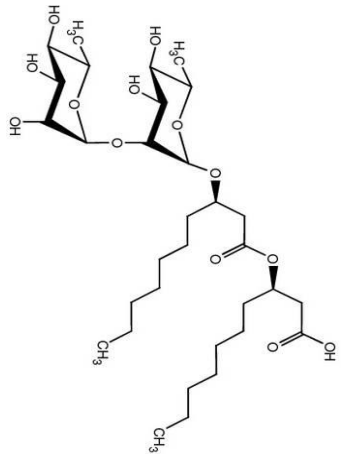
Biosurfactant		Microorganism	Applications in Environmental Biotechnology	References	Structural Examples
Group	**Class**				
Glycolipid	Rhamnolipids	*Pseudomonas aeruginosa*, *Pseudomonas* sp.	Enhancement of the degradat-ion and dispersion of different classes of hydro-carbons; emulsification of hydro-carbons and vegetable oils; removal of metals from soil	Herman et al., 1995; Maier and Soberón-Chávez, 2000; Whang et al., 2008	
	Trehalolipids	*Mycobacterium tuberculosis*, *Rhodococcuserythropolis*, *Arthrobacter* sp., *Nocardia* sp., *Corynebacterium* sp.	Enhancement of the bioavailability of hydrocarbons	Franzetti et al., 2010	
	Mannosylery-thritollipds	*Genus Pseudozyma (yeast)*, *Candida antarctica*, *Ustilagomaydis*		Kitamoto et al., 2002	
	Cellobiolipids	*Ustilagozeae, Ustilagomaydis*		Hewald et al., 2005	

Table 1 (Cont.)

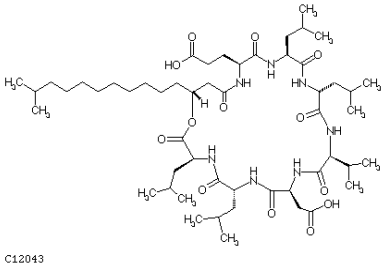
	Sophorolipids	*Torulopsisbombicola Torulopsispetrophilum,T.apicola, Candidabatistae, T. bombicola, C. lypolytica, C. bombicola, T.apicola, T.petrophilum, C. bogoriensis*	Recovery of hydrocarbons from dregs and muds; removal of heavy metals from sediments; enhancement of oil recovery	Pesce, 2002; Whang et al., 2008; Nguyen et al., 2010	
Lipopeptides/ Lipoproteins	Serrawettin	*Serratiamarcescens*		Matsuyama T, 1992	
	Viscosin	*Pseudomonas fluorescens, Pseudomonas. libanensis*		Laycock et al., 1991	
	Surfactin	*Bacillus subtilis; Bacillus pumilus* A	Enhancement of the biodegradation of hydrocarbons and chlorinated pesticides; removal of heavy metals from a contaminated soil, sediment and water; increasing the effectiveness of phytoextraction	Arima et al., 1968; Jenneman et al., 1983; Seydlová and Svobodová, 2008	 C12043
	Lichenysin	*Bacillus licheniformis*	Enhancement of oil recovery	C. P. Thomas, 1993	

Table 1 (Cont.)

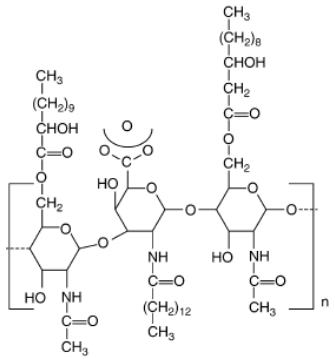
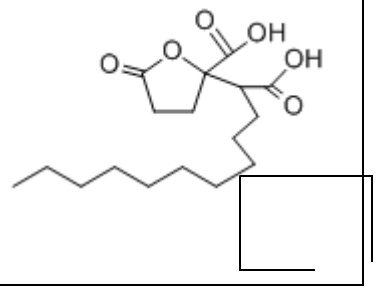
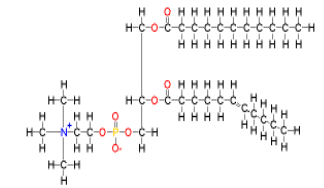
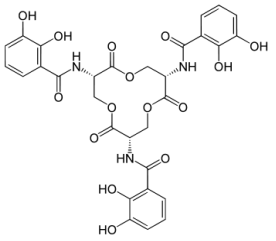
Polymeric biosurfactants	Emulsan	*Acinetobactercalcoaceticus* RAG-1	Stabilization of hydrocarbon-in-water emulsions	Rosenberg and Ron,1999	
	Alasan	*Acinetobacterradioresistens* KA-53		Toren et al., 2001	
	Biodispersan	*Acinetobactercalcoaceticus* A2	Dispersion of limestone in water	Rosenberg et al.,1988; Rosenberg and Ron,1997	
	Liposan	*Candida lipolytica*	Stabilization of hydrocarbon-in-water emulsions	Cirigliano and Carman, 1985	
	Mannoprotein	*Saccharomyces cerevisae, Candida. tropicalis*	Stabilization of hydro-carbon-in-water emulsions; Enhancement of bitumen recovery	Cameron et al., 1988; Rosenberg and Ron,1999	
Fatty acids, phospholipids and neutral lipids	Corynomycolic acid	*Corynebacteriumlepus*	Enhancement of bitumen recovery	Gerson and Zajic, 1978	
	Neutral lipid	*Nocardiaerythropolis*		Kretschmer et al., 1982	
	Spiculisporic acid	*Penicilliumspiculisporum*	Removal of metal ions from aqueous solution; dispersion action for hydrophilic pigments; pre-paration of new Emulsion-type organogels, superfine microcapsules (vesicles or liposomes), heavy metal sequestrants	Ishigami et al., 1983; Ishigami et al., 2000	

Table 1 (Cont.)

	Phospholipid	*Thiobacillusthiooxidans*		(Knickerbocker et al., 2000)	 Phospholipid
	Phosphatidylethanolamine	*Acinetobacter sp.*, *Rhodococcuserythropolis*	Increasing the tolerance of bacteria to heavy metals	Appana et al., 1995	
Siderophores	Flavolipids	*Flavobacterium*		Bodour et al., 2004	

2.1.1 **Glycolipids**

Glycolipids consist of one or more carbohydrate groups linked to long chain aliphatic acid or hydroxyl-aliphatic acid groups. Rhamnolipids (Jarvis and Johnson, 1949) are one of the best studied and most abundant (Desai and Banat, 1997) glycolipids, followed by trehalose lipids and sophorolipids. Typically sophorolipids are partially acetylated 2-O-β-D-glucopyranosyl-D-glucopyranose (sophorose) O-β-glycosidically linked to 17-L-hydroxy-D9-octadecenoic acid (Figure 6). The acyl carboxyl group forms a lactone to the terminal glucosyl residue. Novel sophorolipid characterizations showeda ω-hydroxy-linked acyl group (which is normally α-18-hydroxy-D9-octadecenoate), and occurs predominantly in an anionic form (Price et al., 2012).

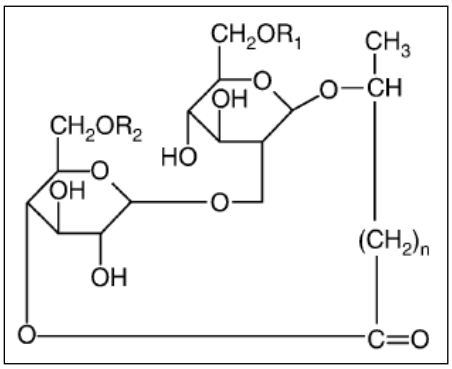


Figure 6 Lactonicsophorolipid (Smyth et al., 2010a).

2.1.2 **Lipoproteins**

The characteristic of this group of surface active agents, which are also called lipopeptides, is a hydrophilic protein moiety attached to fatty acids. The protein moiety are usually between 7 and 10 amino acids long and arranged in a cyclic structure. The most popular representative for this group is the well-studied lipopeptide surfactin, commonly produced by *Bacillus* species (Figure 7).

Surfactin consists of a 7 amino acid cyclic head group connected to a fatty acid consisting of C13 to C16 chain, with the main component of 3-hydroxy-13-methyltetradecanoic (Arima et al., 1968).

Nevertheless, detailed pattern of amino acids and fatty acids depends on substrate and culture conditions (Seydlová et al., 2011).

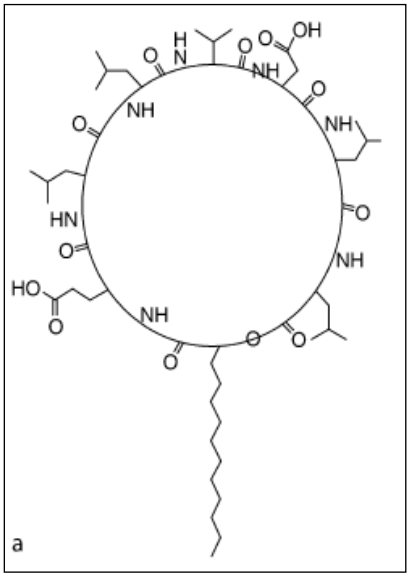


Figure 7 Structure of the cyclic lipopeptide surfactin from *Bacillus* sp. (Smyth et al., 2010b).

Others are for example the iturin and fengycin families which vary in number of amino acids and fatty acid chain length. The two families of lipopeptides were found to have effective antifungal functions (Romero et al., 2007; Liu et al., 2011). Fengycin was described as a lipopeptide composed of I D-Ala (D-Val), 1L-Ile, 1 L-Pro, 1 D-allo-Thr, 3 L-Glx, 1 D-Tyr, 1 L-Tyr, I D-Orn and fatty acid chain length up to C19 (Vanittanakom et al., 1986). Plipastatin another group of lipopeptides produced by *Bacillus* species, was firstly described by Umzawa et al. (1986) exclusively with the function as a phospholipase inhibitor, and structurally characterized. The proposed structure of fatty acids obtained by NMR is as followed: 3(R)-hydroxyhexadecanoic acid and 14(S)-methyl-3(R)-hydroxyhexadecanoic acid, which is connected to a chain of amino acids (obtained by secondary ion mass spectrometry and additional physico-chemical) as follows: B-Hydroxy fatty acid ->L-Glu->D-Orn-> L-Tyr-> D-alto-Thr-->L-Glu-D-Ala(Val)->L-Pro-*L-Gln->D-Tyr- L-Ile • OH (Nishikiori et al., 1986b). Furthermore the The carboxylic group of the L-isoleucine of plipastatinic acid has been revealed to form a lactone linkage with the hydroxyl group of L-tyrosine (Nishikiori et al., 1986a)

In recent researches plipastatin and fengycin biosurfactants has been reported to be structural very similar (Volpon et al., 2000) and later proven to be identical (Soberon-Chavez, 2011; Honma et al., 2012).

Another group of lipoproteins, produced by *Pseudomonas* strains, consists of cyclic lactone peptide chains, belonging to the e.g. viscosin, aphisin, tolaasin or syringomycin groups (Raaijmakers et al., 2006). Two so called pseudofactins have been found to be produced by *Pseudomonas fluorescens* BD5, they consist of a cyclicpeptide head group of 8 amino acids attached to a palmitic acid chain (Janek et al., 2010). A short amino acid chain head group, consisting of 4 amino acids, connected to an octadecanoic methyl ester was reported for *Brevibacteriumaureum* MSA13(Kiran et al., 2010). This group is well known for their antibiotic activity e.g. gramicidin S, which is produced by *Bacillus brevis* (Marahiel et al., 1979).

2.1.3 **Polymeric biosurfactants**

This group of biosurfactants is also called high molecular weight biosurfactants and often contains several of the earlier discussed structural properties (Rosenberg and Ron, 1997) *Acinetobacter* sp., archea as well as fungi are known to produce polymeric biosurfactants (Figure 8) (Rosenberg et al., 1979; Acevedo and McClnerney, 1996; Franzetti et al., 2012).

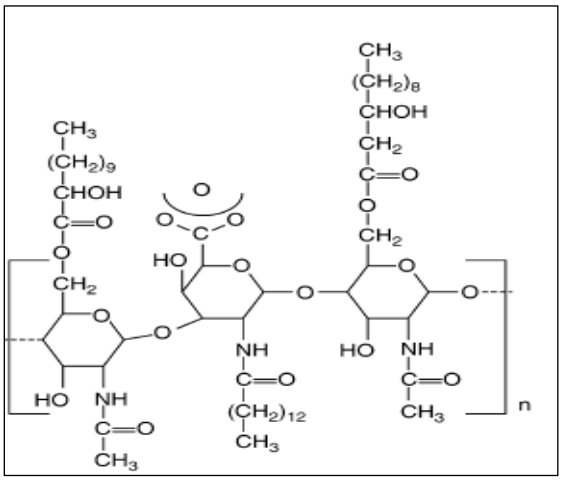


Figure 8 Structure of a biopolymer emulsan from *Acinetobacter* RAG-1 (Smyth et al., 2010b)

3. Biosynthetic pathways and molecular genetics of biosurfactant production

The definition of surfactant biosynthetic pathways and their genes is complicated. Nevertheless, some work to answer those fundamental questions is done and still in progress (Rahim et al., 2001; Satpute et al., 2010b; Sekhon et al., 2011). However, the mystery why biosurfactants are produced by microorganisms is still unknown, but several hypotheses exist, those mainly arguing the survival on hydrophobic substances and desorption from hydrophobic molecules and with it a higher bioavailability of the compounds (Syldatk and Wagner, 1987; Margesin and Schinner, 2001; Oliveira et al., 2003; Gunther et al., 2005).

Repression (as well as induction) of the production of biosurfactants are conditional on the presence of various nutrients and growth factors such as carbon, nitrogen, phosphate, trace elements and multivalent cations (Kitamoto et al., 2002) and environmental factors (Bonilla et al., 2005; Maneerat et al., 2006).

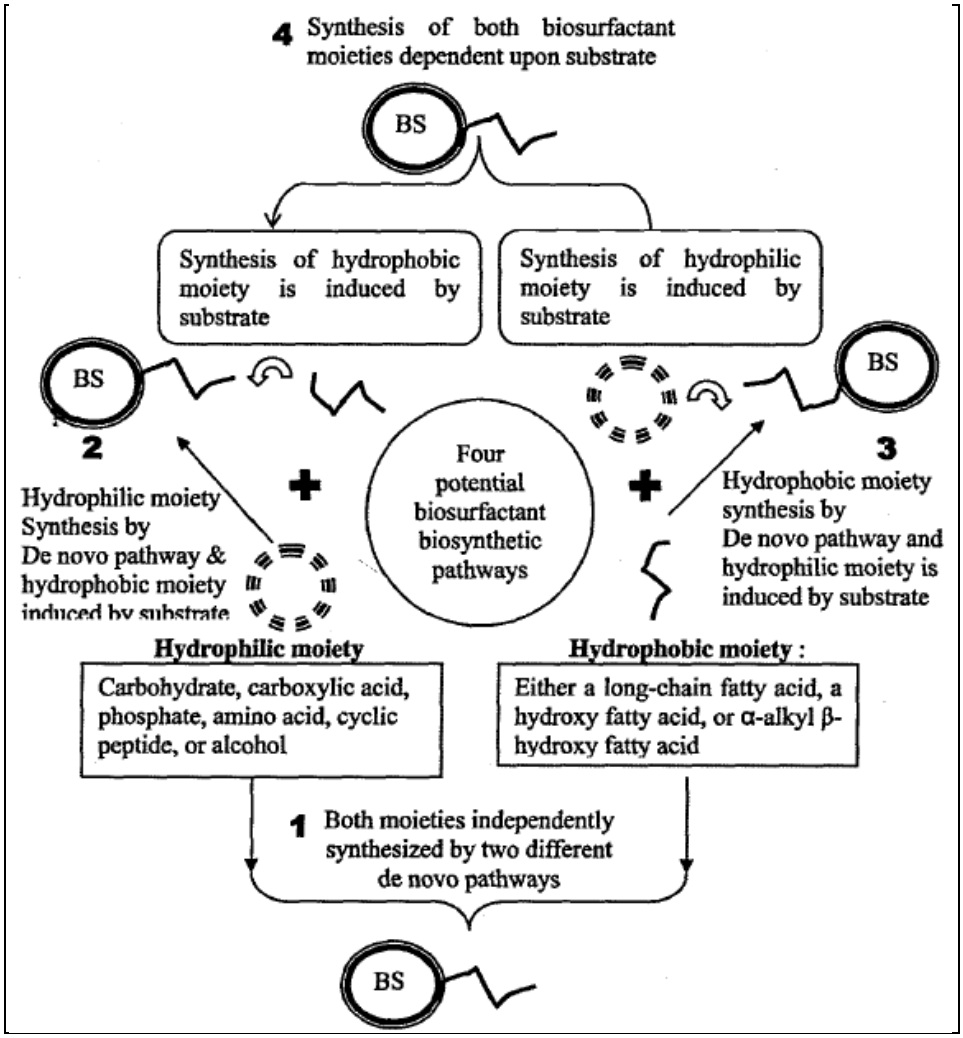


Figure 9 Potential biosynthetic pathways of Biosurfactant production in microorganisms. (Satpute et al., 2010b) based on (Syldatk and Wagner, 1987).

In general, biosurfactant producing genes are located on chromosomal DNA (Stover et al., 2000). Intercellular communication of biosurfactant producing information by quorum sensing is reported and depends on the production of signal molecules (Bosgelmez-Tinaz, 2003)

The genetic regulations of the production of emulsan, rhamnolipids and surfactin by *Acinetobacter calcoaceticus* RAG-1, *Pseudomonas* sp., and *Bacillus* sp. respectively, have been proposed in more detail. Polymer biosynthesis by *A. calcoaceticus* RAG-1 is regulated by a gene cluster of 27 kbp with 20 open reading frames, so called wee regulon (Whitfield and Roberts, 1999; Whitfield and Paiment, 2003; Nakar and

Gutnick, 2001, 2003). First biosynthetic pathways for rhamnolipid production in *Pseudomonas* spp. has been proposed by Hauser, Karnovsky (1957). Later regulatory mechanisms were reported by Ochsner et al. (1994) mainly through quorum sensing systems. The regulation of mono- and dirhamnolipids is regimented by different transferases and its synthesis is coupled with in cell nitrogen limitations (Mulligan and Gibbs, 1989) while phosphate limitations have been found enhancing (Bazire et al., 2005). A better overview of the genetic regulation of rhamnolipids in *Pseudomonas* strains has been summarized by (Satpute et al., 2010a) in Figure 10.

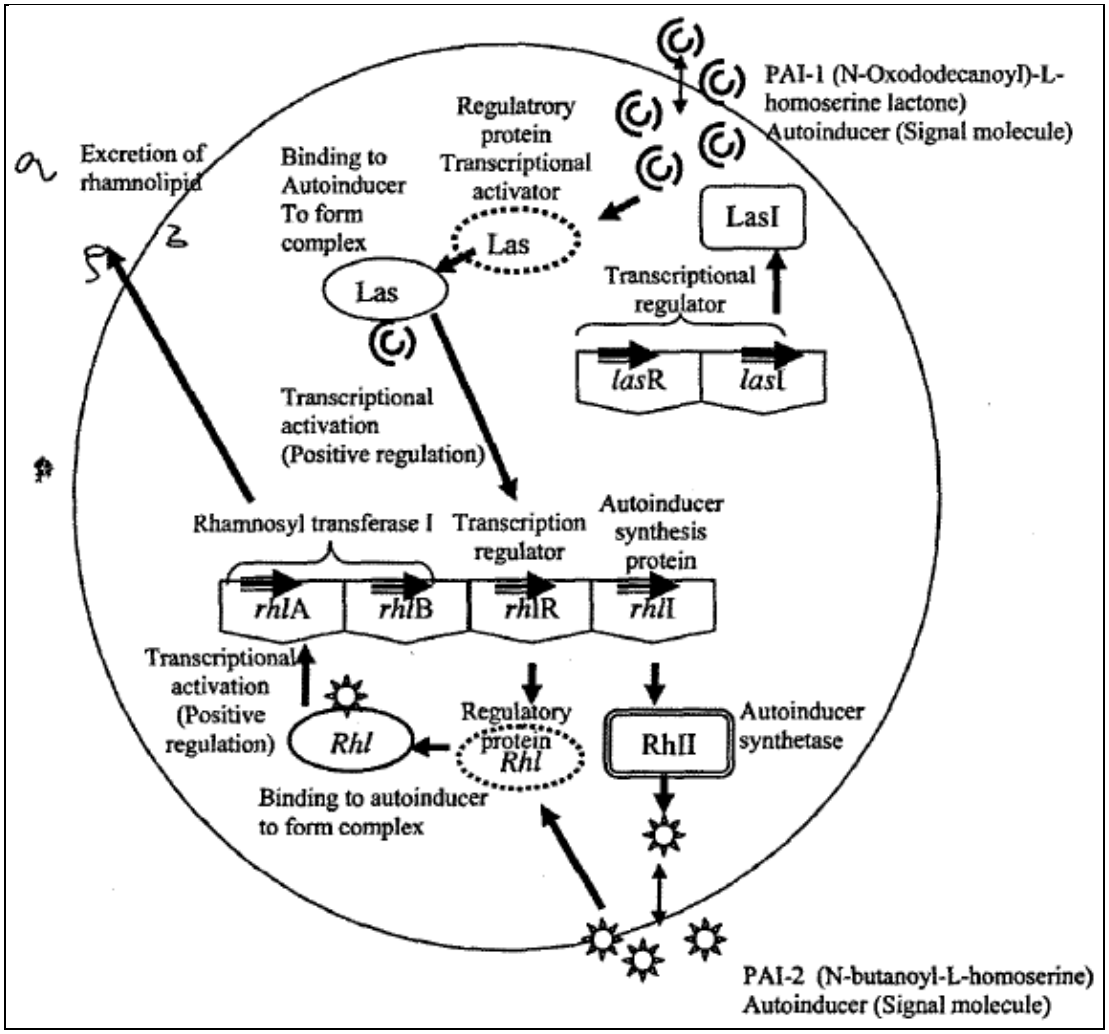


Figure 10 Rhamnolipid synthesis in *Pseudomonas* spp. By two quorum sensing systems: QSS is present at different regions of the chromosome. Thick black bold arrows: genes on chromosome of Pseudomonas; black arrows: protein synthesis from gene; dotted oval indicates inactive regulatory protein; continuous oval: active complex of regulatory protein and autoinducer (Satpute et al., 2010a).

Finally, the genetic regulation of biosurfactants produced by *Bacillus* spp. is discussed. A first non-ribosomal mechanism of surfactin synthetase was proposed by Kluge et al. (1988). The *srfA* operon is under QSS regulation, which involves nonribosomal peptide synthetases with 4 reported open reading frames (D'Souza et al., 1994; Fabret et al., 1995). More parts, e.g. *srfB*, which are necessary for surfactin production, are known, but the release of surfactin however, is still unknown. Stein (2005) proposed the release by passive diffusion across the cytoplasm membrane (Stein, 2005).

Molecular approaches have been developed to screen biosurfactant producers as shown by Tapi et al. (2010). In the mentioned study, a PCR screening method has been developed by using degenerated primers based on the intra operon alignment of adenylation and thiolation nucleic acid domains of all enzymes implicated in the biosynthesis of three lipopeptide families (surfactins, fengycins, iturins) (Tapi et al., 2010).

4. Substrates for biosurfactant production

It is essential for the structure and type of the produced surfactant, which carbon source is used as described in an early study by Robert et al. (1989). where the fatty acid moieties ranged, using different carbon sources such as dodecane, succinate, glucose or mannitol.

Various substrates such as oils, sugars, wastes and alkanes have been studied and found to be suitable for biosurfactant production (Lin, 1996). Main substrates for biosurfactant production are hydrocarbon substrates (Syldatk and Wagner, 1987), but are also produced with soluble carbohydrates as substrates e.g. glucose (Mulligan, 2005; Janek et al., 2010). In the following, the focus lays on the substrates used in this study.

4.1 Molasses

Molasses is a co-product of sugar industry generated during sugar manufacturing from either sugar cane or sugar beet and is a rich carbon and nutrient source ((Makkar et al., 2011). Average values for the constituents of cane molasses (75% dry matter), stated by Saharan et al. (2011) are: 48-56% total sugar, 9-12% organic matter excluding sugar, 2.5% protein, 1.5-5.0% Potassium, 0.4-0.8% Calcium, 0.06% Magnesium, 0.06-2.0% Phosphorus, 1.0-3.0 mg/kg biotin, 15-55 mg/kg pantothenic acid, 2,500-6,000 mg/kg inositol and 1.8 mg/kg thiamine.

A number of studies show effective biosurfactant production using molasses as carbon source, which is a priceless, renewable and valuable resource. (Patel and Desai, 1997; Fusconi et al., 2010; Saimmai et al., 2011). Molasses is used to produce stable biosurfactants by different *Bacillus* strains and was found to be effective in removing crude oil from packed column washing tests (Joshi et al., 2008). A polysaccharide biosurfactant produced by a *Gordonia* strain with molasses as substrate was described as a potential agent to wash contaminated soils (Fusconi et al., 2010). Further studies showed growth and production of lipopetide biosurfactants of *Bacillus subtilis* using molasses as carbon source (Makkar et al., 2011). Patel and Desai (1997) used molasses and corn-steep liquor as the primary carbon and nitrogen source to produce rhamnolipid biosurfactant using *P. aeruginosa* GS3. The biosurfactant production reached a maximum when a combination of 7% (v/v) molasses and 0.5% (v/v) corn-steep liquor waste was used.

4.2 Glycerol and waste/bottom glycerol

Glycerol is the principal byproduct obtained during transesterification of vegetable oils and animal fats (da Silva et al., 2009) and as a component of lipids abundant in nature. It can serve as a substitute for common carbohydrates, such as sucrose, glucose and starch (Bognolo, 1999). Rapid increase in biodiesel production has led to increased accumulation of glycerol as a by-product of this industry. The low cost glycerol could be used as water soluble substrate for biosurfactant production.

At the time being, only a few studies on glycerol and waste glycerol as carbon source for biosurfactant production exists.

(Nitschke et al., 2005) reported the utilization of glycerol as sole carbon source by *Pseudomonas aeruginosa* for synthesis of rhamnolipid. Although the yields were less compared to traditional hydrophobic substrates, the low cost of the substrates (such as waste glycerol) is a clear advantage. In another study 15.4 g/l rhamnolipid was produced by *P. aeruginosa* growing on a basal mineral medium, containing glycerol as the sole carbon source (Zhang et al., 2005). These studies show the feasibility of utilizing glycerol as carbon source for biosurfactant production by microorganisms.

In conclusion, the fact that the carbon source influences the production of the biosurfactant mixtures may result in a selection of effective, priceless and suitable choice of carbon sources which is suitable for different applications (Maier, 2003).

5. Characterization of biosurfactants

Microbial produced biosurfactants have an immense variety of structures, which makes it a difficult task to get detailed information about an exact chemical and physical structure. Thus, only a few biosurfactant structures have been characterized. The process of extraction, purification and analysis is tedious and difficult and often complicated chemical problems may exceed the capacity of a common microbiology laboratory.

Nevertheless, some of the important biosurfactants were successfully characterized such as surfactin, rhamnolipids and emulsan (as already shown above).

Several methods which could point to a structural identification and characterization have been published (Satpute et al., 2010b; Smyth et al., 2010a; Smyth et al., 2010b), ranging from simple colorimetric tests such as anthrone, orcinol, phospho-vanillin tests to very sophisticated mass spectrometry (e.g. MALDI TOF, MS/MS) and NMR with previous purification steps by various chromatography methods (Smyth et al., 2010a; Smyth et al., 2010b). Characterization can be achieved by either analyzing the

intact molecule or break down of the structure into carbohydrates and fatty acid components. To analyze the fatty acids, gas chromatography combined with mass spectroscopy (GC-MS) is a common method (Franzetti et al., 2010). After hydrolysis of the glycolipid mixture, conversion of the lipid portion to fatty acid methyl esters enables the use of GC or GC-MS to determine the structure.

Recent proceedings in characterizations of biosurfactants have been made with trehalose- and sophorolipids (Ratsep and Shah, 2009; Tuleva et al., 2009) as well as flavolipids (Bodour et al., 2004).

Aparna et al. proposed a structure of glycolipid type by Fourier transform infrared (FT-IR) spectroscopy and mass spectroscopy, which showed the presence of carboxyl, hydroxyl and methoxyl functional groups and a detection of dirhamnolipid in abundance with the predominant congener monorhamnolipid (Aparna et al., 2012).

Characterization of two biosurfactants obtained by an arctic freshwater bacterium *Pseudomonas fluorescens* BD5 lead to the structures of pseudofactin I and II. Therefore matrix assisted laser desorption/ionization time of flight (MALDI TOF) mass spectrometry and tandem mass spectrometry (MS/MS) were used. Both compounds are novel cyclic lipopeptides with a palmitic acid connected to the terminal amino group of eighth amino acid in peptide moiety. Pseudofactin II is reported to reduce the surface tension of water from 72 mN/m to 31.5 mN/m at a concentration of 72 mg/l. Its emulsification activity and stability was found to be greater than that of the synthetic surfactants Tween 20® and Triton X-100 (Janek et al., 2010).

A new lipopeptide biosurfactant produced by *B. aureum* MSA13 was characterized by mass spectroscopy. The hydrophobic moiety consisted of an octadecanoic acid methyl ester and a peptide part predicted as a short sequence of four amino acids including pro-leu-gly-gly (Kiran et al., 2010).

6. Applications of biosurfactants

All the mentioned properties and facts draw the basis for a growing interest in special applications of biosurfactants. The advantages and applications are listed in many review papers (Maier, 2003; Pacwa-Płociniczak et al., 2011) with numerous special applications such as cancer treatment (Saini et al., 2008), wound healing (Piljac et al., 2008), pest control in agriculture (Stanghellini and Miller, 1997) and detergent formulation (Bafghi and Fazaelipoor, 2012).

In this part, a focus on environmental applications is described. Examples are petroleum recovery, also called microbial enhanced oil recovery (Donaldson et al., 1989). A study by Klosowska-Chomiczewska et al. (2011), where biosurfactants were tested in an oil-refinery process, showed outstanding results with efficiencies up to 99%. An extensive focus on biosurfactants applications lies in bioremediation of organics (Bai et al., 1997; Mulligan et al., 2001; Ron and Rosenberg, 2002; Nazina et al., 2003; Mulligan, 2005, 2009; Franzetti et al., 2010; Franzetti et al., 2012).

Trehalolipids, especially, have been proposed for bioremediation purposes and microbial enhanced oil recovery (Franzetti et al., 2010). Applications of biosurfactants in the remediation field are therefore aimed at enhancing solubility of organic compounds, either for a soil washing treatment, or to stimulate *in situ* biodegradation. In particular, the application of trehalose lipids generally showed good results in solubilisation and biodegradation experiments with different hydrophobic organic compounds (Franzetti et al., 2010). A rhamnolipid showed high potential for bioremediation of water insoluble compounds such as octadecane, where high dispersion of octadecane with low rhamnolipid concentrations was found (Zhang and Miller-Maier, 1992).

A commercial product (Ekoil) was developed by using *Mycobacterium flavescens* strain EX-91 and tested for the decontamination of an oil-polluted water body, and also proved to be effective in the treatment of the engine oil-contaminated wastewater of a nuclear power station (Ermolenko et al., 1997)

In the following a more detailed focus will be laid on soil washing applications.

7. Soil washing

To release low soluble compounds from soil by aqueous solutions is commonly called soil washing. Utilization of biosurfactants in soil washing technologies is defined by their physic-chemical properties, instead of their effect on metabolic activities or cell-surface properties. (Pacwa-Płociniczak et al., 2011). Soil washing has gained some interest in the literature for treating heavy metal and hydrocarbon contaminated soil (Mulligan et al., 2001; Mulligan, 2009; Gusiatin and Klimiuk, 2012).

The procedure of soil washing is cost and time effective with a potential to treat and recover high amounts of contaminants. The soil washing process has been used in the United States of America to remediate Superfund sites (U.S. EPA, Technology Innovation Office 21/06/1996). Formation of harmful by-products that may be generated during remediation processes is prevented and the application of biosurfactants will further eliminate the fear of generating waste streams, as their release is considered environmentally friendly due to their inherent biodegradability and low toxicity (Urum and Pekdemir, 2004).

A study of removing arsenic in soil using a rhamnolipid biosurfactant (JBR425) showed an effective removal of arsenic in the soil (Wang and Mulligan, 2009).

In a recent study, a rhamnolipid biosurfactant obtained by cultivation with waste frying rice bran oil, showed an effective (up to 74%) washing of copper out of contaminated soil (Venkatesh and Vederaman, 2012).

Torres et al. (2012) reported natural surfactants; mainly polysaccharides (not characterized gums), to be effective washing methyl parathion from soil.

Washing crude oil and hydrocarbons from soils has been the focus of several studies. Rhamnolipids have been found to remove crude oil effectively from soil using soil washing (Urum and Pekdemir, 2004; Urum et al., 2006). Lai et al. (2009) reported a superior performance on total petroleum hydrocarbons of biosurfactants including surfactin and rhamnolipids. A biosurfactant of *Rhodococcus ruber* showed 1.4-2.3 greater removal of crude oil than Tween 60® in a soil washing process (Kuyukina et al., 2005).

A recently published laboratory batch study showed that bioemulsifiers are able to remove hydrocarbons from contaminated soils. The effectiveness of the removal depends strongly on the soil type (Franzetti et al., 2012).

8. Behavior in the environment and toxicity

From the observation that biosurfactants were produced as a response to the presence of hydrophobic substances, suggestions for the possibility for their use in petroleum waste treatment arouse. There are only a few publication devoted to the toxicity and biodegradability of biosurfactants (Klosowska-Chomiczewska et al., 2011). However, the interest remains strongly because biosurfactants are considered as environmentally friendly and compatible. Nevertheless, the always assumed low toxicity has to be ensured before releasing them into the environment (Franzetti et al., 2006).

Glycolipids from a *Rhodococcus* species were tested in toxicity and found to be 50% less toxic in naphthalene solubilization tests than the chemical surfactant Tween 80® (Kanga et al., 1997). Polysacharide emulsifier produced by a fungus was found to be non-toxic in all tests and had a high affinity to soils with high organic matter content (Franzetti et al., 2012). Nevertheless K_d values from 1.3 to 7.3 L/kg indicate a high sorption affinity of the bioemulsifier to the soil particles (Franzetti et al., 2012).

Sophorolipids have been studied in terms of cytotoxicity and biodegradability compared to lipoproteins and chemical block-copolymer nonionic surfactants, and was found to be similar to the tested lipopeptides but higher than the chemical surfactants in terms of cytotoxicity. Biodegradability was tested according to OECD guidelines and can be stated as readily biodegradable chemicals (Hirata et al., 2009).

Biosurfactants as a detergent formulation has been tested for biodegradability, using it as sole carbon source and were found to be easily degradable by soil microorganisms (Bafghi and Fazaelipoor, 2012).

A closer look at the data of toxicity studies shows biosurfactants to have a lower haemolytic activity to human erythrocyte compared to chemically synthesized

cationic surfactants (Klosowska-Chomiczewska et al., 2011). So far no detrimental effects to heart, lung, liver and kidneys have been reported (Das et al., 2008). Luminescence inhibition of *Vibrius fisheri* was found to be 50% in comparable or higher concentration of synthetic surfactants (Ivshina et al., 1998). Moreover, their acute and chronic toxicity is much lower than that of synthetic Triton X-100 (Edwards et al., 2003).

Biodegradability tests in liquid medium and in soil microcosms, performed for five biosurfactants and synthetic SDS, showed that the efficiency of their degradation depends on used bacteria. However, the biodegradability of all biosurfactants in soil did not differ significantly and ranged from 42.5% up to 73.4%, while biodegradability of synthetic SDS during 7 days of incubation was much lower (24.8%) (Lima et al., 2011).

9. *Bacillus* sp. strain GY19

Bacillus sp. GY19 was previously isolated from planted soil by using bottom glycerol in a basal medium as a carbon source. It forms white round, convex colonies and was found to reduce surface tension up to 29 mN/m and gave an emulsification index with soybean oil of 27% (Lab own studies, yet to be published). In a previous study the obtained biosurfactant from *Bacillus* sp. strain GY19 using bottom glycerol as carbon source exhibited high solubilization of PAHs and crude oil (Tulalamba, 2012).

CHAPTER III

METHODOLOGY

1. Materials

1.1 Chemicals

Bottom glycerol which is a by-product of biodiesel production and crude oil were obtained from Petroleum Authority of Thailand (PTT).

Molasses was used as commercially available.

If not noted differently all chemicals were purchased from Merck Ltd. Darmstadt, Germany.

1.2 Culture media and solutions

All media were prepared freshly and autoclaved before usage at 121˚C and 2 bar for 15 minutes. Detailed recipes are listed in the appendix A.

2. Methods

A) Environmental Applications of biosurfactants produced by *Bacillus* sp. GY19 using renewable substrates

2.1 Biosurfactant production

2.1.1 Inoculum preparation

Bacterial colony on LB agar plates was picked and transferred to 100 ml of 25% LB in 250-ml flask and shaken at room temperature, 200 rpm for 1 day. Then, cell suspensions were centrifuged at 8,000 rpm, 4°C for 20 minutes. Cell pellets were washed with 0.85% NaCl solution twice and re-suspended in 0.85% NaCl solution until an OD_{600} equal to 1 was reached.

2.1.2 Production step

Three percent (%v/v) of inoculum was transferred to an Erlenmeyer flasks containing productive medium and shaken at room temperature, 200 rpm for 5 days. The

concentration of substrate was varied from 3%, 7% and 10% (w/v). After the cultivation, the medium was centrifuged at 8,000 rpm, 4°C for 20 minutes and the supernatant (cell-free broth) was taken for further investigation such as surface tension measurements and crude biosurfactant extraction.

2.1.3 Surface tension measurement

The surface tension of the supernatant was measured by an automatic tensiometer using the Wilhelmy plate method (Adamson and Gast, 1993).

2.1.4 Foamate extraction

In order to reduce the volume of biosurfactant containing supernatant, foam fractionation was done to obtain a foamate. Briefly, air was ducted through the cell free supernatant with an air pump and air sparger (commercially available) in a 5 l reactor (built to order). The emerging foam was collected after passing a condenser column (length = 60 cm). Air flow was constant during procedure. The apparatus is shown in Figure 11.

Figure 11 Experimental set up for foamate extraction.

2.1.5 Extraction of crude biosurfactant

The obtained cell-free broth or foamate was adjusted to a pH of 2 using 6 N HCl and let it precipitate at 4°C overnight (Smyth et al., 2010 a&b). Solvent extraction in a shaking funnel was performed, using chloroform/methanol (2:1) at a ratio of solvent to broth equal to 1:1 for three times. The chloroform/methanol (lower) phase was collected and evaporated by a rotary evaporator under vacuum and 37˚C. Once the solvent was evaporated, methanol was added to re-dissolve the residue, which is from now on called the crude biosurfactant extract.

2.2 Soil sorption and soil washing

Soil washing and soil sorption experimental set ups were adopted from Franzetti et al. (2012). Therefore three types of soil taken from the area of Khao Yai National Park, Thailand, were dried and sieved to a size of ≤ 4 mm. The soils were sent to analyze for chemical properties such as organic carbon content and cation exchange capacity at Kasetsart University, Bangkok, Thailand.

2.2.1 Soil sorption

Erlenmeyer flasks containing 2 g of soil (three different types) were shaken (30 min, 200 rpm) with 20 ml of crude biosurfactant solution in different concentrations. After centrifugation, the residual biosurfactant in the aqueous phase was measured by Bradford method. A calibration was made using the crude extract itself.

2.2.2 Soil washing

2.2.2.a Spiking of soil with crude oil

Soil was spiked with Arabian crude oil (PTT Company). Briefly, 10% (w/w) crude oil in hexane was used to spike soil samples in glass bottles, to obtain a final concentration of 5% (w/w) in soil. The reason of solubilize crude oil in hexane first, is to ensure a homogenous distribution of the crude oil in the soil sample. Batches were left at 35˚C for 2 d, to allow the hexane to evaporate.

2.2.2.b Washing experiments

Soil washing experiments were conducted in small glass bottles containing 2 g of contaminated soil and 20 ml of the washing solution. Washing solutions were the supernatants of incubated broth with 3% pure glycerol and 3% molasses, its foamates, and aqueous solutions of crude biosurfactant extracts with 3% molasses and 3% pure glycerol as substrates, in concentrations of 0.5 g/l; 1 g/l; 1.5 g/l and 2 g/l. As comparison two synthetic surfactants (Tween 80® and SDS) were selected. De-ionized water was used as a control. After the washing step (30 min, 300 rpm) the soil water mixture was centrifuged (8000 rpm, 4°C, 10 min). The soil fraction was rinsed with water two times and dried. After drying the residual oil in the soil, soil was extracted three times with chloroform and measured by TLC-FID described elsewhere (Bharati et al., 1993; Orea et al., 2002).

2.3 Ecotoxicity

2.3.1 Seed germination and root elongation tests

For seed germination, the suggestions of a study by Wang and Keturi (1990), three types of seeds were selected: Lettuce, tomato and cucumber (purchased at local supplier). The tests were performed in petri dishes containing a filter paper (Whatman®, #1) on a placeholder. In this test, solutions of crude biosurfactant (using pure glycerol and molasses as substrate), SDS and Tween® 80 were used in with a concentration of 1 g/l, respectively. Concentration of 1 g/l was selected because it is the mean of the used concentrations for the soil washing experiments. Tap water was used as a control. All solutions were adjusted to a pH of 7. Ten seeds of each plant species were placed in one Petri dish.

Petri dishes were kept in air filled plastic bags, to limit the evaporation and were incubated for 5 days at room temperature in the dark. Germination (yes/no) was investigated every day and root length (mm) after the 5th day of incubation.

2.3.2 MIC and MBC

Minimal inhibition and minimal bactericidal concentrations were estimated using an assay carried out in 96 well microwell plates (Andrews, 2001). Tested compounds were solutions of the crude biosurfactants (using pure glycerol and molasses as

substrate), SDS and Tween® 80. Each well contained initially 50 µl of 0.9% NaCl solution. 150 µl of each testing solution with the initial concentration of 10 g/l were present in the first well of each row and subsequently 50 µl were pipetted to the next wells, respectively with a multichannel pipette to achieve a dilution row. In the following 50 µl of a Nutrient Broth solution, containing 2% of a mixed soil culture inoculum (preparation described further down) was pipetted to the submitted dilutions. The inoculum was prepared by adding 10 mg of wet soil (of three different types as described in 2.2) to 200 ml LB broth, which was incubated overnight at room temperature. 100 µl were transformed from these inoculums to a new batch of 100 ml LB broth to minimize the amount of soil in the inoculum. The second batch was used to adjust 20 ml of 0.9 NaCl solution to an $OD_{600} = 0.2$. Each test contains one negative control (only sterile NaCl solution and NB broth, no inoculum, no test solution) and one positive control (NaCl solution and inoculum, no test solution).

The microwell plate, prepared as described was incubated at RT for 24 h and analyzed by a microwell plate reader at 595 nm.

The concentration where no growth occurred was defined as minimal inhibition concentration (MIC). Based on MIC determination content of the wells MIC+2 and MIC-2 were streaked on LB agar to determine whether bacterial growth was only inhibited by the tested compound or whether it has bactericidal effects.

In case of no possible determination of a MIC because all wells showed growth, only the highest concentration of test solution was streaked out on LB agar, to confirm no bactericidal effects by the tested compound.

B) Chemical characterization of the biosurfactant and detection of biosurfactant producing genes in *Bacillus* sp. GY19

2.4 Detection of biosurfactant producing genes

To detect biosurfactant producing genes in the DNA of *Bacillus* GY19 was used to conduct PCR with primer pairs obtained from Tapi et al. (2010), followed by gel

electrophoresis. The purified PCR product was then cloned and sequenced to confirm the presence of biosurfactant producing genes.

2.4.1 **DNA extraction**

Bacterial DNA was extracted by the phenol-chloroform extraction method. *Bacillus* sp. GY19 was inoculated into LB broth, and incubated at RT on a rotary shaker with 200 rpm for 24 hr. 1.5 ml of the culture broth was transferred into an eppendorf tube and centrifuge at 7,000 rpm for 2 min. After the supernatant was discarded this step was repeated two times. Cells were resuspended in 510 µl Tris-EDTA buffer, pH 8 (TE buffer) and vortexed. 50 µl of lysozyme solution (60 mg/ml in TE buffer) were added and incubated at 37°C for 30 min. 30 µl of 10% sodium dodecyl sulphate (SDS) and 10 µl of proteinase K solution (10 mg/ml in sterilize distilled water) were added, mixed and incubated at 37°C for 30 min. In the following step 120 µl of 5 M NaCl were added and gently mixed by inverting. Then subsequently 200 µl of hexadecyl trimethyl ammonium bromide/sodium chloride solution (CTAB/NaCl solution), which was prewarmed at 65°C was added and mixed. After incubation at 65°C for 10 min, the equal volume of phenol:chloroform (25:25) was added and mixed. After centrifugation at 13,000 rpm for 5 min. the upper phase (aqueous phase) is collected and transferred into a new eppendorf tube. The previous step was repeated two times and finally 0.6 times (of the collected volume) of isopropanol was added. The DNA precipitate was collected by centrifugation at 13,000 rpm, for 5 min. 70% ethanol was added to wash the extracted DNA pellet. After a final centrifugation step at 13,000 rpm for 5 min, the pellet was dried at room temperature. In the last step the pellet was dissolved in 100 µl of TE buffer containing 0.2 µl of DNA free RNase solution (10 mg/ml in 0.01 M sodium acetate pH 5.2) to remove RNA (incubated at 37˚C for 60 min). DNA was stored at -20°C until used.

2.4.2 **PCR**

PCR mixtures were prepared with goTaq Master Mix (Promega) according to the protocol. The following Primer Pairs were used: Af2-F/Tf1-R; Ap1-F/Tp1-R; As1-F/Ts2-R; Am1-F/Tm1-R. Polymerase Chain Reaction was carried out in a thermocycler (G-Storm GS482) with conditions as described by Tapi et al. (2010).

2.4.3 **Cloning and sequencing**

2.4.3.a **Ligation**

The purified PCR products were ligated with a pGEM-T Easy Vector (Promega, USA). Ligation mixture was prepared as following:

2X ligation buffer	5 µl
pGEM-T Easy Vector (50 ng)	1 µl
PCR product (100 ng)	1 µl
T4 DNA Ligase (3 U)	1 µl
Deionized water	2 µl

The mixture was incubated overnight at 4 ºC and transferred to a competent cell.

2.4.3.b **Transfer into competent cell**

The competent cells (*E.coli* JM 109) were thawed on ice and 2 µl of the ligated recombinant plasmid was added (to 50 µl of competent cells broth), mixed and incubated on ice for 20 minutes. The cells were heat shocked at 42°C for 45-50 seconds and placed on ice immediately for 2 minutes. After the heat shock, 950 µl of SOC broth was added and incubated at 37°C for at least 1 hour. The solution was spread on LB agar plates (containing IPTG, X-Gal, Ampicillin) and incubated at 37 ºC for 16 – 24 hours. White colonies were picked and transferred into LB broth and incubated at 37 ºC overnight.

2.4.3.c **Plasmid extraction and digestion**

The plasmid was extracted by using QIAprep Spin Miniprep Kit (Qiagen, Germany), according to the manufacturer's instructions. The extracted plasmid was digested with *Eco*RI restriction enzyme to confirm the presence of the inserted fragment. The following digestion mixture was prepared and pipeted into an eppendorf tube and incubated at 37°C overnight:

Plasmid (pGEM-T Easy Vector)	1 µl
10x Buffer	1 µl
*Eco*RI enzyme (0.5 U)	0.5 µl
Sterile water	7.5 µl

After incubation the digested mixture was checked for the size of the inserted product by gel electrophoresis, using a 2% agarose gel at 100 V for 30 min.

If the mixture contained the fragment with the right size the plasmid was sent to Base Co., Ltd., Malaysia for sequencing. Sequence results were analyzed by blastx program to obtain the percentage of similarity to sequenced genes of the database.

2.5 Chemical characterization of crude biosurfactant

To obtain a rough characterization of the obtained crude extract, colorimetric tests were carried out to get information of the biosurfactant type. Lipid, protein and sugar contents were determined by sulpho-phospho-vanillin test (Saifer and Feldmann, 1971; Izard and Limberger, 2003), Bradford assay (Satpute et al., 2010b) and sulphoric acid test (Dubois et al., 1956), respectively.

2.5.1 Sulpho-phospho-vanillin test

This method was selected to estimate the lipid content of the biosurfactant (Saifer and Feldmann, 1971)}(Izard and Limberger, 2003). To a 100 µl sample in a test tube, 2 ml of conc. H_3SO_4 (18 M) were added and the mixture was incubated after mixing for 20 min in 90-100˚C in a water bath. After the samples were cooled down for 5 min at RT, 5 ml P-V reagent was added and incubated again for 15 min in a water bath of 37˚C. The Absorbance of samples was measured at a wavelength of 530 nm by a spectrophotometer after cooling down for 10 min against a blank reference with water. Glyceryl trioleate (Sigma-Aldrich, USA) was used as a standard for calibration.

2.5.2 Bradford assay

Bradford assay or Coomassi blue method was adopted from Bradford (1976) to assess the protein content in the biosurfactant samples (Smyth et al., 2010b). Briefly, 1 ml of sample was added to 5 ml of Bradford reagent. After a reaction time of 20 min. the mixture was taken to measure the absorbance at 595 nm with a spectrophotometer. BSA solutions were used as standard for calibration.

2.5.3 Sulphuric acid test

This test was set up to determine the total carbohydrate content of the biosurfactant samples (Dubois et al., 1956). To 162 µl of the sample, 27 µl of a 5% phenol solution were added and mixed, followed by the addition of 810 µl of concentrated sulfuric acid. After 30 min. the absorbance at 490 nm was measured. Glucose solutions were prepared for a calibration.

2.5.4 TLC autographic method for detection of amino acids

To confirm the presence of amino acids, TLC autography (Friedman, 2004) was applied. Briefly, BSF crude extract was hydrolyzed with 2 M NaOH at 80˚C for 3 hrs. After cooling down and resuspension in methanol, products were spotted on silica sheets (Merck, Darmstadt) and developed in a tank containing a solvent system of 4:1:1 butanol - acetic acid - water, and sprayed with a ninhydrin solution (1.5 g ninhydrin, 100 ml butanol, 3 ml acetic acid). For color development the TLC sheet (Silica gel 60 F254, Merck, Darmstadt, Germany) was kept at 60˚C for 10 h.

2.5.5 Analytics of fatty acid components in lipids

Lipid components can be analyzed by GC after a conversion of the fatty acids to their methyl esters (Christie, 1989; Liu, 1994). Therefore fatty acids have to be esterified first. The obtained nonpolar methyl esters can be analyzed by gas chromatography according their retention in the GC column.

2.5.5.a Preparation of FAME

The first step was hydrolysis or saponification of the sample to get free fatty acids. Approximately 150 mg of the crude BSF sample (obtained with 3% pure glycerol as substrate) were solved in 0.5 M NaOH in methanol and heated under reflux for 1.5 h at 60˚C. After hydrolysis, water was added and partitioned with dichloromethane. The aqueous layer, containing the saponifiable matter, was collected, acidified with 6M HCl and partitioned with dichloromethane. The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness, yielding free fatty acids. The actual esterification is explained in the following step. The obtained free fatty acids

were heated (60˚C) for 2 h, with an excess of anhydrous methanol containing sulfuric acid as a catalyst. Anhydrous methanol was prepared according the Lund & Bjerrum method (Armarego and Chai, 2012).

2.5.5.b Gas chromatography of FAME

The prepared FAME, dissolved in hexane, was injected into a GC-FID (Agilent Technologies 6890N), containing a capillary column Omegawax^{TM} 250 (Supelco Analytical, Bellafonte, USA). The sample and a standard FAME mix (SupelcoTM 37 Component FAME Mix, SUPELCO, Bellafonte, USA) were injected according the protocol received from the supplier (140˚C to 240˚C at 4˚C/min, 1 µl split injection).

2.5.6 Nuclear Magnetic Resonance Spectroscopy

^{1}H and ^{13}C NMR spectra were recorded ($CDCl_3$ and CD_3OD as solvent) at 400 MHz and 100 MHz, respectively, (Varian Mercury^{+} 400 NMR and Bruker (Avance)) Tetramethylsilane (TMS) was used as an internal standard. Chemical shifts are reported in ppm downfield from TMS. The required amount of sample is 2-5 mg and 5-10 mg dissolved in deuterated solvent for ^{1}H and ^{13}C spectra, respectively.

2.6 Statistical analysis

All statistical analyses were performed using MS Office Excel 2007. Two-way ANOVA significance with replication, one factor ANOVA and t-tests were run by the Data-Analysis add-in of the mentioned software using $\alpha = 0.05$. The performed test was selected according the number of factors and proposed hypothesis, respectively.

CHAPTER IV

RESEARCH RESULTS

A) Environmental Applications of biosurfactants produced by *Bacillus* sp. GY19 using renewable substrates

1. Biosurfactant production by different renewable substrates

1.1 Screening of renewable substrates

Reduction of surface tension was calculated in percent from the uninoculated media as control. All used substrates showed high surface tension reduction (up to 28.5 mN/m), nevertheless in comparison to the control (uncultivated broth with substrate) only molasses and clear glycerol decreased the surface tension significantly (up to 40.1% and 54.7% respectively) as shown in Figure 12.

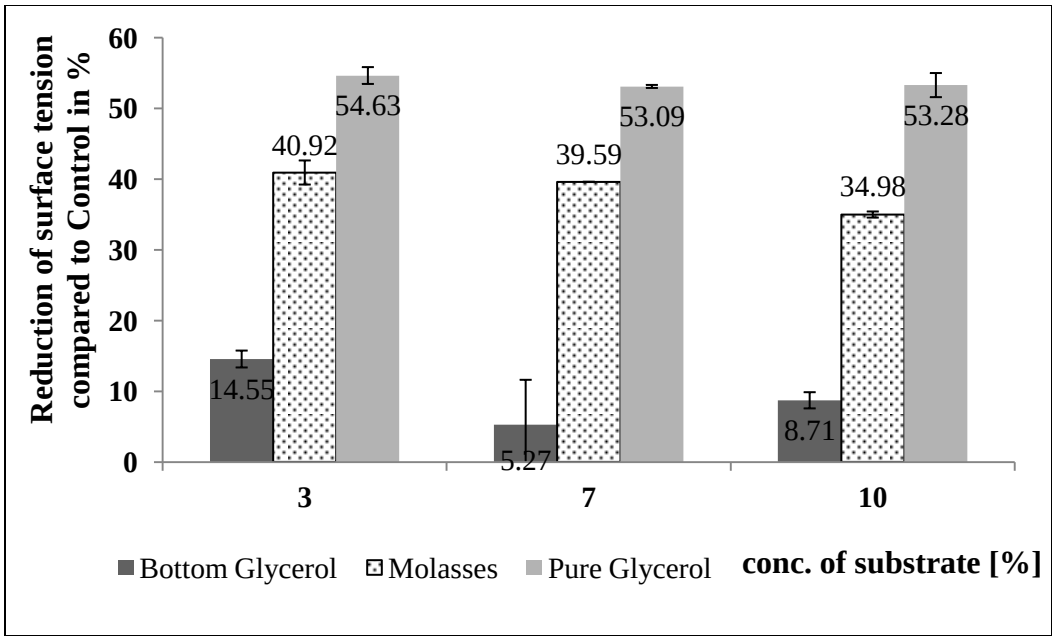


Figure 12 Surface tension reduction of supernatant after cultivating *Bacillus* sp. GY19 with three different substrates at different concentrations added to the production media (media with substrate = control)

Thus, batches cultivated with molasses and glycerol (3% & 7%), were selected for further extraction to obtain the crude biosurfactant yields, due to highest surface

tension reduction compared to control. Substrates with concentration of 10% were not considered for further extraction, because no better decreasing of the surface tension (of the supernatant), compared to lower substrate concentrations, could be observed. The crude extracts have a brown sticky appearance and smell sweet. Obtained yields ranged from 135 mg/l of supernatant (7% pure glycerol) to 742 mg/l (7% molasses) and are shown in Figure 13.

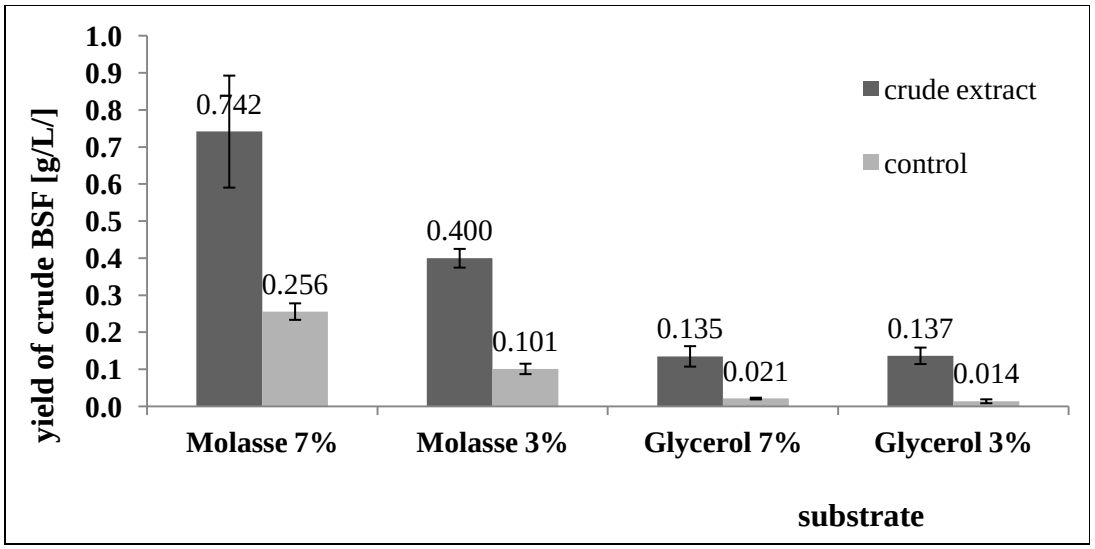


Figure 13 Yields after extraction of the crude biosurfactant from the supernatant produced by *Bacillus* sp. GY19 using different substrates and concentrations.

Considering the controls (extracts of uncultured broth with substrate) 3% molasses and 3% glycerol gave the highest yields, and were selected for further chemical characterization and soil washing experiments.

1.2 Critical Micelle Concentrations

CMC values were obtained by plotting surface tension against concentration of crude extracts in aqueous solutions, resulting in values of 23.2 mg/l for the crude biosurfactant using 3% pure glycerol as substrate and 24.8 mg/l for the crude extract obtained from 3% molasses containing culture media. Table 2 shows the obtained CMC values compared to the synthetic surfactants SDS and Tween 80®. Figure 14 is showing the graph of surface tension at different concentrations of crude biosurfactant

extracts. The graph in logarithmic scale and original data can be viewed in the appendix A.

Table 2 Comparison of bio- and synthetic surfactants in terms of critical micelle concentrations.

Surfactant	CMC	Reference
SDS	0.0082 M = 240 mg/l	Puvvada and Blankschtein (1990)
Tween 80 ®	0.00112 mmol/l = 16 mg/l	Thermo Scientific (2013)
BSF [3% molasses] of *Bacillus* sp. GY19	24.8 mg/l	This study
BSF [3% pure glycerol] of *Bacillus* sp. GY19	23.2 mg/l	This study

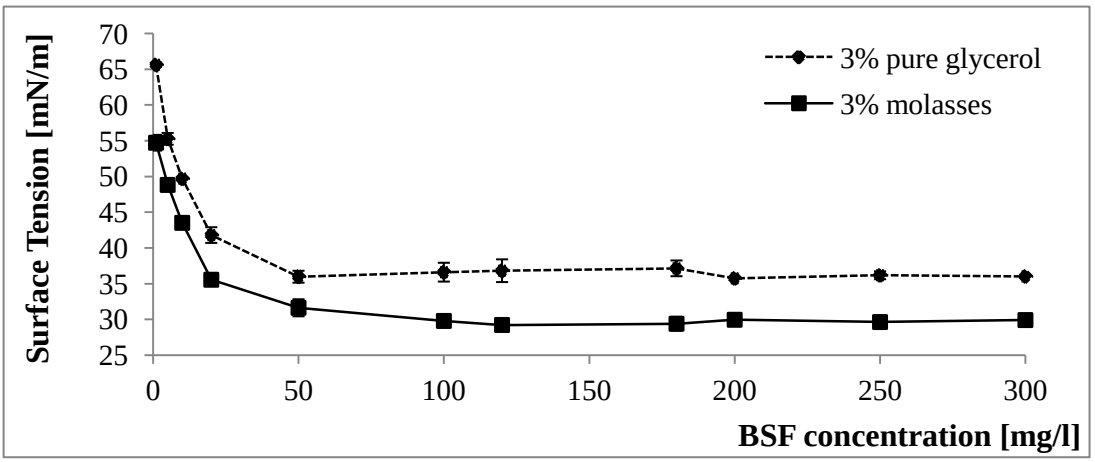


Figure 14 Surface tensions [mN/m] at different concentrations of the crude biosurfactant using 3% pure glycerol and 3% molasses as substrate, respectively. Results are represented as the means +/- standard deviation (n=3).

2. Soil sorption and soil washing

The used soils were characterized and selected parameters are shown in Table 3. The three collected soils were identified as 2 sandy clay loams and 1 sandy loam. The detailed analytical report can be found in the appendix B.

Table 3 Selected Soil Characteristics – OM: Organic Matter; Avai P: Available Phosphorous; Avai K: Available Potassium; OC: Organic Carbon; N: Nitrogen; CEC: Cation Exchange Capacity.

Soil	pH	OM [%]	Avai P [ppm]	Avai K [ppm]	OC [%]	N [%]	CEC [Cmol/kg]
Sandy clay loam 1	7.8	6.44	475	616	3.74	0.32	9.2
Sandy clay loam 2	8.1	1.44	69	127	0.84	0.07	22.9
Sandy loam 3	7.6	4.40	2	38	2.55	0.22	4.6

2.1 Soil sorption

The affinity of the different crude BSF to sorb to three different soil types was determined. Three sorption models were used to calculate the partition constant $K_{d,}$ the best fitting isotherms ($R^2 > 0.95$) are shown in Figure 15 and Figure 16. Table 4 shows the results of each applied model as well as resulting K_d values. The partitioning coefficient K_d shows the affinity of the tested surfactant to sorb to the tested soil.

Table 4 R^2 and K_d values of 3 different sorption models

BSF	soil	Linear isotherm		Freundlich isotherm		Langmuir isotherm		
		K_d	R^2	K_F	R^2	K_L	R^2	K_d
3% PG	sandy clay loam 1	510.34	0.98	682.02	0.96	0.74	0.98	256.41
3% PG	sandy clay loam 2	173.23	0.96	222.08	0.93	1.29	0.81	42.55
3% PG	sandy loam	416.20	0.98	474.24	0.99	1.33	0.88	238.10
3% M	sandy clay loam 1	504.66	0.98	460.04	0.98	1.33	0.99	1111.11
3% M	sandy clay loam 2	290.32	0.97	343.00	0.99	1.62E-06	0.99	0.00029
3% M	sandy loam	460.80	0.99	430.92	0.99	3.81E-05	1.00	0.04231

The best fitting isotherms for the crude BSF obtained with 3% pure glycerol as substrate could be described with the linear model, whereas the best fitting isotherm of the crude biosurfactant of 3% molasses as substrate, were given with the Langmuir model. Nevertheless, the highest sorption affinity of the crude extracts are to the sandy clay loam 1 and sandy loam and lowest sorption was achieved on sandy clay

loam 2. Furthermore, sorption of biosurfactant obtained with 3% molasses as substrate showed higher sorption (higher K_d values) to soils than crude biosurfactant extract obtained with 3% pure glycerol as substrate. The more organic content in the soil, the higher the K_d value of the crude biosurfactant extract, indicating a dependency of soil sorption to organic matter.

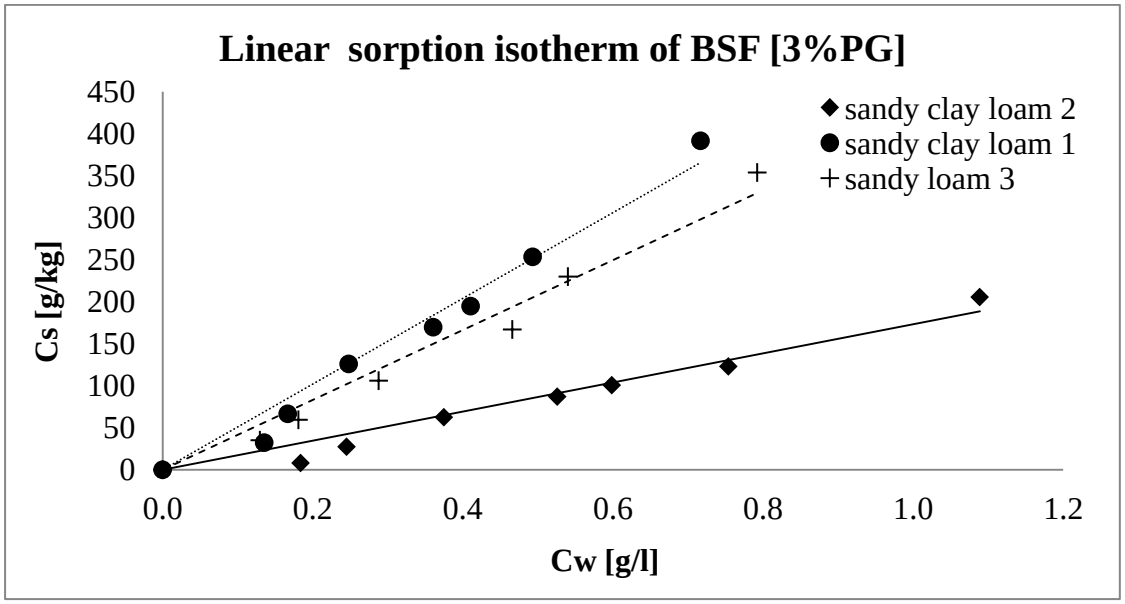


Figure 15 Concentration of BSF (3% PG) in adsorbed to soil (Cs) plotted versus concentration of BSF (3% PG) in aqueous solution (Cw).

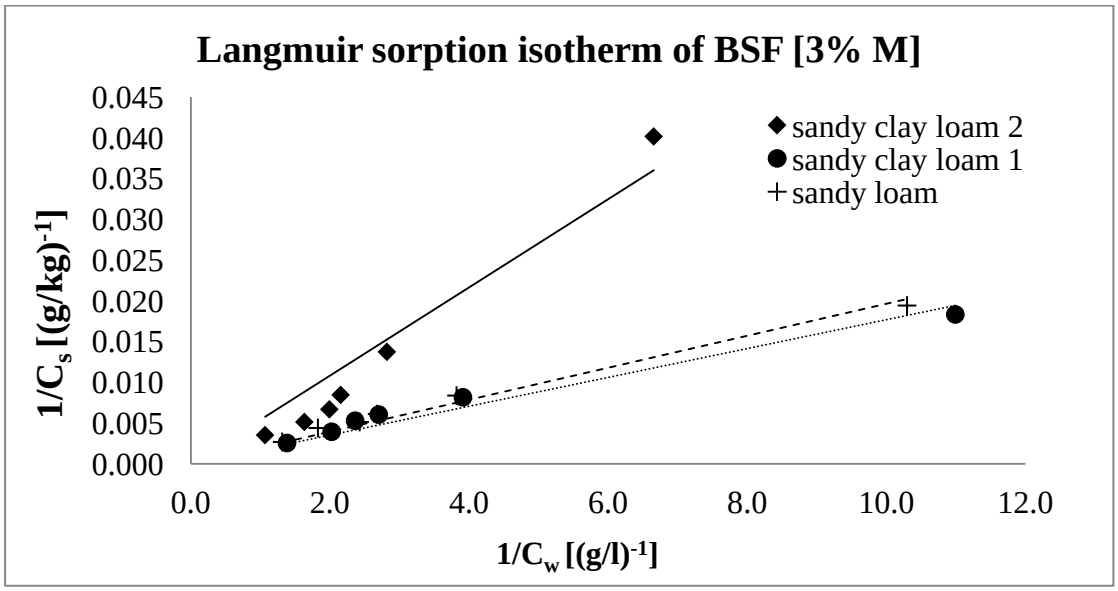


Figure 16 Concentration of BSF (3% molasses) in adsorbed to soil (Cs) plotted versus concentration of BSF (3% molasses) in aqueous solution (Cw) according to Langmuir sorption model.

2.2 Soil washing

The results of soil washing experiments are shown in Figure 17 to 19. Crude oil removal from soil was calculated as the difference [%] of crude oil concentration in the tested batches (washed with different solutions) to a control (unwashed soil, spiked with crude oil).

Statistical results showed significant difference between the samples running a two-way ANOVA with replication, where α was selected to $\alpha = 0.05$. Thus p values $p < 0.05$ rejected the null hypothesis (no significant differences between washing solutions, soil types and both parameters in interaction). Hence there is an effect of the removal of crude oil dependent on washing solution and type of soil. Results of statistical test can be found in the appendix B.

2.2.1 Sandy clay loam 1

Figure 17 shows the crude oil removal of different washing solutions on sandy clay loam 1. All washing solutions achieved a significant higher crude oil removal than water. Highest removal was reached by the washing solution containing 2 g/l of crude BSF [3% pure glycerol] and BSF [3% molasses] with 47.73% and 45.76%, respectively. Solutions containing SDS performed better than washing solutions with Tween 80®, however, chemical surfactants SDS and Tween 80® indicated to be less effective in crude oil removal than crude biosurfactants.

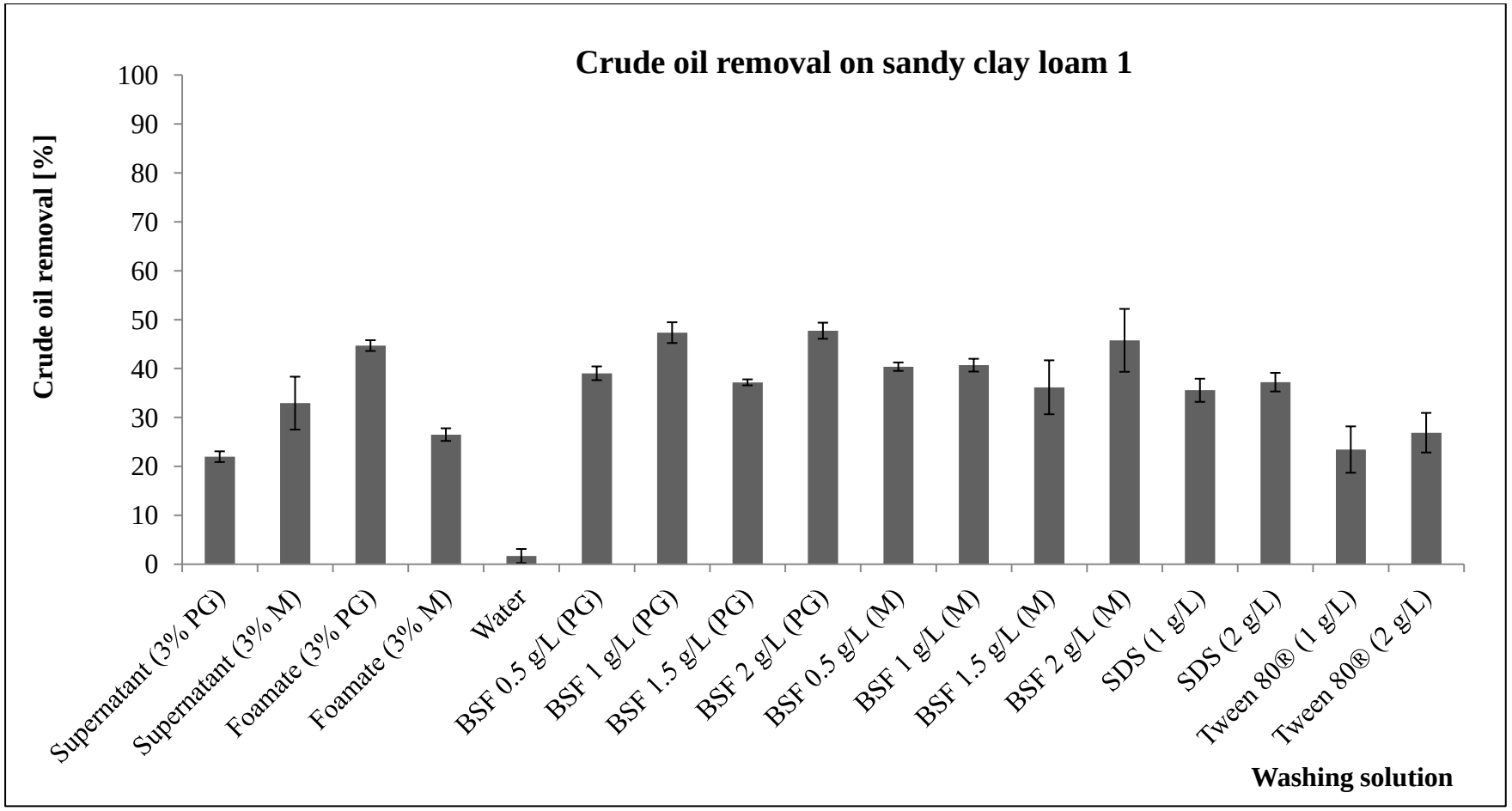


Figure 17 Removal of crude oil [in % to control] of sandy clay loam 1(OC=3.74%, CEC=9.2 cmol/kg) by different washing solutions (BSF = crude biosurfactant, M = molasses, PG = pure glycerol).

2.2.2 **Sandy clay loam 2**

Figure 18 shows the crude oil removal of different washing solutions on sandy clay loam 2. All washing solutions achieved a significant higher crude oil removal than water. Highest removal (90.79%) was reached by the washing solution containing 2 g/l of crude BSF (3% pure glycerol).

The crude oil removal by the washing solution containing crude BSF extract from pure glycerol is increasing with the concentration, which is different with the other tested soils, indicating that there must be other parameters effecting the washing efficiency, than tested by this experiments.

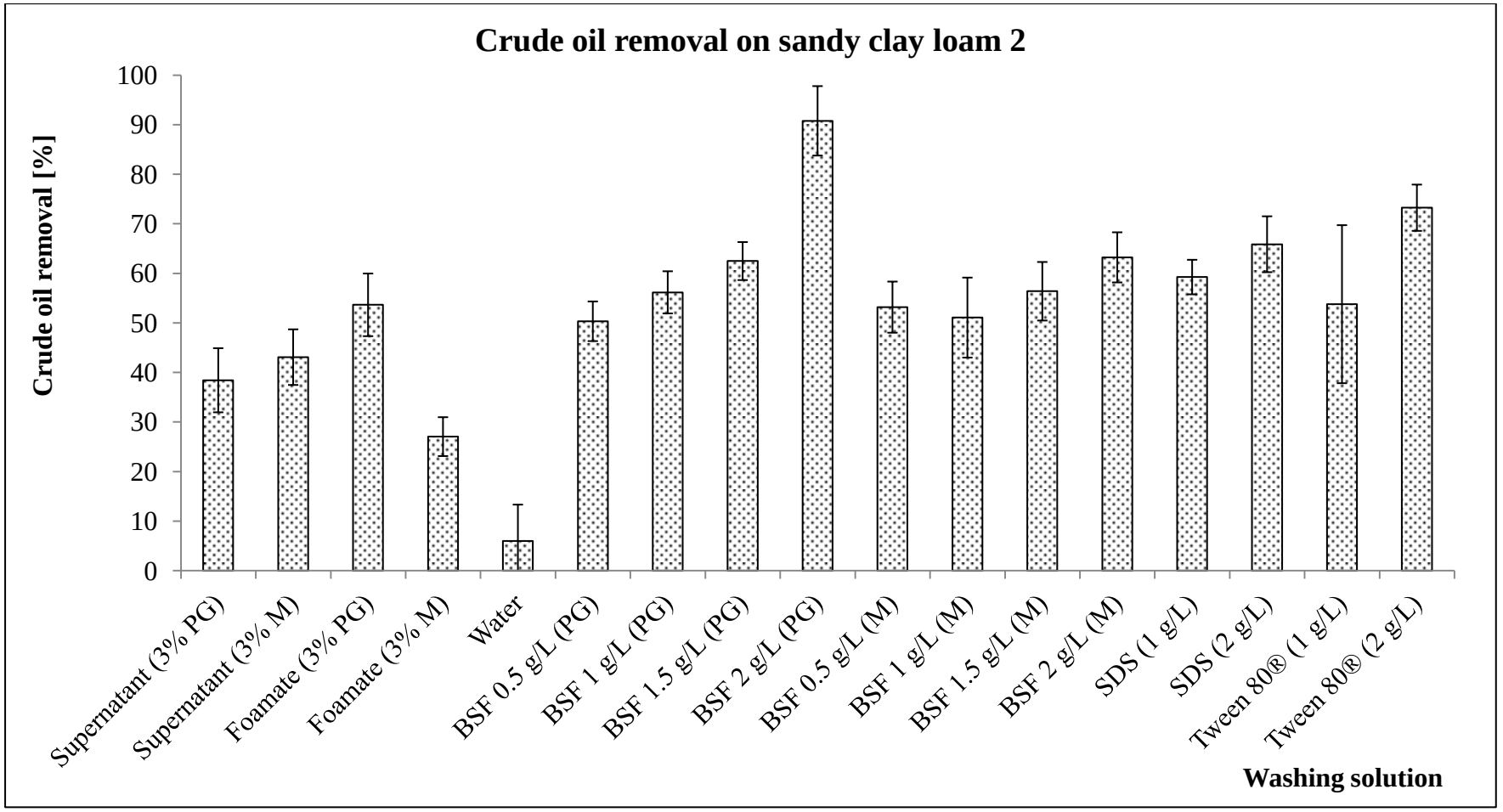


Figure 18 Removal of crude oil [in % to control] of sandy clay loam 2(OC=0.84%, CEC=22.9 cmol/kg) by different washing solutions (BSF = crude biosurfactant, M = molasses, PG = pure glycerol).

2.2.3 **Sandy loam 3**

Figure 19 shows the crude oil removal of different washing solutions on sandy clay loam 2. All washing solutions achieved a significant higher crude oil removal than water. Highest removal (51.33%) was reached by the washing solution containing 1.5 g/l of crude BSF (3% molasses).

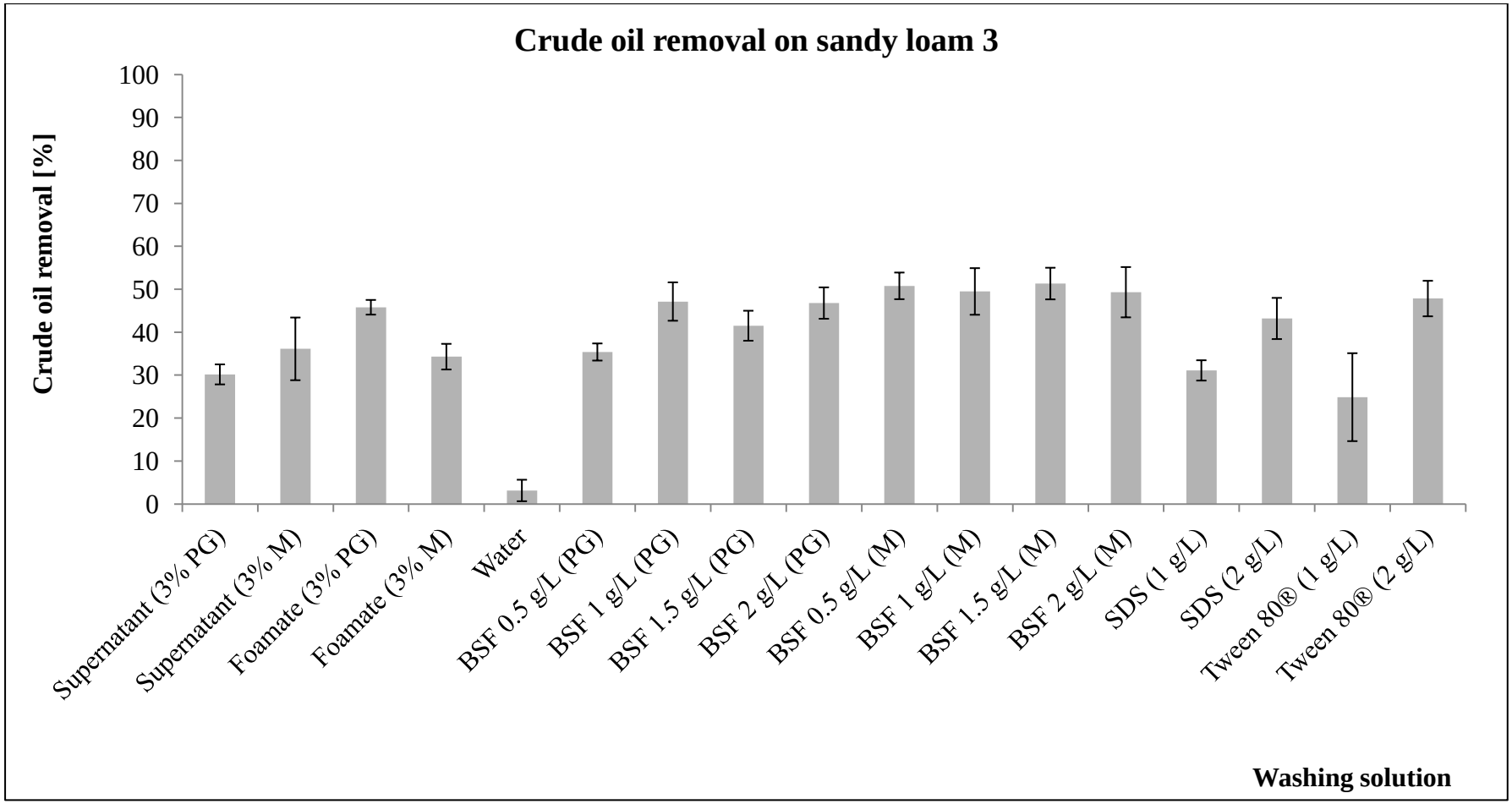


Figure 19 Removal of crude oil [in % to control] of sandy loam 3(OC=2.55%, CEC=4.6 cmol/kg) by different washing solutions (BSF = crude biosurfactant, M = molasses, PG = pure glycerol).

2.2.4 **Summary of soil washing results**

All tested washing solutions showed better removal of crude oil from soil, than water in a range between 22% up to 91%. The best removal was achieved by a washing solution containing 2 g/l of crude biosurfactant (obtained from 3 % pure glycerol as substrate) on sandy clay loam 2 (91% removal of crude oil). Moreover, it is apparent that generally removal of crude oil is better on sandy clay loam 2 than with other tested soils. On sandy clay loam 1 and sandy loam crude biosurfactant washing solutions performed better or similar to the washing solutions containing synthetic surfactants. Crude oil removal was higher from foamate of broth cultivated with 3% pure glycerol and slightly higher than washing crude oil from sandy loam soil 2 with 0.5 g/l of crude BSF (3% pure glycerol as substrate). Having a closer look on the concentration of the crude BSF in the washing solution, it shows a concentration dependence on the washing performance of the crude BSF obtained from 3% PG on sandy loam soil 2 whereas the washing performance of solutions containing crude BSF obtained from 3% molasses only show small increase with concentration.

Concentrations calculated in times of CMC of each washing solution, are presented in Table 5. When concentrations are calculated in times of CMC, the washing solution containing 0.5 g/l BSF (pure glycerol) and 1 g/l SDS solution are almost at the same value (5.82 and 4.17, respectively). Crude oil removal gave similar results for this two washing solutions, meaning that double of the amount of SDS is needed to reach the washing effectiveness of the BSF.

Table 5 Concentration of BSF calculated in times of CMC (conc. of BSF divided through CMC from Table 2).

Washing solution	x CMC
Supernatant (3% PG)	5.82
Supernatant (3% M)	16.13
BSF 0.5 g/L (PG)	21.55
BSF 1 g/L (PG)	43.10
BSF 1.5 g/L (PG)	64.66
BSF 2 g/L (PG)	86.21
BSF 0.5 g/L (M)	20.16
BSF 1 g/L (M)	40.32
BSF 1.5 g/L (M)	60.48
BSF 2 g/L (M)	80.65
SDS (1 g/L)	4.17
SDS (2 g/L)	8.33
Tween 80® (1 g/L)	62.50
Tween 80® (2 g/L)	125.00

3. Ecotoxicity

3.1 Seed germination

Figure 20 is showing the total number of germinated seeds after 5 days. Single factor ANOVA of each plant species says there is no significant difference of seed germination using different test solutions with tomato and cucumber seeds but shows significant difference within the test solutions of lettuce seed germination. T-tests showed significant difference between SDS solution and tab water of seed germination of lettuce.

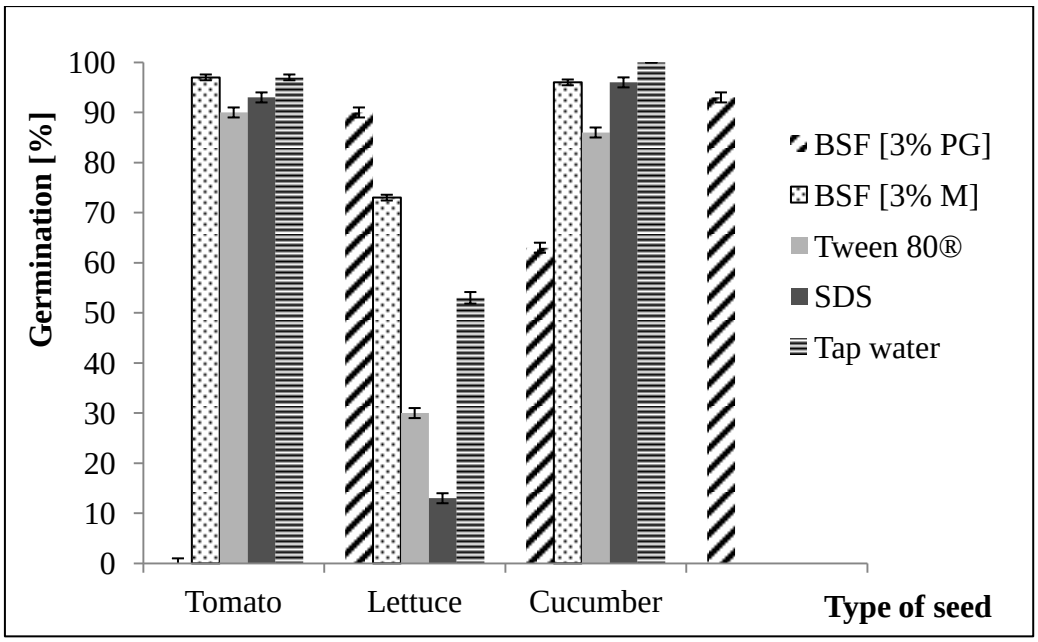


Figure 20 Germination in % after 5 days of three different plant species and different test solutions, concentration = 1 g/l; (BSF [3% PG] – crude biosurfactant extract with 3% pure glycerol as substrate; BSF [3% M] – crude biosurfactant with 3% molasses as substrate).

Figure 21 to Figure 23 show the number of germinated seeds over time of the three investigated plant species tomato, lettuce and cucumber, respectively. Tomato seeds with SDS solution germinated slower than other solutions and tap water; whereas Cucumber seeds didn't show a big difference in the germination with time.

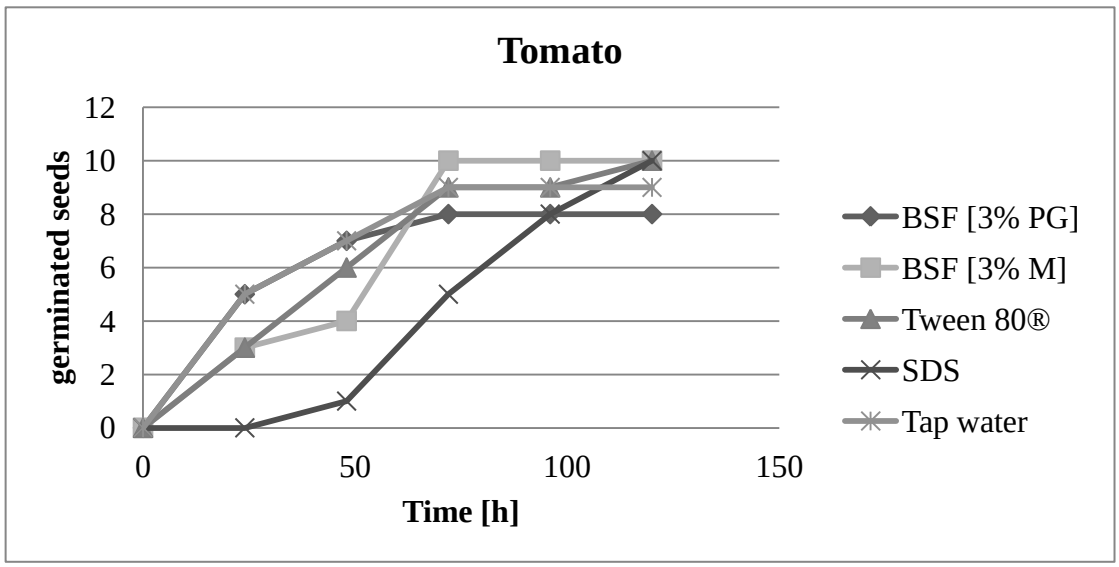


Figure 21 Germination of tomato seeds over investigated time with different test solutions, concentration = 1 g/l; (BSF [3% PG] – crude biosurfactant extract with 3% pure glycerol as substrate; BSF [3% M] – crude biosurfactant with 3% molasses as substrate).

Lettuce shows a greater sensitivity than cucumber and tomato seeds. Biosurfactants show a higher number of germinated seeds than tap water and synthetic surfactants SDS and Tween 80®.

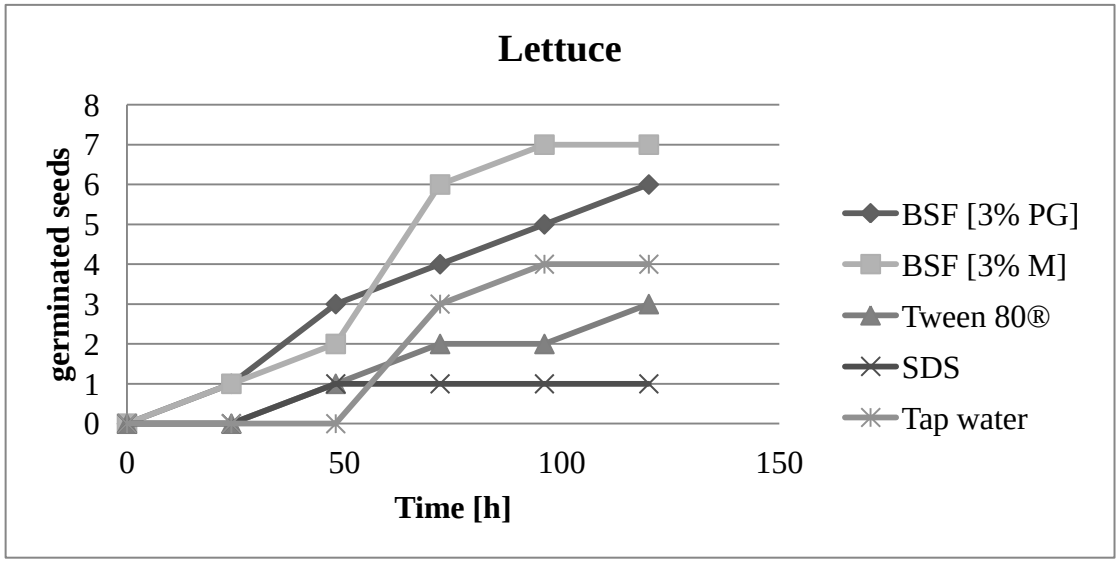


Figure 22 Germination of lettuce seeds over investigated time with different test solutions, concentration = 1 g/l; (BSF [3% PG] – crude biosurfactant extract with 3% pure glycerol as substrate; BSF [3% M] – crude biosurfactant with 3% molasses as substrate).

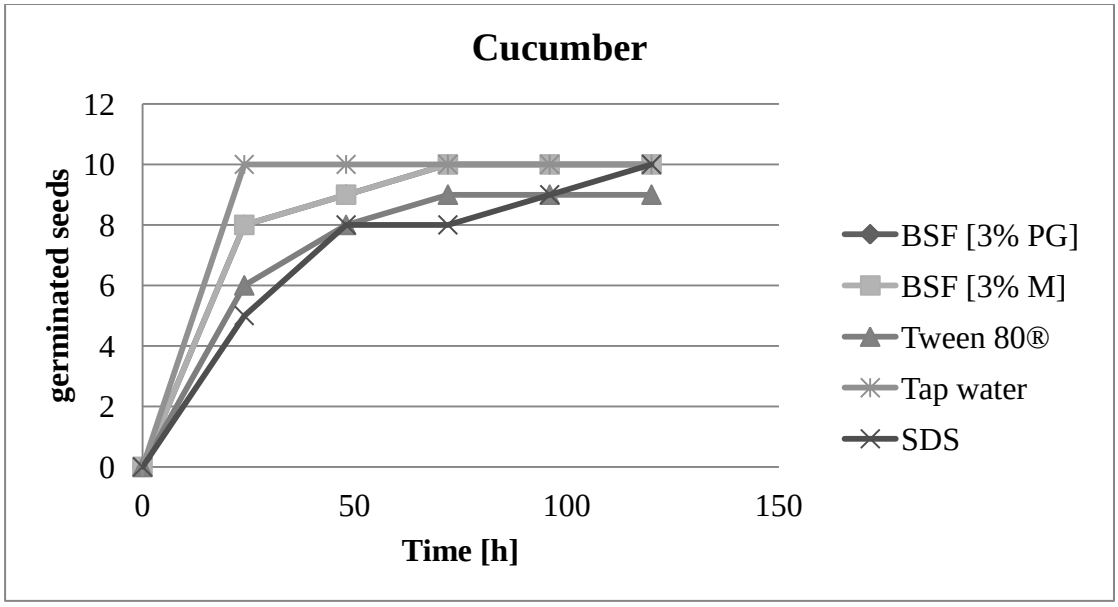


Figure 23 Germination of cucumber seeds over investigated time with different test solutions, concentration = 1 g/l; (BSF [3% PG] – crude biosurfactant extract with 3% pure glycerol as substrate; BSF [3% M] – crude biosurfactant with 3% molasses as substrate).

3.2 Root elongation

Root elongation results are shown in Figure 24 and Table 6. Root lengths differ depending on the type of seed. Having a closer look on cucumber seeds, the root lengths showed significant differences ($p < 0.05$) to the control (tap water) only with the synthetic surfactant solutions SDS and Tween 80® as test solution. Seeds of tomato and lettuce showed significant differences in root lengths only with SDS solution. Hence, there is no toxicity of crude BSF solutions towards tomato, cucumber and lettuce plants. Statistical results can be viewed in the appendix B.

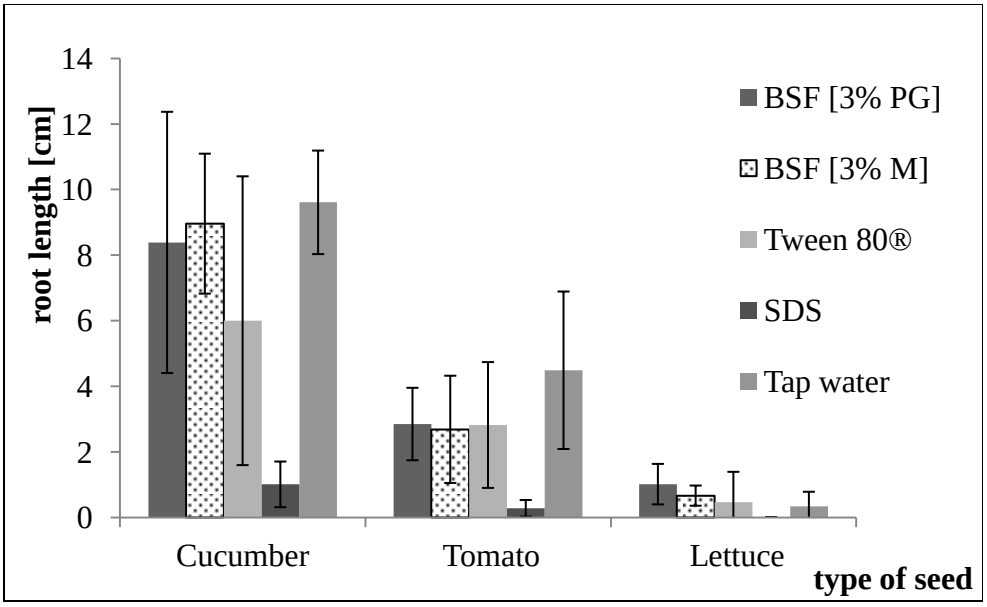


Figure 24 Presented are the means of root length of germinated seeds (Cucumber, Tomato, and Lettuce) with different test solutions, concentration = 1 g/l.

Table 6 Means (M) of root length in cm and standard deviation (SD) of three species of plant seeds with different test solutions.

		BSF [3% PG]	BSF [3% Molasses]	Tween 80®	SDS	Tap water
Cucumber	**M**	8.388	8.960	6.000	1.010	9.61
	SD	3.983	2.134	4.403	0.697	1.58
Tomato	**M**	2.850	2.688	2.820	0.280	4.49
	SD	1.105	1.635	1.918	0.253	2.40
Lettuce	**M**	1.017	0.667	0.470	0.000	0.34
	SD	0.618	0.308	0.923	0.000	0.45

3.3 MIC and MBC

No toxicity to the mixed soil culture of crude surfactants and Tween 80® could be observed using concentrations from 10 g/l to 0.005 g/l. Only SDS solution showed bacterial growth inhibition at a concentration of 1.25 g/l and had bactericidal effects at a concentration of 10 g/l. Results are shown in Table 7.

Table 7 Results of soil bacteria toxicity assay of four test solutions; N.D. – not detectable.

Test solution	MIC [g/l]	MBC [g/l]
BSF [PG 3%]	N.D.	N.D.
BSF [M 3%]	N.D.	N.D.
SDS	1.25	10.00
Tween 80	N.D.	N.D.

B) Chemical characterization of the biosurfactant and detection of biosurfactant producing genes in *Bacillus* sp. GY19

1. Detection of biosurfactant producing genes

PCR products after proceding a gel electrophoresis are shown in Figure 25. The loading scheme can be viewed at Table 9. PCR products obtained with the primer pairs Af2-F/Tf1-R, Ap1-F/Tp1-R, and As1-F/Ts2-R showed positive results after running a gel electrophoresis. In total 5 bands were detected and are presented in Table 8 and are framed red and numbered in Figure 25.

Table 8 PCR products obtained from different primer pairs specific for BSF producing genes.

Band No.	Band size [bp]
1	1150
2	950
3	400
4	290
5	490

These 5 bands were selected for cloning and sequencing to confirm that the amplified fragments are encoding biosurfactant producing genes.

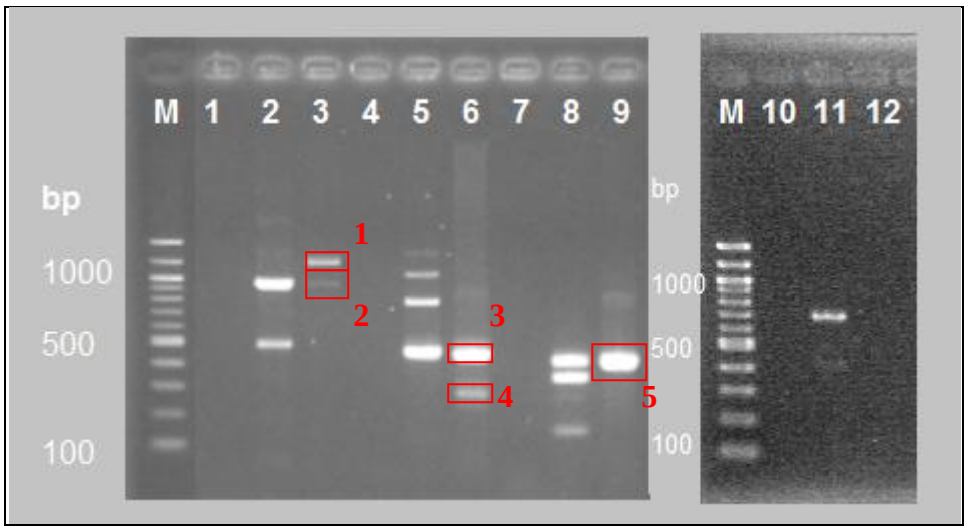


Figure 25 Agarose gel after running gel electrophoresis with PCR products using specific primer pairs for biosurfactant producing genes; loading scheme is presented in Table 9 .

Table 9 Loading scheme of agarose gel presented in Figure 25

Well No.	Description	Primer pair
M	Marker	
1	Negative Control	Ap1-F/Tp1-R
2	Positive Control (*B. licheniformis* ATCC 14580)	
3	*Bacillus* GY 19	
4	Negative Control	Af2-F/Tf1-R
5	Positive Control (*B. subtilis* ATCC 6633)	
6	*Bacillus* GY 19	
7	Negative Control	As1-F/Ts2-R
8	Positive Control (*B. subtilis* ATCC 6633)	
9	*Bacillus* GY 19	
10	Negative Control	Am1-F/Tm1-R
11	Positive Control (*B. subtilis* ATCC 6633)	
12	*Bacillus* GY 19	

Table 10 is showing the results after the procedure of purifying and cloning the earlier obtained PCR products. Sequences of band number 2 and 4 did not give biosurfactant producing genes as result after using blast x program. Hence, three BSF producing

genes could be detected in *Bacillus* GY19. Sequences of the purified and cloned PCR products are presented in appendix D.

Table 10 Similarity of PCR products to biosurfactant encoding genes ([1]shown is the name of the biosurfactant encoded by the gene, which could be detected by the corresponding primer pair; [2]accession number of the sequenced gene compared with the database using blastx program) Numeration of bands referred to bands numbered in Figure 25.

Band No.	Primer Pair	Gene[1]	Result	Accession[2]
1	Ap1-Tp1	Plipastatin	92% plipastatin synthetase [*Bacillus* sp. JS]	YP_006231790.1
3	Af2-Tf1	Fengycins	80% fengycinsynthetase *FenE* [*Bacillus subtilis*]	YP_006231792.1
5	As1-Ts2	Surfactin	73% surfactinsynthetase [*Bacillus*sp. JS]	YP_006230161.1

2. Chemical characterization of the crude biosurfactant

2.1 Colorimetric tests

Colorimetric tests were carried out showing a large part of the crude extract has a lipid structure, followed by protein and carbohydrate properties. Results are shown in Table 11.

The crude extract using pure glycerol as substrate showed 35% lipid, 1% carbohydrates and 10% of protein content, whereas the extract originated from molasses containing supernatant showed a slightly different amount of tested substances (29%, 2% and 4% as lipid, carbohydrates and protein contents, respectively). Standards curves are shown in appendix C.

Table 11 Summary of colorimetric tests: lipid, sugar and protein contents in % of two studied crude BSF.

Sample	Lipid [%]	Sugar [%]	Protein [%]	Total [%]
BSF [3% Molasses]	29.17	1.80	4.32	35.29
BSF [3% PG]	34.77	0.51	9.64	44.91

2.2 TLC with Ninhydrin staining

Friedman (2004) reported the development of Ruhmans purple of ninhydrin with amino acids, peptides etc. In this study TLC sheets showed purple spots after hydrolysis of the sample and development with ninhydrine reagent. In contrast, the controls (unhydrolyzed sample) did not generate purple spots (Figure 26). Hence, amino acids are present in a bounded form such as peptides.

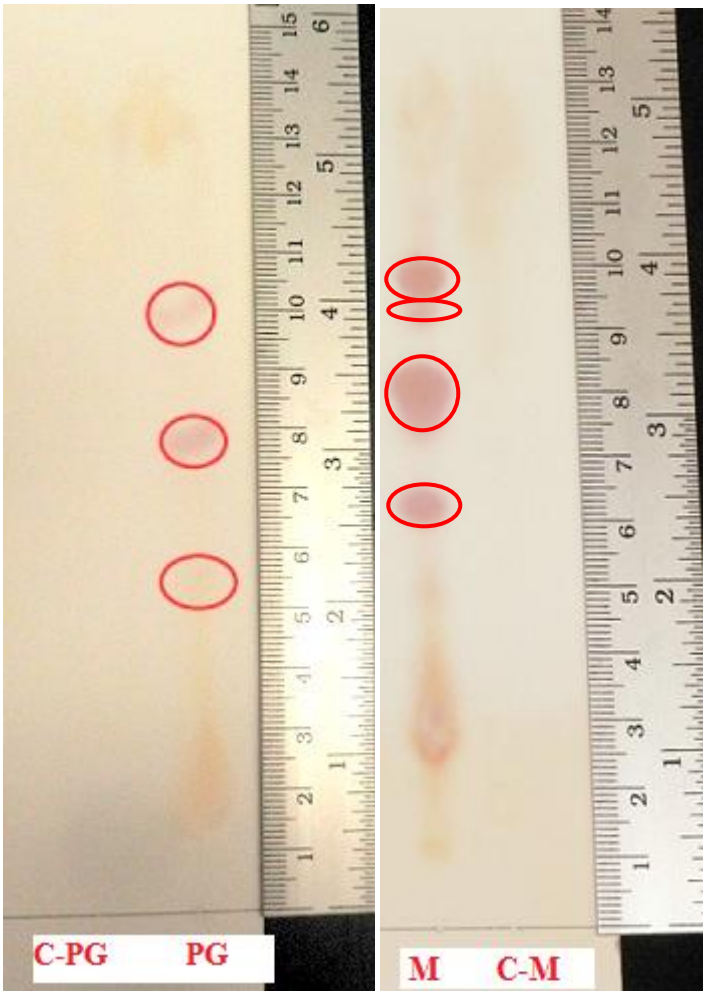


Figure 26 Silica-TLC sheet with spotted sample of hydrolyzed crude BSF (3% M as substrate shown as M, 3% PG as substrate shown as PG), control: unhydrolyzed sample (shown as C-M and C-PG, respectively); solvent system: 4:1:1 ButOH – CH_3COOH – H_2O.

2.3 Analysis of FAME

Analysis of fatty acid methyl esters could not lead to a full characterization of fatty acids in the crude extract of BSF using 3% pure glycerol by *Bacillus* sp. GY19. The standard FAME mixture used in this experiment contained mixtures of fatty acids ranging from short chain (C4) to long chain (C21) hydrocarbons. The GC chromatogram of crude BSF sample tentatively indicated the presence of medium (C14) to long (C18) chain fatty acids. However, exact qualification and quantification of fatty acids could not be pointed out because of particular peak overlapping.

Therefore, GC conditions need to be optimized to ensure that fatty acid peaks of both standard FAME and crude BSF are well separated. Table 12 shows the detected retention times of the standard FAME mixture, Table 13 shows the retention times of the crude BSF sample. Chromatograms can be viewed in the appendix C.

Table 12 Retention times of standard FAME mixture of GC-FID.

Peak No	Standard	Retentiontime [min]
1	solvent	3.205
2	butyric acid ME	3.343
3	caproic acid ME	3.897
4	caprylic acid ME	5.256
5	capric acid ME	8.219
6	undecanoic acid ME	10.382
7	lauric acid ME	12.863
8	tridecanoic ME	15.505
9	myristic acid ME	18.194
10	myristoleic acid ME	19.241
11	pentadecanoic acid ME	20.846
12	cis-10-pentadecanoic ME	21.881
13	palmitic acid ME	23.438
14	palmitoleic acid ME	24.155
15	heptadecanoic acid ME	25.927
16	cis-10heptadecanoic acid ME	26.634
17	stearic acid ME	28.339
18	elaidic acid ME	28.886
19	oleic acid ME	30.006
20	linolelaidic acid ME	30.214
21	linoleic acid ME	30.774
22	arachidic acid ME	31.611
23	y-linolenic acid ME	33.189
24	heneicosanoic acid ME	33.839

Table 13 Retention times of FAME of crude biosurfactant of *Bacillus* sp. GY19 using 3% pure glycerol as substrate of GC-FID.

Peak No	similar to Standard	Retention time [min]
1	solvent	3.025
2	solvent	3.068
3		3.196
4		3.246
5		3.522
6	myristoleic acid ME	19.578
7		19.989
8		22.194
9	palmitic acid ME	23.427
10	palmitoic acid ME	24.15
11	heptadecanoic acid ME	25.89
12		27.419
13	stearic acid ME	28.331
14	elaidic acid ME	28.976
15	linolelaidic acid ME	30.205
16		31.391
17		31.704
18		31.831

2.4 NMR

^{13}C NMR signals with high intensity at δ_C 10-40 ppm, indicated the presence of saturated alkanes. Other peaks with δ_C 130-115 ppm appeared due to the presence of olefinic carbons bonds.

In addition NMR spektra gave chemical shifts in the range of δ_C 40-50 ppm and δ_H 4-5 ppm with low intensity, specific for quaternary carbon and α-hydrogen atoms, respectively, which are present in amino acids and δ_C 169-170 ppm for C=O bonds in peptides.

Two signals with chemical shift of ~157 ppm and 120 ppm could indicate phenolic or p-substituted aromatic structure as in aromatic amino acids.

^{1}H NMR spectra can be found in the appendix C. A proposed structure of the lipopeptide biosurfactant is shown in Figure 27.

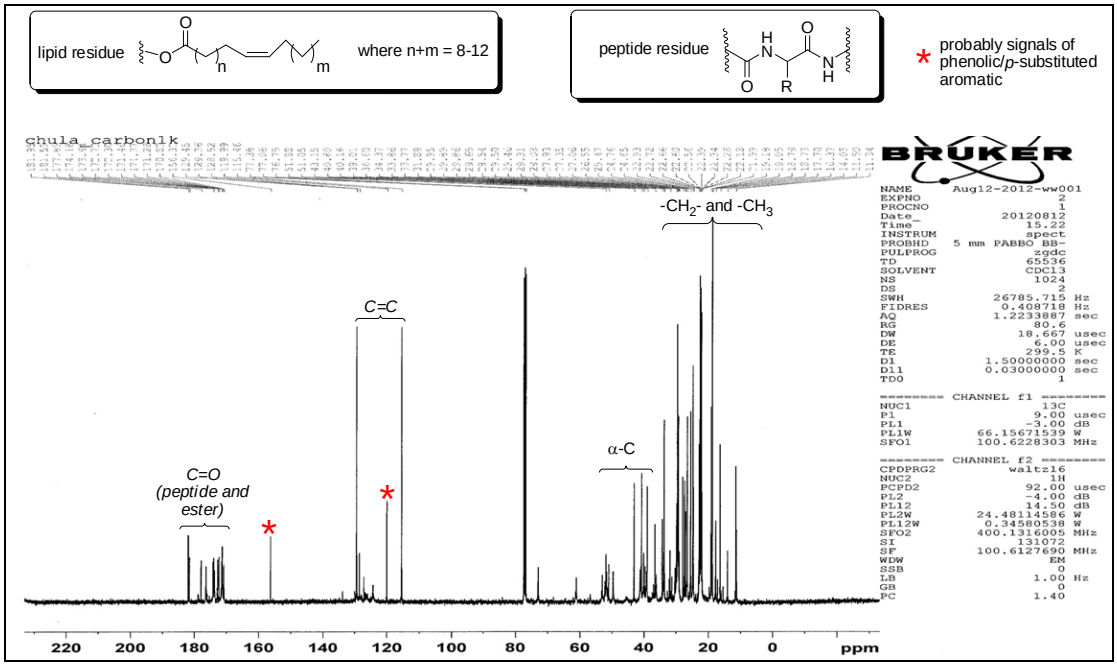


Figure 27 ^{13}C-NMR of crude biosurfactant extract obtained with 3% pure glycerol as substrate.

CHAPTER V

DISCUSSION & CONCLUSION

1. Discussion

1.1 Renewable substrates

The question whether biosurfactants are worth to be considered as competitive to synthetic surfactants, starts with the substrate used to cultivate the selected strain. Environmental friendly, renewable and economic efficient substrates have to be selected.

This study showed the feasibility of *Bacillus* GY 19 to produce surface active compounds using molasses and glycerol. Despite the low yields obtained by this study, more potential for biotechnological production could be achieved by a subsequent optimization of cultivation e.g. through substrate mixtures or additives (Kiran et al., 2010) or other methods e.g. optimizing carbon-nitrogen ratio (Mukherjee et al., 2006). Several studies show increased biosurfactant production due to optimization of the production media (Abdel-Mawgoud et al., 2008; Kiran et al., 2010; Najafi et al., 2010; Thavasi et al., 2010; Ghribi et al., 2011). The rather low focus on bottom glycerol in this study is justified by the high content of fatty acids already present in the substrate, leading to a high surface tension reduction in a control batch (no inoculum). The results obtained with pure glycerol; show that waste/bottom glycerol could be a low cost substitute (Nitschke et al., 2005).

Biosurfactants produced by *Bacillus* sp. GY19 using pure glycerol and molasses as substrates are competitive with the commercially produced surfactants Tween 80® and sodiumdodecylsulfate (SDS). Critical micelle concentrations of produced crude extracts with 23 mg/l and 25 mg/l are lower than that of SDS (0.0082 M = 2.4 g/l Puvvada and Blankschtein, 1990) and not far from the CMC of Tween 80® (0.00112 mmol/l = 16 mg/l Thermo Scientific, 2013). An overview is given in Table 2 .

1.2 Applications in soil washing and soil sorption

Crude oil removal of contaminated sites is a decent problem nowadays, where almost all products rely on petrochemical sources. The utilization of biosurfactants for soil washing was focus in this work. Therefore, details in the behavior of the biosurfactant in soil are necessary to plan effective removal procedures. Sorption is one factor influencing soil washing effectiveness. Simple sorption experiments should lead to an understanding of what type of soil can be successfully washed with solutions containing biosurfactants.

The sorption experiments of the biosurfactants by *Bacillus* sp. GY19 showed the highest affinity of sorption to sandy clay loam 1, followed by sandy loam and the least sorption could be observed with sandy clay loam 2. These conclusions can be drawn after plotting the measured concentrations according to different sorption models (as shown in Chapter IV. Different sorption can be explained by the different characteristics of the used soils. It is conspicuous that the highest sorption was achieved on soils with the highest organic content, which is a known factor for influencing sorption behavior (Schwarzenbach et al., 2005). Figure 28 is showing the relationship between the organic carbon content and obtained K_d values for the crude biosurfactant extracts, indicating the higher the OC [%] in the soil, the higher sorption of the crude biosurfactant (K_d) to the soil.

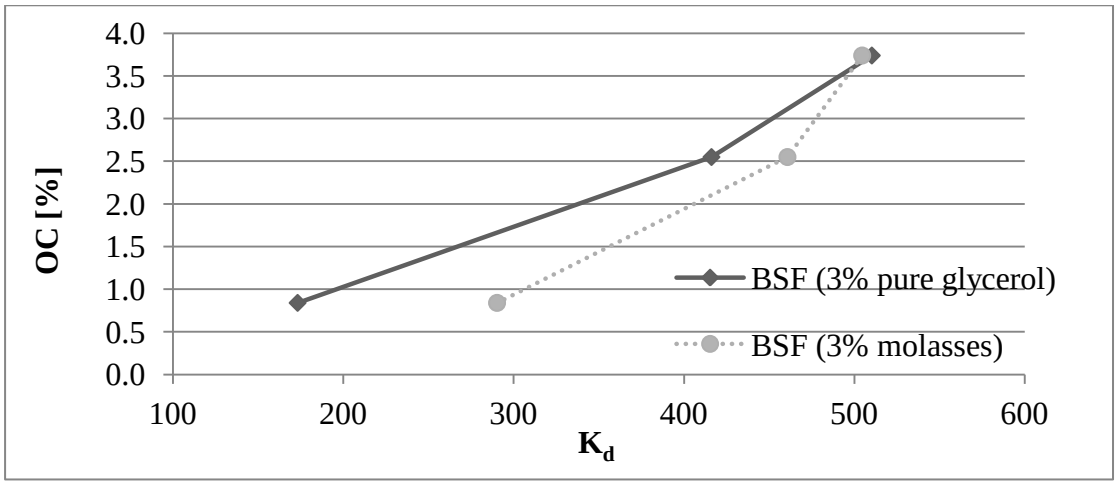


Figure 28 Relationship between organic carbon content and K_d values of crude biosurfactant extracts.

Sorption experiments showed slightly higher sorption of crude BSF of 3% molasses than BSF of 3% pure glycerol. This can be an incident of different structural properties.

For the application in soil washing, crude oil removal was best with sandy clay loam 2, which can be explained by the low amount of organic content and organic matter compared to the other used soils, leading to less sorption of the biosurfactant to the soil (referring to sorption experiments in this study). In terms of pure glycerol as substrate, supernatant and foamate washing solutions seem to be less efficient than a 2 g/l crude extract solution in terms of removing crude oil from sandy clay loam 2, Indicating that the concentration of BSF in the washing solution is important for the washing efficiency. The concentration of the BSF in the supernatant can be estimated to be 0.14 g/l (from yield experiments), which is lower than the tested washing solutions containing crude BSF extracts. The concentration of BSF in foamate solutions is unknown and can therefore not be used to compare it with the other washing solutions, concentration wise. Soil sorption experiments confirmed the soil washing results. Highest sorption affinity of the used crude biosurfactant was on sandy clay loam 1 and sandy loam. The same two soils showed less effective soil washing results. Additionally, the crude extract obtained with 3% molasses as substrate shows higher sorption on all soils leading to lower removal of crude oil in the conducted experiments. This can be explained by the higher K_d values on all tested soils, showing a higher sorption affinity than BSF using 3% pure glycerol as substrate. Concerning the composition of the three soil types, soil with highest sorption affinity (sandy clay loam 1 and sandy loam) contain more organic carbon than sandy clay loam 2 (best washing results, lowest obtained K_d value). Similar results were reported by Franzetti et al. (2012), where a dependence of soil washing efficiency to the organic content and other properties of soil could be observed.

The soil washing performance of the used biosurfactants showed competitive results, compared to SDS and Tween 80®. Washing solutions containing SDS and Tween 80® lead to even lower crude oil removal of soils with higher organic content. In other studies similar trends were shown. Urum et al. (2006) showed a better removal of

crude oil by biosurfactant than plant surfactant saponin. Removal efficiency was similar to synthetic surfactant SDS (Urum and Pekdemir, 2004; Urum et al., 2005). In another study biosurfactant was even more effective to remove hexadecane from soil than SDS and Tween 80® (Bai et al., 1997). This shows that the produced biosurfactant by *Bacillus* sp. GY19 is comparable to those of other studies.

Comparing the crude oil removal efficiency of the different washing solutions containing crude BSF extract to the synthetic surfactants SDS and Tween 80® in terms of CMC, it shows, that the biosurfactant containing solutions reach better crude oil removal with less xCMC (times CMC), indicating a better effectiveness of washing crude oil from soil than the tested synthetic surfactants.

A possible improvement of the washing effectiveness could be reached by increasing the concentration of BSF in the washing solutions, but only the BSF of pure glycerol shows significant increase of the crude oil removal with an increasing concentration.

Nevertheless, the selection of the washing solution should consider the costs and efforts of production as well as the efficiency, since it is time consuming and more expensive to obtain a crude extract. Furthermore, this study only shows results of a single washing step, whereas multiple washing steps could lead to different results. In addition it might not always be necessary to remove 91% of the contaminant, since the question of how to treat the washing solutions afterwards remains.

1.3 Ecotoxicity

If biosurfactants are suggested to be used in soil remediation they have to be harmless to the environment and its living organisms. Many synthetic surfactants are known to cause delicate to severe health effects, either to animal, plants or microorganisms, or to human beings.

Toxicological assays could not detect any negative effects of BSF on the germination and root elongation of the three tested plant species. Furthermore, the tested biosurfactants did not inhibit growth or show bactericidal effects on mixed cultures obtained from soil. Hence, the two tested biosurfactants produced by *Bacillus* sp.

GY19, using molasses and pure glycerol as substrate, can be considered biodegradable. In contrast to the results obtained with solutions containing BSF, SDS solutions showed toxic effects. These results indicates that the biosurfactants produced by *Bacillus* GY19 using pure glycerol and molasses are non toxic and can be released into the environment without worrying. Only a few publications are dedicated to the ecotoxicity or biodegradability of biosurfactants. However, studies focusing on this issue showed a rather low toxicological effect and high biodegradability when compared to synthetic surfactants (Klosowska-Chomiczewska et al., 2011). A study where a bioemulsifier produced by *Variovorax paradoxus* 7bCT5 was tested in a germination test and toxicity test using earthworms, showed no toxicity towards the tested organisms (Franzetti et al., 2012).

Nonetheless, with this study only a limited range of possible toxic effects was considered, for example no statements according accumulation in environment can be made (Whitehouse, 1998; Schwarzenbach et al., 2005). Furthermore there was only one concentration (1 g/l) tested in seed germination, possible toxic effects could occur with higher concentrations.

1.4 Characterization

The above discussed experiments gave an idea for potential applications the produced biosurfactant of *Bacillus* sp. GY19 could be useful. To make detailed suggestions for applications and behavior of the BSF in the environment for example, the chemical structure and properties have to be elucidated. In this study only preliminary results could be obtained.

Colorimetric tests didn’t give a complete amount of components in the biosurfactant after having a closer look on the mass balance. This could be a result of using crude extracts instead of purified biosurfactants. Additionally the used standards for calibration are representatives for the detected class of components e.g. glucose for carbohydrates. In fact biosurfactants contain more complex molecules than used for calibration and exist in mixtures rather than single compounds, thus responses within

the tests are different than those of the standard compounds (Bradford, 1976). Thus, the results describe the trend of the tested compounds, rather than the actual amount present in the obtained biosurfactants (Satpute et al., 2010b).

Although the analysis of fatty acid analysis by GC-FID could not reveal all components, it can be proposed that fatty acids, whether free or bound in lipids consists of chain length between 14C to 18C which is accounted for common natural occurring fatty acids such as palmitic and stearic acid. However quantitative analyses of these fatty acids need to be performed in further studies.

TLC sheets showed purple spots after hydrolysis of the sample whereas, in contrast, the controls (unhydrolyzed sample) did not generate purple spots, thus amino acids are present in form of peptide bonds.

The high lipid content, determined by colorimetric methods, and the presence of amino acids in peptides, indicates that the obtained biosurfactant is a lipopeptide type biosurfactant. NMR results supported the hypothesis with chemical shifts in the range of δ_C 160-170 and δ_H 4-5 specific for quaternary carbon and α-hydrogen atoms respectively. Moreover, the detection of three biosurfactant producing genes, all belonging to the class of lipopeptides, shows the ability of *Bacillus* sp. GY19 to produce the mentioned biosurfactant. Nevertheless, using different primer pairs for PCR or different biosurfactant extraction techniques could reveal other possible biosurfactant types.

All in all, the characterization revealed the type of the biosurfactant as one of the class of lipopeptides, but could not differ in detailed structural questions. Therefore the hypothesis that different BSF are produced by using different substrates could not be proved. In fact there is different sorption as well as soil washing performance of the two focused BSF leading to the assumption of structural differences.

2. Conclusions

This study showed the feasibility of molasses and pure glycerol to produce biosurfactants, which can be used for soil washing applications. Soil washing efficiency of the biosurfactant is dependent on the type of soil and type of substrate used. Result showed no toxicity of the crude extracts towards germination of cucumber, tomato and lettuce seeds and no bactericidal effects towards mixed soil cultures. Furthermore, characterization experiments revealed the biosurfactant by *Bacillus* sp. GY19 as lipopeptide type. Biosurfactant producing genes of fengycin, plipastatin and surfactin family could be detected. Slightly different chemical properties (content of carbohydrates, protein and lipids; cmc) by the obtained crude extracts using different substrates (molasses and pure glycerol) were observed; however, the hypothesis of different chemical characteristics could not be fully stated.

3. Recommendations

Structural characterization is still not complete and requires a good and easy purification step. LC-MS or MALDI-TOF-MS is suggested for detailed information.

In terms of detection of biosurfactant producing strains, molecular methods could be useful, because no time consuming production is required. Therefore more research on specific genes and primer design is necessary.

In order to produce enough BSF for an actual use in the field the production has to be optimized. The focus on renewable and waste products could be promising.

Interesting would be the question how to enhance the biosurfactant activity/effectiveness in terms of soil washing.

Further studies should focus on sorption in more detail for example with column tests rather than batch experiments. Having a focus on the relation of sorption and structural properties would make more detailed suggestions for applications possible.

Furthermore aging and weathering of contaminated soil should be considered by using contaminated soil, rather than spike soil.

To make sure that, there is no possible toxic effect of biosurfactant to the environment and living organisms, more specific test (e.g. fish egg test) have to be conducted. A closer focus on biodegradation of the studied compounds could help.

REFERENCES

Abdel-Mawgoud, A.; Mabrouk Aboulwafa, M. and Hassouna Abdel-Haleem, Nadia (2008) Optimization of Surfactin Production by Bacillus subtilis Isolate BS5. Applied Biochemstry & Biotechnology 150: 305–325.

Acevedo, G. Trebbau de and McClnerney, M. J. (1996) Emulsifying activity in thermophilic and extremely thermophilic microorganisms. Journal of Industrial Microbiology 16: 1–7.

Adamson, Arthur W.; Gast, Alice P. (1993) Physical Chemistry of Surfaces. 5th ed. New York: John Wiley & Sons.

Alvarez, P.J.J and Vogel, T.M (1991) Substrate Interactions of Benzene, Toluene, and para-Xylene during Microbial Degradation by Pure Cultures and Mixed Culture Aquifer Slurries. Applied and Environmental Microbiology 57: 2981–2985.

Andrews, J. M. (2001) Determination of minimum inhibitory concentrations. Journal of Antimicrobial Chemotherapy 48: 5–16.

Aparna, A.; Srinikethan, G. and Smith, H. (2012) Production and characterization of biosurfactant produced by a novel *Pseudomonas* sp. 2B. Colloids and Surfaces B: Biointerfaces 95: 23–29.

Appana, Visu D.; Finn, Hugh and St. Pierre, Micheal (1995) Exocellular phosphatidylethanolamine production and multiple-metal tolerance in *Pseudomonas fluorescens*. FEMS Microbiology Letters 131: 53–56.

Arima, K.; Kahinuma, A. and Tamura, G. (1968) Surfactin, a crystalline peptide lipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. Biochemical and Biophysical Research Communications 31: 488–494.

Armarego, W.L.F; Chai, Christina (2012) Purification of Laboratory Chemicals. 7th ed.: Butterworth-Heinemann.

Bafghi, M.K and Fazaelipoor, M.H (2012) Application of Rhamnolipid in the Formulation of a Detergent. Journal of Surfactantants & Detergents 15: 679–684.

Bai, G.; Brusseau, M.L and Miller-Maier, R.M (1997) Biosurfactant enhanced removal of residual hydrocarbon from soil. Journal of Contaminant Hydrology 25: 157–170.

Bazire, Alexis et al. (2005) Osmotic stress and phosphate limitation alter production of cell-to-cell signal molecules and rhamnolipid biosurfactant by *Pseudomonas aeruginosa*. FEMS Microbiology Letters 253: 125–131.

Bharati, Sunil; Rostum, Geier Arne and Loberg, Rita (1993) Calibration and standardization of latroscan (TLC-FID) using standards derived from crude oils. Advances in Organic Geochemistry 22: 835–862.

Bodour, Adria A. et al. (2004) Structure and Characterization of Flavolipids, a Novel Class of Biosurfactants Produced by *Flavobacterium* sp. Strain MTN11. Applied and Environmental Microbiology 70: 114–120.

Bognolo, G. (1999) Biosurfactants as emulsifying agents for hydrocarbons. Colloids and Surfaces A: Physicochemical and Engineering Aspects 152: 41–52.

Bonilla, M.; Olivaro, C.; Corona, M.; Vazquez, A. and Soubes, M. (2005) Production and characterization of a new bioemulsifier from *Pseudomonas putida* ML2. Journal of Applied Microbiology 98: 456–463.

Bosgelmez-Tinaz, Gülgün (2003) Quorum Sensing in Gram-Negative Bacteria. Turkish Journal of Biology 27: 85–93.

Bradford, M.M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248–254.

C. P. Thomas, M. L. Duvall E. P. Robertson K. B. Barrett G. A. Baia (1993) Surfactant-Based EOR Mediated by Naturally Occurring Microorganisms. SPE Reservoir Engineering 8: 285–291.

Cameron, D.R; Cooper, David G. and Neufeld, R.J (1988) The mannopreotein of *Saccharomyces cerevisiae* Is an Effective Bioemulsifier. Applied and Environmental Microbiology 54: 1420–1425.

Christie, William W. (1989) Gas Chromatography and Lipids. A Practical Guide. Bridgwater, Somerset: The Oily Press. Available online at http://lipidlibrary.aocs.org/gc_lipid/gc_lip.html.

Cirigliano, M.C and Carman, G.M (1985) Purification and Characterization of Liposan, a Bioemulsifier from *Candida lipolytica*. Applied and Environmental Microbiology 50: 846–850.

da Silva, Gervásio Paulo; Mack, Matthias and Contiero, Jonas (2009) Glycerol: A promising and abundant carbon source for industrial microbiology. Biotechnology Advances: 30–39.

Das, Palashipriya; Mukherjee, Soumen and Sen, Ramkrishna (2008) Genetic Regulations of the Biosynthesis of Microbial Surfactants: An Overview. Biotechnology and Genetic Engineering Reviews 25: 165–186.

Desai, A.J and Banat, Ibrahim M. (1997) Microbial Production of Surfactants and Their Commercial Potential. Microbiology and Molecular Biology Reviews 61: 47–64.

Donaldson, Erle C.; Chilingarian, George V.; Yen, Teh Fu (Eds.) (1989) Microbial enhanced oil recovery. Amsterdam, Oxford, New York, Tokyo: Elsevier.

D'Souza, C.; Nakano, M.M and Zuber, Peter (1994) Identification of *comS*, a gene of the *srfA* operon that regulates the establishment of genetic competence in *Bacillus subtilis*. PNSA 91: 9397–9401.

Dubois, M.; Gilles, K.A; Hamilton, J.K; Rebers, P.A and Smith, F. (1956) Colorimetric Method for determination of sugars and related substances. Analytical Chemistry 28: 350–356.

Edwards, Katherine R.; Lepo, Joe Eugene and Lewis, Michael A. (2003) Toxicity comparison of biosurfactants and synthetic surfactants used in oil spill remediation to two estuarine species. Marine Pollution Bulletin 46: 1309–1316.

Ermolenko, Z.M; Kholodenko, V.P; Chungunov, V.A; Zhirkova, N.A and Raulova, G.E (1997) A mycobacterial strain isolated from the oil of the Ukhtinskoe oil fiel: identification and degradative properties. Microbiology 66: 542–545.

Fabret, Céline; Quentin, Yves; Guiseppi, Annick; Busuttil, Jeanine; Haiech, Jacques and Denizot, Francois (1995) Analysis of errors in finished DNA sequences: the surfactin operon of *Bacillus subtilis* as an example. Microbiology 141: 345–350.

Franzetti, Andrea et al. (2012) Environmental fate, toxicity, characteristics and potential applications of novel bioemulsifiers produced by *Variovorax paradoxus* 7bCT5. Bioresource Technology 108: 245–251.

Franzetti, Andrea; Di Gennaro, Patrizia; Bevilacqua, Alessandro; Papacchini, Maddalena and Bestetti, Giuseppina (2006) Environmental features of two commercial surfactants widely used in soil remediation. Chemosphere 62: 1474–1480.

Franzetti, Andrea; Gandolfi, Isabella; Bestetti, Giuseppina; Smyth, Thomas J. P. and Banat, Ibrahim M. (2010) Production and applications of trehalose lipid biosurfactants. European Journal of Lipid Science and Technology 112: 617–627.

Friedman, Mendel (2004) Applications of the Ninhydrin Reaction for Analysis of Amino Acids, Peptides, and Proteins to Agricultural and Biomedical Sciences. Journal of Agricultural and Food Chemistry 52: 385–406.

Fusconi, Roberta; Nascimento Assunção, Rosana Maria; Moura Guimarães, Renata de; Filho, Guimes Rodrigues and da Hora Machado, Antonio Eduardo (2010) Exopolysaccharide produced by *Gordonia polyisoprenivorans* CCT 7137 in GYM commercial medium and sugarcane molasses alternative medium: FT-IR study and emulsifying activity. Carbohydrate Polymers 79: 403–408.

Gerson, O.F and Zajic, J.E (1978) Surfactant production from hydrocarbons by *Corynebacterium lepus* sp. nov. and *Pseudomonas asphaltenicus* sp. nov. Dev. Ind. Microbiol. 19: 577–599.

Ghribi, Dhouha; Mnif, Ines; Boukedi, Hanen; Kammoun, Radhouane and Ellouze-Chaabouni, Semia (2011) Statistical optimization of low-cost medium for economical production of Bacillus subtilis biosurfactant, a biocontrol agent for the olive moth Prays oleae. African Journal of Microbiology Research 5: 4927–4936.

Gusiatin, Zygmunt Mariusz and Klimiuk, Ewa (2012) Metal (Cu, Cd and Zn) removal and stabilization during multiple soil washing by saponin. Chemosphere 86: 383–391.

Gunther, N.W; Nuñez, A.; Fett, W. and Solaiman, D.K.Y (2005) Production of Rhamnolipids by *Pseudomonas chlororaphis*, a Nonpathogenic Bacterium. Applied and Environmental Microbiology 71: 2288–2293.

Hauser, G. and Karnovsky, M.L (1957) Rhamnose and rhamnolipid biosynthesis by *Pseudomonas aeruginosa*. Journal of Biology and Chemistry: 91–105.

Herman, D.C; Artiola, J.F and Miller, Raina M. (1995) Removal of cadmium, lead, and zinc from soil by a rhamnolipid biosurfactant. Environmental Science & Technology 29: 2280–2285.

Hewald, S.; Josephs, K. and Bolker, M. (2005) Genetic Analysis of Biosurfactant Production in Ustilago maydis. Applied and Environmental Microbiology 71: 3033–3040.

Hirata, Yoshihiko et al. (2009) Novel characteristics of sophorolipids, yeast glycolipid biosurfactants, as biodegradable low-foaming surfactants. Journal of Bioscience and Bioengineering 108: 142–146.

Holliger, C. et al. (1997) Contaminated environments in the subsurface and bioremediation: organic contaminants. FEMS Microbiology Reviews 20: 517–523.

Honma, Miho; Tanaka, Kazuaki; Konno, Katsuhiro; Tsuge, Kenji; Okuno, Toshikatsu and Hashimoto, Masaru (2012) Termination of the structural confusion between plipastatin A1 and fengycin IX. Bioorganic & Medicinal Chemistry 20: 3793–3798.

IHS Chemical (2010) Specialty Chemicals Update Program - Surfactants. Available online at http://www.ihs.com/products/chemical/planning/scup/surfactants.aspx.

Ishigami, Yutaka; Yamazaki, S. and Gama, Y. (1983) Surface active properties of biosoap from spiculisporic acid. Journal of Colloid and Interface Science 94: 131–139.

Ishigami, Yutaka; Zhang, Yuejun and Faxiang, Ji (2000) Spiculisporic acid. Functional development of biosurfactant. Chimica Oggi 18.

Ivshina, I.B; Kuyukina, M.S; Philip, J.C and Christofi, N. (1998) Oil desorption from mineral and organic materials using biosurfactant complexes produced by *Rhodococcus* species. World Journal of Microbiology and Biotechnology 14: 711–717.

Izard, Jacques and Limberger, Ronald J. (2003) Rapid screening method for quantitation of bacterial cell lipids from whole cells. Journal of Microbiological Methods 55: 411–418.

Janek, Tomasz; Łukaszewicz, Marcin; Rezanka, Tomas and Krasowska, Anna (2010) Isolation and characterization of two new lipopeptide biosurfactants produced

by Pseudomonas fluorescens BD5 isolated from water from the Arctic Archipelago of Svalbard. Bioresource Technology 101: 6118–6123.

Jarvis, F. G. and Johnson, M. J. (1949) A glyco-lipid produced by *Pseudomonas aeruginosa*. American Journal of Chemistry.

Jenneman, G.E et al. (1983) Halotolerant, biosurfactant-producing *Bacillus* species potentially useful for enhanced oil recovery. Dev. Ind. Microbiol.: 485–492.

Joshi, Sanket; Bharucha, Chirag; Jha, Sujata; Yadav, Sanjay; Nerurkar, Anuradha and Desai, Anjana J. (2008) Biosurfactant production using molasses and whey under thermophilic conditions. Bioresource Technology 99: 195–199.

Kanga, S.A; Bonner, J.S; Page, C.A; Mills, M.A and Autennrieth, R.L (1997) Solubilization of Naphthalene and Methyl-Substituted Naphthalenes from Crude Oil Using Biosurfactants. Environmental Science Technology: 556–561.

Kibron Inc. (2012) Measurement techniques of surface tension. Kibron Inc. Available online at http://www.kibron.com/surface-tension/measurement-techniques#wilhelmy-plate.

Kiran, Seghal G.; Thomas, Anto T.; Selvin, Joseph; Sabarathnam, B. and Lipton, A.P (2010) Optimization and characterization of a new lipopeptide biosurfactant produced by marine *Brevibacterium aureum* MSA13 in solid state culture. Bioresource Technology 101: 2389–2396.

Kitamoto, Dai; Isoda, Hiroko and Nakahara, Tadaatsu (2002) Functions and Potential Applications of Glycolipid Biosurfactants. from Energy-Saving Materials to Gene Delivery Carriers. Journal of Bioscience and Bioengineering 94: 187–201.

Klosowska-Chomiczewska, I.E; Medrzycka, K. and Karpenko, E. (2011) Biosurfactants - Biodegradability, Toxicity, Efficiency in comparison with synthetic surfactants. Available online at http://www.wis.pk.edu.pl/media/file/konferencje/Klosowska-Chomiczewska.pdf.

Kluge, Britta; Vater, Joachim and Salnikow, Johann Eckart Klaus (1988) Studies on the biosynthesis of surfactin, a lipopeptide antibiotic from *Bacillus subtilis* ATCC 21332. Federation of European Biochemical Societies Letters 231: 107–110.

Knickerbocker, C.; Nordstrom, D.K and Southam, G. (2000) The role of ‘‘blebbing’’ in overcoming the hydrophobic barrier during biooxidation of elemental sulfur by *Thiobacillus thiooxidans*. Chemical Geology 169: 425–433.

Kretschmer, A.; Bock, H. and Wagner, F. (1982) Chemical and physical characterization of interfacial active lipids from *Rhodococcus erythropolis* grown on normal alkanes. Applied and Environmental Microbiology 44: 864–870.

Kuyukina, Maria S.; Ivshina, Irena B.; Makarov, Sergey O.; Litvinenko, Ludmila V.; Cunningham, Colin J. and Philp, James C. (2005) Effect of biosurfactants on crude oil desorption and mobilization in a soil system. Environment International 31: 155–161.

Kvenvolden, K. A. and Cooper, C. K. (2003) Natural seepage of crude oil into the marine environment. Geo-Marine Letters 23: 140–146.

Lai, Chin-Chi; Huang, Yi-Chien; Wei, Yu-Hong and Chang, Jo-Shu (2009) Biosurfactant-enhanced removal of total petroleum hydrocarbons from contaminated soil. Journal of Hazardous Materials 167: 609–614.

Laycock, M.V; Hildebrand, P.D; Thibault, P.; Walter, J.A and Wright, J.L.C (1991) Viscosin, a potent peptidolipid biosurfactant and phytopathogenic mediator produced by a pectolytic strain of *Pseudomonas fluorescens*. Journal of Agriculture & Food Chemistry 39: 483–489.

Lebron-Paler, Ariel (2008) Solution and interfacial characterization of rhamnolipid biosurfactant from *P. aeruginosa* ATCC 9027. Dissertation. University of Arizona.

Lima, T.M.S; Procopio, L.C; Brandao, F.D; Carvalho, A.M.X; Totola, M.R and Borges, A.C (2011) Biodegradability of bacterial surfactants. Biodegradation 22: 585–592.

Lin, S.C (1996) Biosurfactants: Recent advances. Journal of Chemistry, Technology and Biotechnology 66: 109–120.

Liu, Jun et al. (2011) Functions of Lipopeptides Bacillomycin D and Fengycin in Antagonism of *Bacillus amyloliquefaciens* C06 towards *Monilinia fructicola*. Journal of Molecular Microbiology and Biotechnology 20: 43–52.

Liu, Ke-Shun (1994) Preparation of fatty acid methyl esters for gas-chromatographic analysis of lipids in biological materials. Journal of the American Oil Chemists' Society 71: 1179–1187.

Maier, Rainer M. (2003) Biosurfactants: Evolution and Diversity in Bacteria. Advances in Applied Microbiology 52: 101–121.

Maier, Rainer M. and Soberón-Chávez, Gloria (2000) *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential applications. Applied Microbiology and Biotechnology 54: 625–633.

Makkar, Randhir S.; Cameotra, Swaranjit S. and Banat, Ibrahim M. (2011) Advances in utilization of renewable substrates for biosurfactant production. Applied and Industrial Microbiology (AMB Express) 1: 5.

Maneerat, S.; Bamba, T.; Harada, K.; Kobayashi, A.; Yamada, H. and Kawai F. (2006) A novel crude oil emulsifier excreted in the culture supernatant of a marine bacterium, Myroides sp. strain SM1. Applied Microbiology & Biotechnology 70: 254–259.

Marahiel, M.A; Danders, Wolf; Krause, Michael and Kleinkauf, Horst (1979) Biological Role of Gramicidin S in Spore Functions. Studies on Gramicidin-S-

Negative Mutants of Bacillus brevis ATCC 9999. European Journal of Biochemistry 99: 49–55.

Margesin, R. and Schinner, F. (2001) Bioremediation (Natural Attenuation and Biostimulation) of Diesel-Oil-Contaminated Soil in an Alpine Glacier Skiing Area. Applied and Environmental Microbiology 67: 3127–3133.

Marinescu, Mariana; Lacatusu, Anca; Dumitru, M. and Marinescu, M. (2012) Dynamic of Petroleum Hydrocarbon Concentration in Soil Polluted with Crude Oil. Research Journal of Agricultural Science 44: 240–243.

Matsuyama T, Kaneda K. Nakagawa Y. Isa K. Hara-Hotta H. Yano I. (1992) A novel extracellular cyclic lipopeptide which promotes flagellum-dependent and -independent spreading growth of *Serratia marcescens*. Journal of Bacteriology 174: 1769–1776.

Mukherjee, Soumen; Das, P. and Ramkrishna Sen (2006) Towards commercial production of microbial surfactants. Trends in Biotechnology 24: 509–515.

Mulligan, Catherine N. (2005) Environmental applications for biosurfactants. Environmental Pollution: 183–198.

Mulligan, Catherine N. (2009) Recent advances in the environmental applications of biosurfactants. Current Opinion in Colloid & Interface Science 14: 372–378.

Mulligan, Catherine N. and Gibbs, B.F (1989) Correlation of nitrogen metabolism with biosurfactant production by *Pseudomonas aeruginosa* biosurfactant. Applied and Environmental Microbiology 55: 3016–3019.

Mulligan, Catherine N.; Yong, R.N and Gibbs, B.F (2001) Surfactant-enhanced remediation of contaminated soil: a review. Engineering Geology 60: 371–380.

Najafi, A.R; Rahimpour, M.R; Jahanmiri, A.H; Roostaazad, R.; Arabian, D. and Ghobadi, Z. (2010) Enhancing biosurfactant production from an indigenous strain of *Bacillus mycoides* by optimizing the growth conditions using a response surface methodology. Chemical Engineering Journal 163: 188–194.

Nakar, David and Gutnick, D.L (2001) Analysis of the wee gene cluster responsible for the biosynthesis of the polymeric bioemulsifier from the oil-degrading strain *Acinetobacter lwoffii* RAG-1. Microbiology 147: 1937–1946.

Nakar, David and Gutnick, D.L (2003) Involvement of a Protein Tyrosine Kinase in Production of the Polymeric Bioemulsifier Emulsan from the Oil-Degrading Strain *Acinetobacter lwoffii* RAG-1. Journal of Bacteriology 185: 1001–1009.

Nazina, T.N; Sokolova, D.Sh; Grigoŕyan, A.A; Xue, Y.-F; Belyaev, S.S and Ivanov, M.V (2003) Production of Oil-Releasing Compounds by Microorganisms from the Daqing Oil Field, China. Microbiology 72: 173–178.

Nguyen, Thu T.; Edelen, Ashley; Neighbors, Bridgett and Sabatini, David A. (2010) Biocompatible lecithin-based microemulsions with rhamnolipid and sophorolipid biosurfactants: Formulation and potential applications. Journal of Colloid and Interface Science: 498–504.

Nguyen, Thu T.; Youssef, Noha H.; McInerney, Michael J. and Sabatini, David A. (2008) Rhamnolipid biosurfactant mixtures for environmental remediation. Water Research 42: 1735–1743.

Nishikiori, T.; Naganawa, Hiroshi; Muraoka, Y.; Aoyagi, T. and Umezawa, H. (1986a) Plipastatins: new inhibitors of phospholipase A2 produced by *Bacillus cereus* BMG 302-fF67. III. Structural elucidation of plipastatins. The Journal of Antibiotics 39: 755–761.

Nishikiori, T.; Naganawa, Hiroshi; Muraoka, Y.; Aoyagi, T. and Umezawa, H. (1986b) Plipastatins: new inhibitors of phospholipase A2 produced by *Bacillus cereus* BMG 302-fF67. II. Structure of fatty acid residue and amino acid sequence. The Journal of Antibiotics 39: 745–754.

Nitschke, M.; Costa, S.G and Contiero, Jonas (2005) Rhamnolipid surfactants: an update on the general aspects of these remarkable biomolecules. Biotechnology Progress: 1593–1600.

Ochsner, Urs A.; Koch, Andreas K.; Fiechter, Armin and Reiser, Jakob (1994) Isolation and Characterization of a Regulatory Gene Affecting Rhamnolipid Biosurfactant Synthesis in Pseudomonas aeruginosa. Journal of Bacteriology 176: 2044–2054.

Oliveira, N.L; Commendatore, M.G; Delgado, O. and Esteves, J.L (2003) Microbial characterization and hydrocarbon biodegradation potential of natural bilge waste microflora. Journal for Industrial Microbiology and Biotechnology: 542–548.

Orea, Miguel A.; Alberdi, Margarita; Ruggiero, Armando and Colaiocco, Silvia (2002) Alternative Calibration and Standardization Procedure of Iatroscan TLC-FID for Sara Hydrocarbon Class Quantification: Application to TAR-MAT Zone Identification. Fuel Chemistry Division Preprints 47: 652–656.

Pacwa-Płociniczak, Magdalena; Płaza, Grażyna A.; Piotrowska-Seget, Zofia and Cameotra, Swaranjit Singh (2011) Environmental Applications of Biosurfactants: Recent Advances. International Journal of Molecular Sciences 12: 633–654.

Patel, Martin (2003) Surfactants Based on Renewable Raw Materials. Journal of Industrial Ecology 7: 47–62.

Patel, R.M and Desai, A.J (1997) Biosurfactant production by Pseudomonas aeruginosa GS3 from molasses. Letters in Applied Microbiology: 91–94.

Pesce, L.A biotechnological method for the regeneration of hydrocarbons from dregs and muds on the base of biosurfactants. App. no. 495. Patent no. 02/062.

Piljac, A.; Stipcević, T.; Piljac-Zegarac, J. and Piljac, G. (2008) Successful treatment of chronic decubitus ulcer with 0.1% dirhamnolipid ointment. Journal of cutaneous medicine and surgery: 142–146.

Price, Neil P.J; Ray, Karen J.; Vermillion, Karl E.; Dunlap, Christopher A. and Kurtzman, Cletus P. (2012) Structural characterization of novel sophorolipid

biosurfactants from a newly identified species of *Candida* yeast. Carbohydrate Research: 33–41.

Puvvada, Sudhakar and Blankschtein, Daniel (1990) Molecular-thermodynamic approach to predict micellization, phase behavior and phase separation of micellar solutions. I. Application to nonionic surfactants. Journal of Chemical Physics 92.

Raaijmakers, Jos M.; Bruijn, Irene de; de Kock and Maarten J. D. (2006) Cyclic Lipopeptide Production by Plant-Associated Pseudomonas spp.: Diversity, Activity, Biosynthesis and Regulation. Molecular Plant-Microbe Interactions 19: 699–710.

Rahim, Rahim et al. (2001) Cloning and functional characterization of the Pseudomonas aeruginosa *rhlC* gene that encodes rhamnosyltransferase 2, an enzyme responsible for dirhamnolipid biosynthesis. Molecular Microbiology 40: 708–718.

Ratsep, Peter and Shah, Vishal (2009) Identification and quantification of sophorolipid analogs using ultra-fast liquid chromatography–mass spectrometry. Journal of Microbiological Methods 78: 354–356.

Rendell, N.B; Taylor, Graham W.; Somerville, M.; Todd, H.; Wilson, R. and Cole, P.J (1990) Characterisation of *Pseudomonas rhamnolipids*. Biochemica et Biophysica Acta: 189–193.

Robert, M.; Mercadé, M.E; Bosch, M.P; Parra, J.L; Espuny, M.J and Manresa, M.A Guinea J. (1989) Effect of the carbon source on biosurfactant production by *Pseudomonas aeruginosa* 44T1 11: 871–874.

Romero, Diego et al. (2007) The Iturin and Fengycin Famillies of Lipopeptides are Key Factors in Antagonism of *Bacillus subtilis* towards *Podosphaera fusca*. Molecular Plant-Microbe Interactions 20: 430–440.

Ron, Eliora Z. and Rosenberg, Eugene (2001) Natural roles of biosurfactants. Environmental Microbiology 3: 229–236.

Ron, Eliora Z. and Rosenberg, Eugene (2002) Biosurfactants and oil bioremediation. Current Opinion in Biotechnology 13: 249–252.

Rosenberg, Eugene and Ron, Eliora Z. (1997) Bioemulsans: microbial polymeric emulsifiers. Current Opinion in Biotechnology 8: 313–316.

Rosenberg, Eugene and Ron, Eliora Z. (1999) High- and low-molecular-mass microbial surfactants. Applied Microbiology & Biotechnology 52: 154–162.

Rosenberg, Eugene; Rubinovitz, C.; Legmann, r. and Ron, Eliora Z. (1988) Purification and Chemical Properties of Acinetobacter calcoaceticus A2 Biodispersan. Applied and Environmental Microbiology 54: 323–326.

Rosenberg, Eugene; Zuckerberg, A.; Rubinovitz, C. and Gutnick, D.L (1979) Emulsifier of *Anthrobacter* RAG-1: Isolation and Emulsifying Properties. Applied and Environmental Microbiology 37.

Rosen, Milton J.; Kunjappu, Joy T. (2012) Surfactants and Interfacial Phenomena. 4.th ed. Hoboken, New Jersey: John Wiley & Sons.

Royal Society of Chemistry (2003) Surfactants: the ubiquitous amphiphiles. United Kingdom of Greatbritain. Available online at http://www.rsc.org/chemistryworld/Issues/2003/July/amphiphiles.asp.

Saharan, B.S; Sahu, R.K and Sharma, D. (2011) A Review on Biosurfactants: Fermentation, Current Developments and Perspectives. Genetic Engineering and Biotechnology Journal.

Saifer, A. and Feldmann, N. (1971) The photometric determination of gangliosides with the sulfo-phospho-vanillin reaction. Journal of Lipid Research 12: 112–115.

Saimmai, A.; Sobhon, V. and Maneerat, S. (2011) Molasses as a Whole Medium for Biosurfactants heir Applications. Applied Biochemstry & Biotechnology 165: 315–335.

Saini, H.S et al. (2008) Efficient Purification of the Biosurfactant Viscosin from *Pseudomonas libanensis* Strain M9-3 and its Physicochemical and Biological Properties. Journal of natural Products: 1011–1015.

Satpute, S.K et al. (2010a) Molecular Genetics of Biosurfactant Synthesis in Microorganisms. In Ramkrishna Sen (Ed.): Biosurfactants: 14–41, vol. 672. New York: Springer Science (Advances in Experimental Medicine and Biology).

Satpute, S.K; Banpurkar, A.G; Dhakephalkar, P.K; Banat, Ibrahim M. and Chopade, B.A (2010b) Methods for investigating biosurfactants and bioemulsifiers: a review. Critical Reviews in Biotechnology 30: 127–144.

Schwarzenbach, Rene P.; Gschwend, Philip M.; Imboden, Dieter M. (2005) Environmental Organic Chemistry: John Wiley & Sons.

Sekhon, Kamaljeet; Khanna, Sunil and Cameotra, Swaranjit (2011) Enhanced biosurfactant production through cloning of three genes and role of esterase in biosurfactant release. Microbial Cell Factories 10: 49.

Seydlová, Gabriela; Cabala, Radomir and Svobodova, Jaroslava (2011) Surfactin - Novel Solutions for Global Issues. In M.A Komorowska, S. Olsztynska-Janus (Eds.): Biomediacl Engineering, Trends, Research and Technologies: 305-.

Seydlová, Gabriela and Svobodová, Jaroslava (2008) Review of surfactin chemical properties and the potential biomedical applications. Central European Journal of Medicine 3: 123–133.

Smyth, T. J. P.; Perfumo, A.; Marchant, R. and Banat*, I. M. (2010a) Isolation and Analysis of Low Molecular Weight Microbial Glycolipids. In K.N Timmis (Ed.): Handbook of Hydrocarbon and Lipid Microbiology: 3705–3723. Hydocarbons, Oils and Lipids; Diversity, Properties and Formation. 1 volume. Berlin - Heidelberg: Springer Verlag.

Smyth, Thomas J. P.; Perfumo, A.; McClean, S.; Marchant, R. and Banat, Ibrahim M. (2010b) Isolation and Analysis of Lipopeptides and High Molecular Weight Biosurfactants. In K.N Timmis (Ed.): Handbook of Hydrocarbon and Lipid Microbiology: 3687–3704. Hydocarbons, Oils and Lipids; Diversity, Properties and Formation. 1 volume. Berlin - Heidelberg: Springer Verlag.

Soberon-Chavez, Gloria (Ed.) (2011) Biosurfactants (Microbiology Monographs, 20). Berlin - Heidelberg: Springer-Verlag.

Soberon-Chavez, Gloria and Maier, Rainer M. (2011) Biosurfactants - A General Overview. In Gloria Soberon-Chavez (Ed.): Biosurfactants. Berlin - Heidelberg: Springer-Verlag (Microbiology Monographs, 20).

Stanghellini, Michael E. and Miller, Raina M. (1997) Biosurfactants: Their Identity and Potential Efficacy in the Biological Control of Zoosporic Plant Pathogens. Plant Disease 81: 4–12.

Stein, Torsten (2005) Bacillus subtilis antibiotics: structures, syntheses and specific functions. Molecular Microbiology 56: 845–857.

Stover, C.K et al. (2000) Complete genome seequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature.

Syldatk, C. and Wagner, F. (1987) Production of biosurfactants. In N. Kosaric, W.L Cairns, N.C.C Gray (Eds.): Biosurfactants and Biotechnology: 89–120. New York: Marcel Dekker.

Tapi, Arthur; Chollet-Imbert, Marlène; Scherens, Bart and Jacques, Philippe (2010) New approach for the detection of non-ribosomal peptide synthetase genes in *Bacillus* strains by polymerase chain reaction. Applied Microbiology and Biotechnology 85: 1521–1531.

Thavasi, Rengathavasi; Jayalakshmi, Singaram and Banat, Ibrahim M. (2010) Biosurfactants from marine bacterial isolates. Applied Microbiology and Microbial Biotechnology: 1367–1373.

Thermo Scientific (2013) Tween-80 Detergent Solution. Thermo Scientific. Available online at http://www.piercenet.com/browse.cfm?fldID=6403AF4C-5056-8A76-4E45-B7648E03C63B.

Toren, A.; Navon-Venezia, S.; Ron, E. Z. and Rosenberg, E. (2001) Emulsifying Activities of Purified Alasan Proteins from *Acinetobacter radioresistens* KA53. Applied and Environmental Microbiology 67: 1102–1106.

Torres, L.G; Ramos, F.; Avila, M.A and Ortiz, I. (2012) Removal of methyl parathion by surfactant-assisted soil washing and subsequent wastewater biological treatment. Journal of Pesticide Science 37: 240–246.

Tuleva, Borjana K.; Christova, Nelly E.; Cohen, Rashel; Antonova, Daniela; Todorov, Tihomir and Stoineva, Ivanka (2009) Isolation and characterization of trehalose tetraester biosurfactants from a soil strain *Micrococcus luteus* BN56. Process Biochemistry 44: 135–141.

U.S. EPA, Technology Innovation Office (21/06/1996)King of Prussia Technical Corporation Superfund Site Cost and Performance Report. Available online at http://clu-in.org/download/remed/kop.pdf, checked on 15/01/2013.

Umezawa, H. et al. (1986) Plipastatins: new inhibitors of phospholipase A2 produced by *Bacillus cereus* BMG302-fF67. I. Taxonomy, production, isolation and preliminary characterization. Journal of Antibiotics 39: 737–744.

Urum, Kingsley; Grigson, Steve; Pekdemir, Turgay and McMenamy, Sean (2006) A comparison of the efficiency of different surfactants for removal of crude oil from contaminated soils. Chemosphere 62: 1403–1410.

Urum, Kingsley and Pekdemir, Turgay (2004) Evaluation of biosurfactants for crude oil contaminated soil washing. Chemosphere 57: 1139–1150.

Urum, Kingsley; Pekdemir, Turgay; Ross, David and Grigson, Steve (2005) Crude oil contaminated soil washing in air sparging assisted stirred tank reactor using biosurfactants. Chemosphere 60: 334–343.

Vanittanakom, Nongnuch; Loeffler, Wolfgang; Koch, Ulrike and Jung, Guenther (1986) Fengycin- A Novel Antifungal Lipopeptide Antibiotic produceed by *Bacillus subtilis* F-29-3. Journal of Antibiotics 39: 888–901.

Venkatesh, Narayana Murthy and Vederaman, Nagarajan (2012) Remediation of soil contaminated with copper using Rhamnolipids produced from *Pseudomonas aeruginosa* MTCC 2297 using waste frying rice bran oil. Ann Microbiology 62: 85–91.

Volpon, Laurent; Besson, Francoise and Lancelin, Jean-MArc (2000) NMR structure of antibiotics plipastatin A and B from *Bacillus subtilis* inhibitors of phospholipase A2. FEBS Letters 485: 76–80.

Wang, Suiling and Mulligan, Catherine N. (2009) Rhamnolipid biosurfactant-enhanced soil flushing for the removal of arsenic and heavy metals from mine tailings. Process Biochemistry 44: 296–301.

Wang, Wuncheng and Keturi, Paul H. (1990) Comparative seed germination tests using ten plant species for toxicity assessment of a metal engraving effluent sample. Water, Air and Soil Pollution 52: 369–376.

Waritha Tulalamba (2012) Efficiency of Biosurfactant from *Bacillus* sp. GY19 on Enhancement of Pyrene Solubilization and Biodegradation. Master Thesis. Chulalongkorn University, Bangkok.

Whang, Liang-Ming; Liu, Pao-Wen G.; Ma, Chih-Chung and Cheng, Sheng-Shung (2008) Application of biosurfactants, rhamnolipid, and surfactin, for enhanced biodegradation of diesel-contaminated water and soil. Journal of Hazardous Materials 151: 155–163.

Whitehouse, P. Wilkinson M. Fawell J. and Sutton A. (1998) Proposed Environmental Quality Standards for Nonylphenol in Water.

Whitfield, Chris and Paiment, Anne (2003) Biosynthesis and assembly of Group 1 capsular polysaccharides in *Escherichia coli* and related extracellular polysaccharides in other bacteria. Carbohydrate Research 338: 2491–2502.

Whitfield, Chris and Roberts, Ian S. (1999) Structure, assembly and regulation of expression of capsules in *Escherichia coli.* Molecular Microbiology 31: 1307–1319.

Zhang, Guo-liang; Wu, Yue-ting; Qian, Xin-ping and Meng, Qin (2005) Biodegradation of crude oil by *Pseudomonas aeruginosa* in the presence of rhamnolipids. Journal of Zhejiang University SCIENCE 6: 725–730.

Zhang, Yimin and Miller-Maier, R.M (1992) Enhanced Octadecane Dispersion and Biodegradation by a *Pseudomonas* Rhamnolipid Surfactant (Biosurfactant). Applied and Environmental Microbiology 58: 3276–3282.

APPENDICES

APPENDIX A

1. Solutions and media

a) Luria Bertani medium

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Aqua dest.	1000 ml

b) Productive medium

Glucose	1.00 g
Beef Extract	0.50 g
K_2HPO_4	1.00 g
KH_2PO_4	0.14 g
$NaNO_3$	5.00 g
$CaCl_2$	0.04 g
NaCl	0.04 g
$MgSO_4 \times 7H_2O$	0.30 g
$FeSO_4 \times 7H_2O$	0.10 g

Filled up to 1 l with aqua dest. pH = 7,5, autoclaved for 15 min at 110˚C

c) Tris-EDTA buffer

1 M Tris-HCl pH 8	1 ml
0.5 M EDTA pH 8	0.2 ml

Filled up to 100 ml with aqua dest.

d) 10% SDS

SDS	10 g
Aqua dest.	70 ml

Dissolved at 70^{o}C and brought up with aqua dest. to 100 ml.

e) CTAB in 0.7M NaCl

NaCl	4.1 g
Distilled water	80 ml
CTAB	10 g

Slowly added under stirring, while heating at 65˚C
Filled up with aqua dest. to 100 ml

f) 5 M NaCl

NaCl 14.61 g
Filled up to 50 ml with aqua dest.

g) Phenol : Chloroform solution

Phenol 25 ml
Chloroform 25 ml
Equilibrated by extraction several times with 0.1 M Tris-HCl (pH 7.6)
The equilibrated mixture under equal volume of 0.01 M Tris-HCl (pH 7.6) will be stored in a dark glass bottle.

h) Phospho-Vanillin Reagent (P-V Reagent)

Vanillin 120 mg
Aqua dest. 20 ml
Filled up to 100ml with phosphoric acid

i) Bradford Reagent

Coomassi Blue 25 mg
Phosphoric acid 50 ml
Filled up to 500 ml with aqua dest.

APPENDIX B

Figure B- 1 Graph with logarithmic scale of BSF concentration plotted to the measured surface tension, to calculate cmc values. 89

Figure B- 2 ANOVA two factor with replication of soil washing test. 93

Figure B- 3 Single factor ANOVA of germinated seeds of lettuce. 96

Figure B- 4 t-Test of SDS solution and tap water with seed germination of lettuce 97

Figure B- 5 Single factor ANOVA of germinated seeds of cucumber. 97

Figure B- 6 t-Test of SDS solution and tap water with seed germination of cucumber. 98

Figure B- 7 t-Test of Tween 80® solution and tap water with seed germination of cucumber. 98

Figure B- 8 Single factor ANOVA of germinated seeds of tomato. 99

Figure B- 9 t-Test of SDS solution and tap water with seed germination of tomato. 99

Table B- 1 Surface tensions for CMC calculation of BSF of *Bacillus* GY 19 with 3% pure glycerol as substrate. 89

Table B- 2 Surface tensions for CMC calculation of BSF of *Bacillus* GY 19 with 3% Molasses as substrate. 90

Table B- 3 Calculation of CMC, where m = slope, k = y-intercept 90

Table B- 4 Soil analysis report as obtained from Kasetsart University 'Agricultural Chemistry Group'; 09.10.2012 91

Table B- 5 Percentage of removal and standard deviation of different washing solutions on 3 types of soil 92

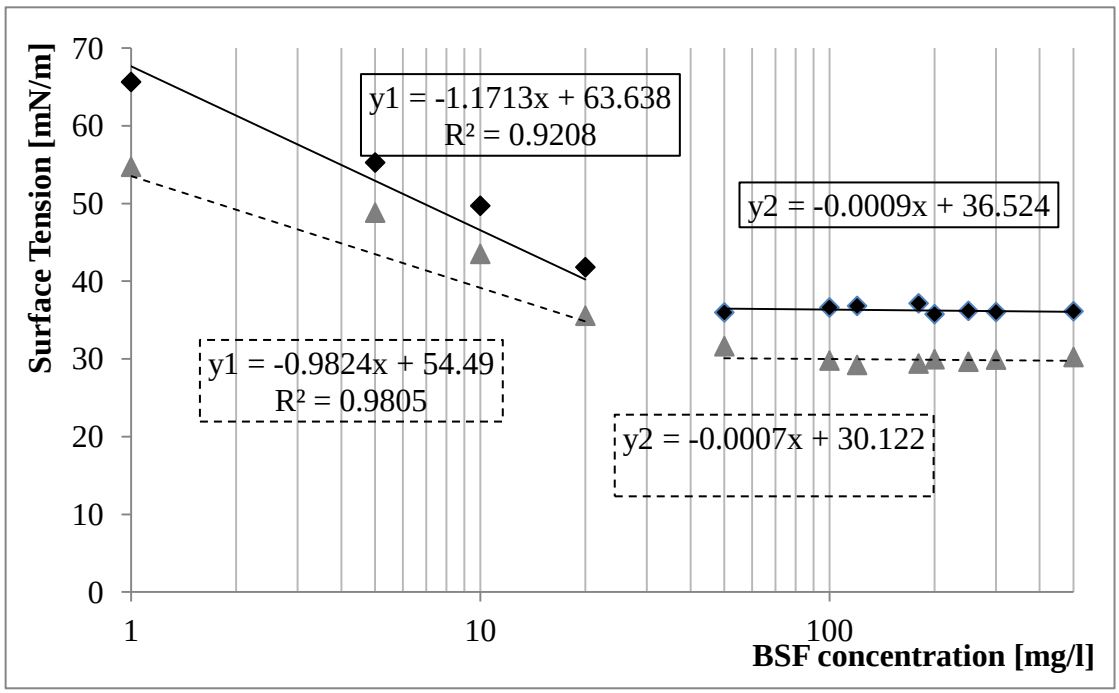


Figure B- 1 Graph with logarithmic scale of BSF concentration plotted to the measured surface tension, to calculate cmc values.

Table B- 1 Surface tensions for CMC calculation of BSF of *Bacillus* GY 19 with 3% pure glycerol as substrate.

Conc. [mg/l]	Surface tension [mN/m]			MW	SD
	1	2	3		
1	65.873	65.442	65.577	65.63	0.30
5	55.774	54.603	55.404	55.26	0.83
10	49.711	49.493	49.888	49.70	0.15
20	40.979	42.532	41.887	41.80	1.10
50	35.377	36.557	35.988	35.97	0.83
100	35.549	37.409	36.877	36.61	1.32
120	35.198	37.447	37.799	36.81	1.59
180	37.997	36.439	37.003	37.15	1.10
200	35.778	35.653	35.822	35.75	0.09
250	36.137	35.356	37.034	36.18	0.55
300	35.939	35.736	36.388	36.02	0.14
500	36.336	35.998	36.035	36.12	0.24

Table B- 2 Surface tensions for CMC calculation of BSF of *Bacillus* GY 19 with 3% Molasses as substrate.

Conc. [mg/l]	Surface tension [mN/m]			MW	SD
	1	2	3		
1	54.021	55.988	54.145	54.72	1.10
5	48.876	49.021	48.553	48.82	0.24
10	43.633	42.766	44.133	43.51	0.69
20	35.524	35.152	35.977	35.55	0.41
50	30.677	32.967	31.231	31.63	1.19
100	29.549	29.673	30.132	29.78	0.31
120	29.198	29.422	29.045	29.22	0.19
180	29.133	28.588	30.466	29.40	0.97
200	29.778	30.311	29.766	29.95	0.31
250	29.137	29.440	30.324	29.63	0.62
300	29.939	30.311	29.475	29.91	0.42
500	29.336	31.533	29.846	30.24	1.15

Table B- 3 Calculation of CMC, where m = slope, k = y-intercept

	m1	k1	m2	k2	CMC (mg/l)
BSF [3% PG]	-0.0009	36.5240	-1.1713	63.6380	23.1664
BSF [3% M]	-0.0007	30.122	-0.9824	54.4900	24.8222

Table B- 4 Soil analysis report as obtained from Kasetsart University 'Agricultural Chemistry Group'; 09.10.2012

Soil	pH	water cont.	EC	OM	Avai P	Avai K	Ca	Mg	OC	N	CEC	WHC	Sand	Silt	Clay	Texture
		%	dS/m	%	ppm	ppm	ppm	ppm	%	%	Cmol/kg	%	%	%	%	
1	7.8	26.19	0.112	6.44	475	616	5023	216	3.74	0.32	9.2	68.19	57.2	18.0	24.8	sandy clay loam
2	8.1	16.81	0.069	1.44	69	127	3724	118	0.84	0.07	22.9	37.07	55.2	16.6	28.2	sandy clay loam
3	7.6	2259	0.388	4.40	2	38	5871	82	2.55	0.22	4.6	65.59	53.2	34.8	12.0	sandy loam

Table B- 5 Percentage of removal and standard deviation of different washing solutions on 3 types of soil

	sandy clay loam 1		sandy clay loam 2		sandy loam 3	
	% removal	SD	% removal	SD	% removal	SD
Supernatant (3% PG)	21.97	1.10	38.43	6.47	30.17	2.33
Supernatant (3% M)	32.92	5.41	43.09	5.61	36.12	7.30
Foamate (3% PG)	44.69	1.10	53.65	6.32	45.80	1.71
Foamate (3% M)	26.49	1.29	27.05	3.92	34.30	2.99
Water	1.66	1.43	5.98	7.34	3.14	2.50
BSF 0.5 g/L (PG)	39.02	1.41	50.33	4.00	35.39	1.99
BSF 1 g/L (PG)	47.34	2.12	56.17	4.25	47.14	4.47
BSF 1.5 g/L (PG)	37.17	0.61	62.48	3.83	41.51	3.49
BSF 2 g/L (PG)	47.73	1.65	90.79	7.01	46.78	3.65
BSF 0.5 g/L (M)	40.38	0.87	53.18	5.16	50.79	3.11
BSF 1 g/L (M)	40.69	1.30	51.08	8.07	49.49	5.43
BSF 1.5 g/L (M)	36.16	5.51	56.39	5.91	51.33	3.68
BSF 2 g/L (M)	45.76	6.43	63.23	5.06	49.32	5.86
SDS (1 g/L)	35.55	2.37	59.26	3.49	31.10	2.36
SDS (2 g/L)	37.21	1.90	65.88	5.64	43.20	4.80
Tween 80® (1 g/L)	23.42	4.74	53.78	15.95	24.87	10.24
Tween 80® (2 g/L)	26.87	4.05	73.25	4.67	47.84	4.14

Anova: Two-Factor With Replication

SUMMARY	black	red	brown	Total
Water				
Count	3.00	3.00	3.00	9.00
Sum	260.05	317.29	297.16	874.50
Average	86.68	105.76	99.05	97.17
Variance	1.07	60.30	6.15	87.13
Supernatant [3% PG]				
Count	3.00	3.00	3.00	9.00
Sum	206.06	218.22	214.22	638.51
Average	68.69	72.74	71.41	70.95
Variance	1.21	47.86	2.77	16.16
Foamate [3% PG]				
Count	3.00	3.00	3.00	9.00
Sum	146.01	156.42	166.28	468.71
Average	48.67	52.14	55.43	52.08
Variance	1.21	10.86	0.90	11.81
Supernatant [3% M]				
Count	3.00	3.00	3.00	9.00
Sum	177.13	192.06	195.99	565.18
Average	59.04	64.02	65.33	62.80
Variance	29.27	12.92	22.76	24.48
Foamate [3% M]				
Count	3.00	3.00	3.00	9.00
Sum	194.12	246.20	201.56	641.88
Average	64.71	82.07	67.19	71.32
Variance	1.66	10.35	4.03	70.14
BSF [3% PG 0.5 g/l]				
Count	3.00	3.00	3.00	9.00
Sum	161.02	167.62	198.22	526.86
Average	53.67	55.87	66.07	58.54
Variance	1.98	5.00	1.73	35.01

Figure B- 2 ANOVA two factor with replication of soil washing test.

BSF [3% PG 1 g/l]				
Count	3.00	3.00	3.00	9.00
Sum	139.06	147.91	162.18	449.15
Average	46.35	49.30	54.06	49.91
Variance	4.51	4.40	5.83	15.02
BSF [3% PG 1.5 g/l]				
Count	3.00	3.00	3.00	9.00
Sum	165.92	126.61	179.45	471.97
Average	55.31	42.20	59.82	52.44
Variance	0.37	2.62	4.36	64.61
BSF [3% PG 2 g/l]				
Count	3.00	3.00	3.00	9.00
Sum	138.02	31.09	163.26	332.38
Average	46.01	10.36	54.42	36.93
Variance	2.71	0.53	3.95	412.06
BSF [3% M 0.5 d/l]				
Count	3.00	3.00	3.00	9.00
Sum	157.45	157.99	150.97	466.41
Average	52.48	52.66	50.32	51.82
Variance	0.75	7.38	2.44	3.91
BSF [3% M 1 g/l]				
Count	3.00	3.00	3.00	9.00
Sum	156.63	165.10	214.95	536.68
Average	52.21	55.03	71.65	59.63
Variance	1.70	19.73	15.14	91.89
BSF [3% M 1.5 g/l]				
Count	3.00	3.00	3.00	9.00
Sum	168.58	147.17	180.63	496.37
Average	56.19	49.06	60.21	55.15
Variance	30.41	8.40	4.92	34.87

Figure B- 2 (Cont.)

BSF [3% M 2 g/l]				
Count	3.00	3.00	3.00	9.00
Sum	143.23	124.08	161.63	428.95
Average	47.74	41.36	53.88	47.66
Variance	41.38	4.38	9.95	43.30
SDS 1 g/l				
Count	3.00	3.00	3.00	9.00
Sum	170.18	137.50	211.38	519.05
Average	56.73	45.83	70.46	57.67
Variance	5.63	2.55	2.78	116.95
SDS 2 g/l				
Count	3.00	3.00	3.00	9.00
Sum	165.81	103.25	174.27	443.33
Average	55.27	34.42	58.09	49.26
Variance	3.62	3.77	7.76	129.17
Tween 80® 1 g/l				
Count	3.00	3.00	3.00	9.00
Sum	202.21	155.98	230.49	588.68
Average	67.40	51.99	76.83	65.41
Variance	22.48	68.76	61.88	156.16
Tween 80® 2 g/l				
Count	3.00	3.00	3.00	9.00
Sum	193.11	90.28	160.03	443.42
Average	64.37	30.09	53.34	49.27
Variance	16.38	1.98	4.87	235.46
Total				
Count	51.00	51.00	51.00	
Sum	2944.60	2684.79	3262.65	
Average	57.74	52.64	63.97	
Variance	107.75	441.22	144.40	

Figure B- 2 (Cont.)

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	25568.12	16.00	1598.01	135.76	3.83E-61	1.74
Columns	3284.79	2.00	1642.39	139.53	6.43E-30	3.09
Interaction	7899.60	32.00	246.86	20.97	4.63E-32	1.56
Within	1200.67	102.00	11.77			
Total	37953.17	152.00				

Figure B- 2 (Cont.) ANOVA two factor with replication of soil washing test.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
PG	3.00	19.00	6.33	0.33
Molasses	3.00	22.00	7.33	0.33
Tween	3.00	9.00	3.00	0.00
SDS	3.00	4.00	1.33	0.33
Tap water	3.00	16.00	5.33	1.33

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	72.67	4.00	18.17	38.93	4.56E-06	3.48
Within Groups	4.67	10.00	0.47			
Total	77.33	14.00				

Figure B- 3 Single factor ANOVA of germinated seeds of lettuce.

t-Test: Two-Sample Assuming Unequal Variances		
	SDS	*Tap water*
Mean	1.33	5.33
Variance	0.33	1.33
Observations	3.00	3.00
Hypothesized Mean Difference	0.00	
df	3.00	
t Stat	-5.37	
P(T<=t) one-tail	0.01	
t Critical one-tail	2.35	
P(T<=t) two-tail	0.01	
t Critical two-tail	3.18	

Figure B- 4 t-Test of SDS solution and tap water with seed germination of lettuce.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
PG	10	64.10	6.41	26.02
Molasses	10	89.60	8.96	4.55
Tween 80®	10	41.00	4.10	29.72
SDS	10	10.10	1.01	0.49
Tap water	10	96.10	9.61	2.49

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	504.70	4.00	126.18	9.97	7.27E-06	2.58
Within Groups	569.37	45.00	12.65			
Total	1074.07	49.00				

Figure B- 5 Single factor ANOVA of germinated seeds of cucumber.

t-Test: Two-Sample Assuming Unequal Variances		
	SDS	*Tap water*
Mean	1.010	9.610
Variance	0.485	2.492
Observations	10.000	10.000
Hypothesized Mean Difference	0.000	
df	12.000	
t Stat	-15.760	
P(T<=t) one-tail	1.10E-09	
t Critical one-tail	1.78	
P(T<=t) two-tail	2.20E-09	
t Critical two-tail	2.179	

Figure B- 6 t-Test of SDS solution and tap water with seed germination of cucumber.

t-Test: Two-Sample Assuming Unequal Variances		
	Tween 80®	*Tap water*
Mean	4.100	9.610
Variance	29.716	2.492
Observations	10.000	10.000
Hypothesized Mean Difference	0.000	
df	10.000	
t Stat	-3.070	
P(T<=t) one-tail	5.92E-03	
t Critical one-tail	1.81	
P(T<=t) two-tail	1.18E-02	
t Critical two-tail	2.228	

Figure B- 7 t-Test of Tween 80® solution and tap water with seed germination of cucumber.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
PG	10	22.80	2.28	2.39
Molasses	10	21.50	2.15	3.36
Tween	10	28.20	2.82	3.68
SDS	10	2.80	0.28	0.06
Tap water	10	44.90	4.49	5.77

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	91.16	4	22.79	7.46	0.00011	2.58
Within Groups	137.38	45	3.05			
Total	228.54	49				

Figure B- 8 Single factor ANOVA of germinated seeds of tomato.

t-Test: Two-Sample Assuming Unequal Variances

	SDS	*Tap water*
Mean	0.280	4.490
Variance	0.064	5.765
Observations	10	10.000
Hypothesized Mean Difference	0	
df	9	
t Stat	-5.514	
P(T<=t) one-tail	1.87E-04	
t Critical one-tail	1.83	
P(T<=t) two-tail	3.73E-04	
t Critical two-tail	2.262	

Figure B- 9 t-Test of SDS solution and tap water with seed germination of tomato.

APPENDIX C

Figure C- 1 Calibration Curve for phospho-vanillin Test: glycerol trioleate as standard measured at 530 nm. 101

Figure C- 2 Calibration curve for Bradford Test: BSA as standard, measured at 495 nm. 102

Figure C- 3 Calibration curve for sulphoric acid test: glucose as standard, measured at 490 nm. 103

Figure C- 4 Chromatogram of Standard FAME mix after proceeding GC-FID . 104

Figure C- 5 GC-FID Chromatogram of FAME of crude BSF produced by *Bacillus* sp. GY19 using 3% pure glycerol as substrate. 105

Figure C- 6 ^{13}C-NMR sprektra from crude biosurfactant using 3% pure glycerol as substrate. 106

Figure C- 7 ^{1}H-NMR sprektra from crude biosurfactant using 3% pure glycerol as substrate. 107

Table C- 1 Raw data of phosphor-vanillin test for 2 crude BSF extracts..... 101

Table C- 2 Raw data of Bradford test for 2 crude BSF extracts..... 102

Table C- 3 Raw data of sulphoric acid test for 2 crude BSF extracts. 103

1. Phospho-Vanillin Test

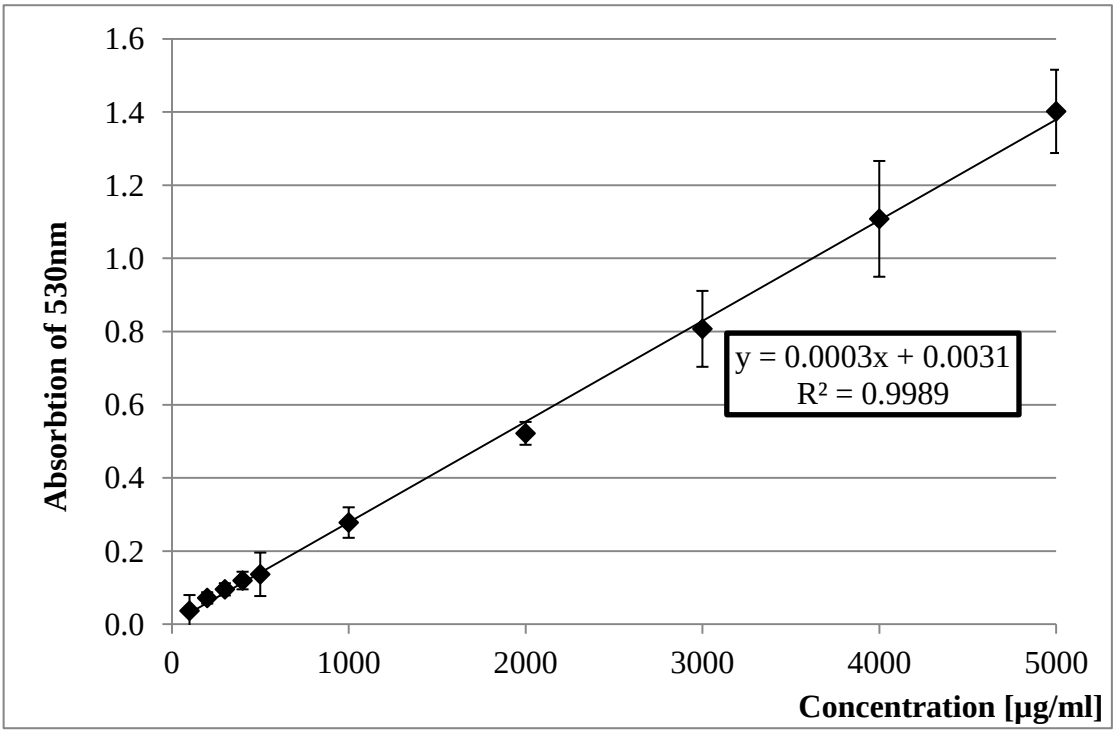


Figure C- 1 Calibration Curve for phospho-vanillin Test: glycerol trioleate as standard measured at 530 nm.

Table C- 1 Raw data of phosphor-vanillin test for 2 crude BSF extracts.

Sample	Dilution factor	Absorption			Mean	Conc. of Lipid [µg/ml]	Conc. of Lipid [mg/ml]	[%]
BSF [3% PG]	10	0.647	0.626	0.699	0.687	23016.67	23.02	34.77
BSF [3% M]	10	0.395	0.444	0.425	0.421	49150.90	14.15	29.17

2. Bradford Test

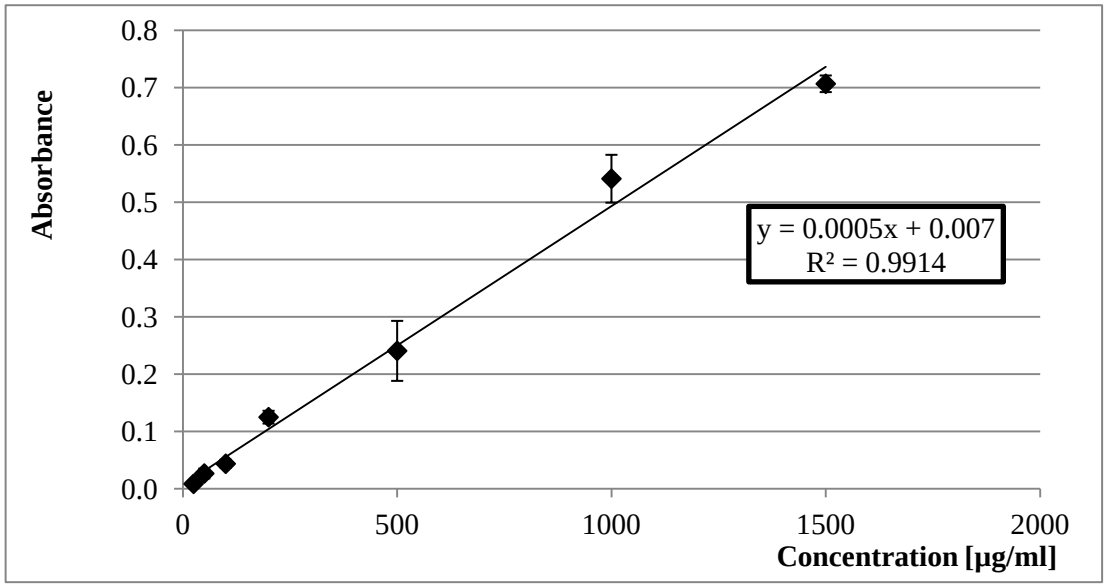


Figure C- 2 Calibration curve for Bradford Test: BSA as standard, measured at 495 nm.

Table C- 2 Raw data of Bradford test for 2 crude BSF extracts.

Sample	Dilution factor	Absorbance			Mean	Conc. of Protein [µg/ml]	[%]
BSF [3% M]	10	0.11	0.13	0.10	0.11	2093.33	4.32
BSF [3% PG]	10	0.33	0.32	0.32	0.33	6380.00	9.64

3. Sulphoric acid Test

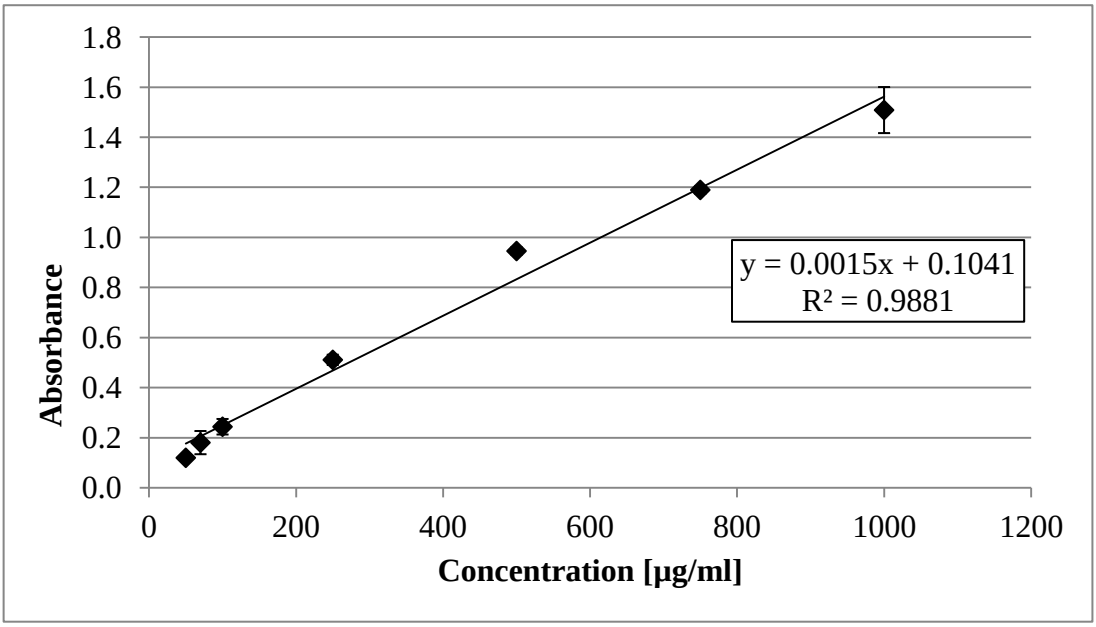


Figure C- 3 Calibration curve for sulphoric acid test: glucose as standard, measured at 490 nm.

Table C- 3 Raw data of sulphoric acid test for 2 crude BSF extracts.

Sample	Dilution factor	Absorption			Mean	Conc. of sugars [µg/ml]	Conc. of sugars [mg/ml]	[%]
BSF [3% M]	10	1.445	1.392	1.342	1.393	874.60	0.8746	**1.803**
BSF [3% PG]	10	0.587	0.596	0.569	0.584	335.27	0.3353	**0.506**

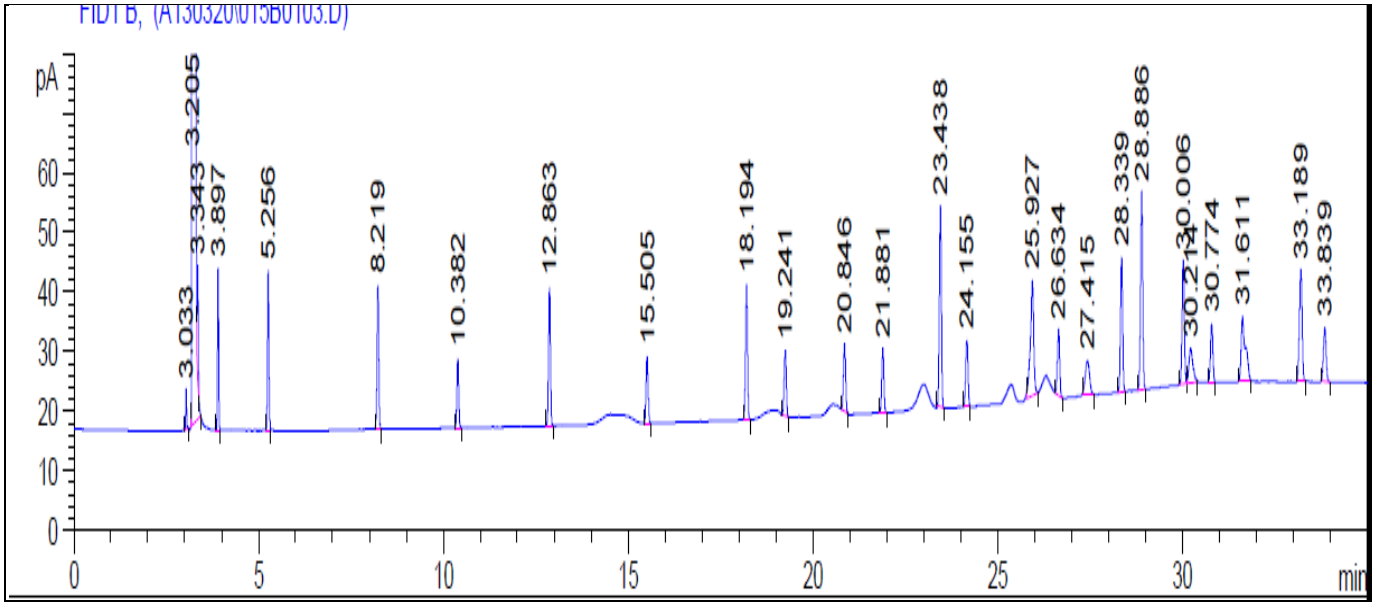


Figure C- 4 Chromatogram of Standard FAME mix after proceeding GC-FID

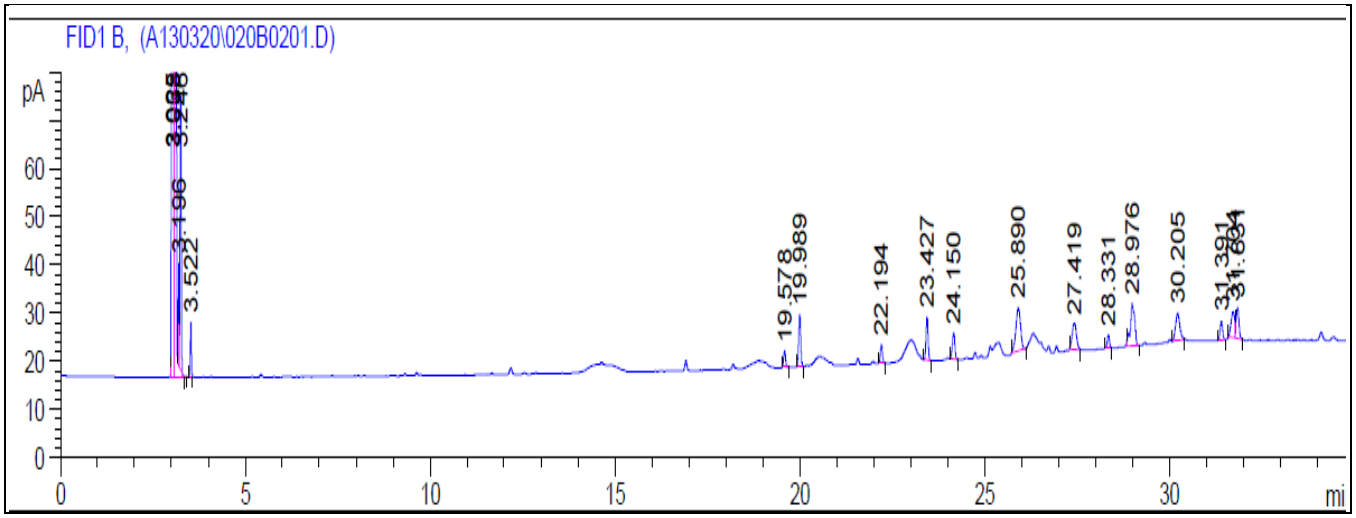


Figure C- 5 GC-FID Chromatogram of FAME of crude BSF produced by *Bacillus* sp. GY19 using 3% pure glycerol as substrate.

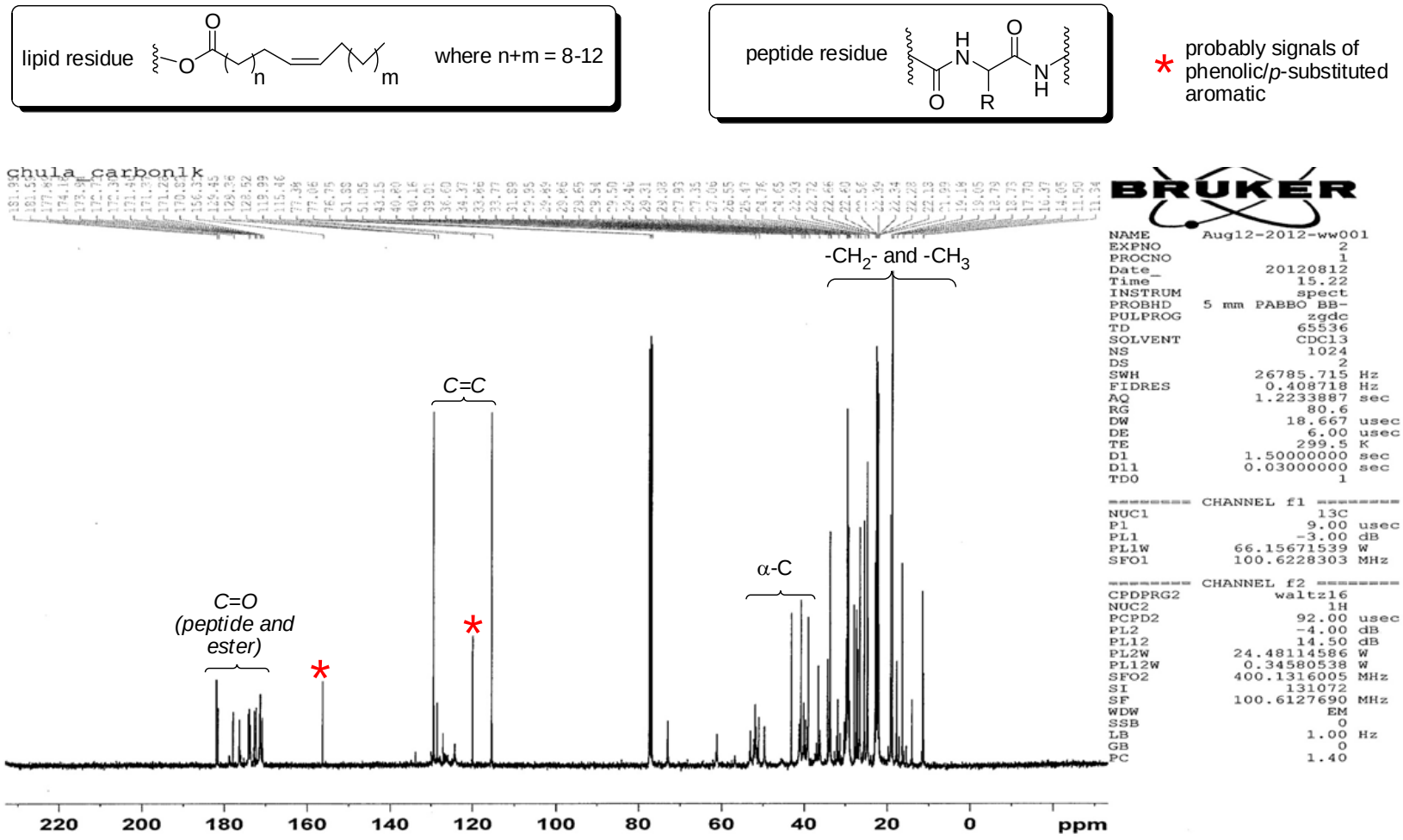


Figure C- 6 ^{13}C-NMR sprektra from crude biosurfactant using 3% pure glycerol as substrate.

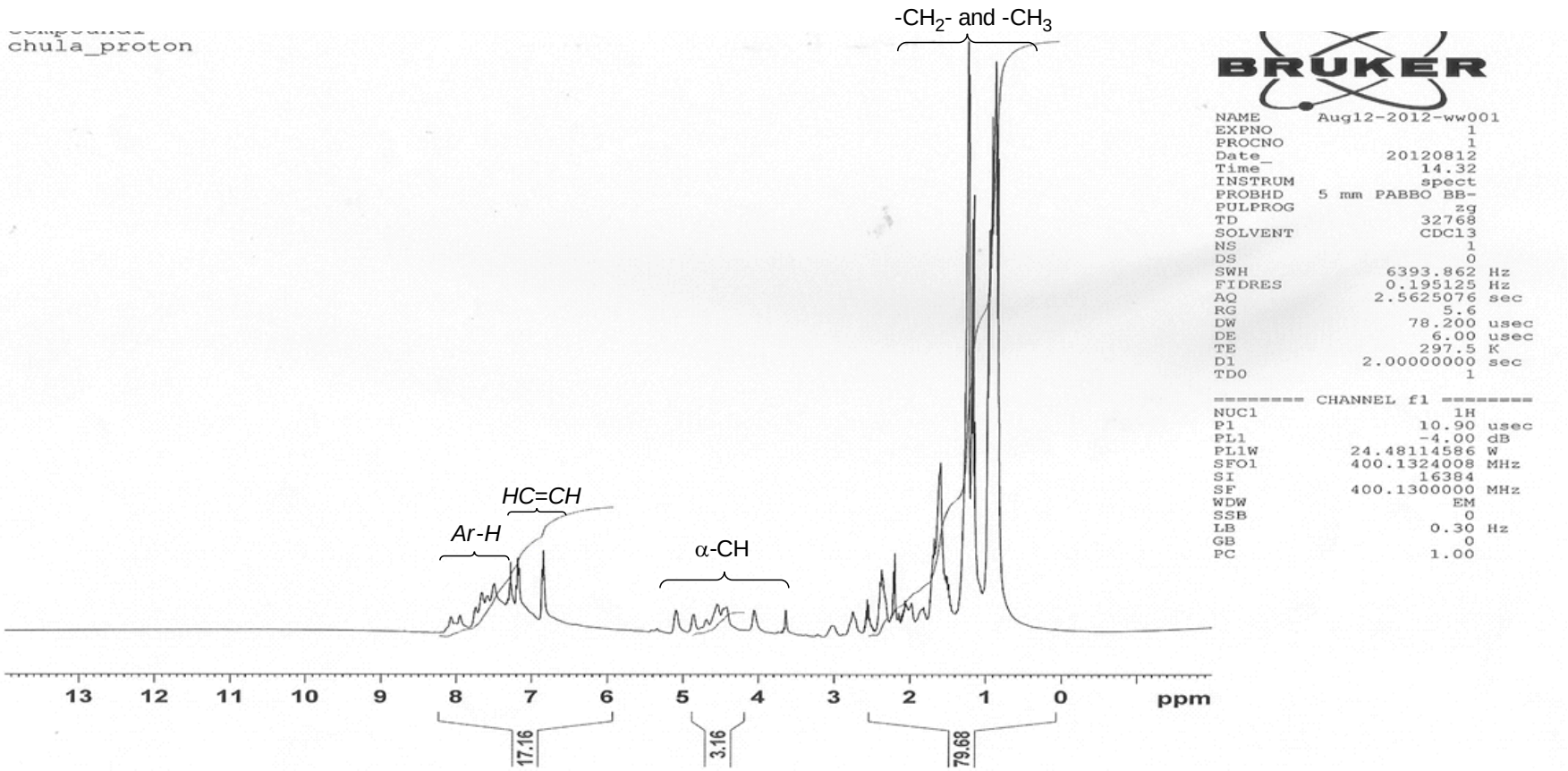


Figure C- 7 ^{1}H-NMR sprektra from crude biosurfactant using 3% pure glycerol as substrate.

APPENDIX D

>nucleotide sequence of Band No. 1 (Figure 23)/Primer pair Ap1-F/Tp1-R

GGCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATT
AGACAGCTGGCCAGATCACCCTGATACGCCAATTGCTGATTTTTCTTTTCT
TGATGCAACAGAAAAAGAGCAGATTGTCACACAGTTCAACAATACAAAA
ACAGAGTATCCAAAGAATCATACAATTATCGATTTATTTCGAGAACAAGC
AGAAAAGACGCCAGACCATATCGCACTTGTGTGTGGGAATTTGACTTTTTC
GTATGAAGAACTTGATAGACGCTCTAATTCACTCGCCAGAGCGTTATATC
AAAAAGGGTTTCGGAAGAACGAGACAGCCGGCATATTGGCTGCTCATTCT
GCCGAATTCATCATCAGTGTGCTTGCCGTTTTAAAAGCAGGGGGAGCATA
CCTCCCGCTTGATGCTGAGCTACCTCCTGAACGAGTCAGCTTTATGCTTGA
GGAAACGCAAGCAAAAATGCTGATTGTTCAAAAGGGACTGGAGCAAAAC
GCTGCGTTCTCAGGAACATGTATCATTTCAGATGCGCAGGGATTGATGGA
AGAGAACGATATCCCAATCAATATCACCTCCAGCCCGGACGATCTTGCAT
ACATCATGTATACCTCAGGATCAACAGGCCGGCCAAAAGGCGTCATGATC
ACCAATCGCAATGTCGTGTCCCTTGTCAAAAACAGCAATTACACGTCTGC
GTCCGTTGATGACCGATTTATTCTGACTGGATCTATCAGCTTTGACGCCGT
CACCTTTGAAATGTTCGGGGCGCTATTAAATGGTGCAAGCCTTCATATCAT
TGATCAGTCGACACTGCTGTCACCTGATCGGTTTGGGGCGTATTTGATTGA
AAATGACATCACCGTGCTATTTTTAACGACAGCTCTTTTTAATCAGCTGGC
ACAGGTACGAGCAGACATGTTCAGCGGGCTCCATACACTATATGTCGGAG
GAGAAGCACTCTCTCCCGCCCTGATGAATGCTGTCAGACATGCCTGTCCTG
ATCTTGCGCTTCATAATATTTACGGACCTACGGGAAAACCCGACATTTT

>nucleotide sequence of Band No. 2 (Figure 23)/Primer pair Ap1-F/Tp1-R

GGGATAGTCCTTATAGGGCGAAATTGGGCCCGACGTCGCATGCTCCCGGC
CGCCATGGCGGCCGCGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGA
GAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTA
AATAGCTTGGCGTATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATC
CGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCC
TGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTG
CCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGC
CAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTC
GCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAG
CTCACTCAAAGGGGTCTCCTGTTTCAACCTGGCGGGGCGGTAATACGGTT
ATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGA
ATTCGATTATGCCTTTCTTTGAATGTCCGCCGGGACCTGGTTGGTCGAGAT
GTGCAGTCGCGCGGGTCCGTTGGGTGCCTGCTGCAGCTCGACCCCCGGAC
CCTTGGGCTGGTTGTAGAACACGAAGTCGTCGTCGGTTCGCACGACGCCG
CGGTCGGTCACGAGCAGTGCCGACACGTCGGCGGCGGTCGCGAGGTCAAC
GGTGAGGACGATCTGGGAGGTCGACAGCGGGCCGTTCTGGCCTTTGGTCA
GCGAGGGCATGATGCTCAGGGTAGTCATCGGTCCCGACGTCCTCCGAATA
CTGTCGCCCGGGCGGCAGATGCGATCGACCGGGCGACAGAGAAGTGTCA
GTCGAGCGGCTTATGCTGCTCGTTATGTCAGGAAGCCGGGTCGGCACGCA
GTTCGGGCCATATCGTCTCGACGCGTTGTTGGGCCGGGGCGGCATGGGCG
AGGTCTACCGGGCCCACGACACCACCAAGGAGCGCACCGTCGCGCTGAA
GCTGCTCAATTCGGTATCCGCGAATCACTAGTGGGCCAGCAAAAGGCCAG
GAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCC
CTGACGAGCATCCCAAAAATCGACGCTCAAGTTCAAAGTTGGCGAAACCC
GACAGGGCTATAAAGATACCAGGCGTTTCCCCCGGGAAACTCCCTCTTTTC
CGGAAACTTGGCGCCTTTTTCTCTTTGGGAGAGAGGGGCGTTTCTTAAATC
ACCCCTCAG

>nucleotide sequence of Band No. 3 (Figure 23)/Primer pair Af2-F/Tf1-R

AAGTAATCCTATAGGGGCGAAATTGGGCCCGACGTCGCATGCTCCCGGCC
GCCATGGCGGCCGCGGGAATTCGATTGCTTTTATTGAATGTCCGCCCAATT
CAAAGAATGAATCCCCTATTCCGATCCTCTGGATGCCTAGAACCTCCTGCC
AGATCAATGCCAGCTGCTCTTCAAGTTCGTTGCGTGGCGGGATGTATGTTT
GTTTAGATGCGAAATCTGGTTCAGGCAGTGCTTTCCGGTTCAATTTGCCGT
TCGATGTGAGCGGAAGATTCTCCATTTCAATGATATACGCCGGAACCATA
TAATTAGGCAGTGAACGGGCGAGAAGAGAACGCACTTGTTCAGCATTCGT
GCCCGCTTTTACGCTGATATAGCCAAACAGCTCTTTGCTCCCTGACTGTCC
TGTACGTGCCAACACCGCCGCTTCTTTGACTCCGTCTATGTGGCGAAGTGC
TGATTCGATCTCACCCAGCTCTACCCGATAGCCTCTGACTTTTACTTGATC
ATCAATACGTCCGATATATTCAATCACTAGTGAATTCGCGGCCGCCTGCAG
GTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTAT
TCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCC
TGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAA
GCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTA
ATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAG
CTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGG
GCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCT
GCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAG
AATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAA
GGCCAGGAACCGTAAAAAAGGCGCGTTTTTTGGGGTTTTTCCTAAGGGCC
CCCCCCCCTGGCGAGAATCACAAAAAACAACGCTCAAATCCAAAGAGGG
GGGAAACCCCCCGGGGGTTTAAAGAAAACCAGGGGTTTCTCCTCGGAGAG
ACCCCCCGGGGGGTTCCCTGTGTCGAACCCAGCGCCTTACCCGGAAACAA
GGTCCGCCTTCTCTCTCTCTGGAAAGAAGGGAGGCGTTTCTATAATTACAC
CCGGAA

>nucleotide sequence of Band No. 4 (Figure 23)/Primer pair Af2-F/Tf1-R

ACATACTCGTATAGGGGCGAAATTGGGCCCGACGTCGCATGCTCCCGGCC
GCCATGGCGGCCGCGGGAATTCGATTAGACAGCTCGCAACATCCCCACCA
GATTTAGCGAGGATGCGCCGCGGGGTGGGTGCCTTGCCCTCAGCGGTGAT
CTCCCGGGCGTACTCCCAGATTTTCGATCGCCACCCGGATAGGGCTTCGG
GGTGGACGCGGTCGATTAGTTCGGTGTCGGGGTTGGTGACAGACAGGATC
GCCGATACCAGGACGAGTTCGTCTTGTTCGGATTCGTAGATCACAGTCGG
CCTTTCAGTGCCTGGACGAGTTCGTCGCGGTTCTCTTTGATCCAGCGGCGG
TGGAAGTCGCGGACGTAGGCGGGCTGGTCGAAGTCGGCGGGGATGGGGT
CGGGCCACCTGATGCAGTCGGGTTTACGGCCAGTTAGTTCGGTGATCTTGC
CAGCTGTCTAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATAT
GGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTC
ACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATT
GTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGT
AAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCG
CTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATG
AATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCG
CTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGG
TATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGAT
AACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACC
GTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACG
AGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGG
ACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCC
TGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTTTCCCTTTGGGA
AACGGGGGGCTTTCTCTAACTCACGCTGGGAGGTCTCAAATTCCGGGGAG
AGGCGTTCCTCCCAAACTGGGGGTGTGTGACAAAACCCCCCGTTTAACCC
ACCGCGTGGCCCTTATCCGGGAAATAATTTCTTTGTGTCCACCCCGGGAGA
AACAACATTATTCCCCT

>nucleotide sequence of Band No. 5(Figure 23)/Primer pair As1-F/Ts2-R

ANNTTNNTCTTAGGGCGATTGGGCCGACGTCGCATGCTCCCGGCCGCCAT
GGCGGCCGCGGGAATTCGATTATGCCTTTCCAGGAATGTCCGCCAGAAAT
AAAAAACCAGCTCGAACACTGTTCGTGTTGCACCATGCGGATGCTTCCAA
ATGGCCGGAAATTCTTCCTCATACAACTTTCTTGGCGTATGTCACACCTGA
ATCATTGGACATATATTTTTGGGATCAGGAATAAAGGAAACCCTGACTTA
TTACATGATCCCGGGCTTCTGGGTAACGACGAGCGAGCTTTGGGTTACGA
TCATATAGTCTGGGACCGCAAAGGCTTGCCTGAACGGACATCCTTTTCTGT
AAGTTTGGAATCAAAGCGCCGAGACCGACATGGTGTCCCTGCTTGCCGGT
TCTGGCATTGACGTGCTTGGAATGGCTGAACCGGTGTTCCGATAATTTCTT
CTCCCTGGGCGGACATTCCAAATAAGGAATCATCAATCACGAATTCATTG
CCGCCTGCCTGCCGACCATATGGGAGAGCTCCCTCGCGTTGGATGCAAAG
CTTGAGTATTCTATTCTATCACGTCACATAGCTTAGCTTAATCATGTCCAG
AGCTGTTTCCTGTGCGGAATTAAATTGCGATCACATTTCCACTCCACATAC
CAGACGGAAGCGTAACGTGTAAAGTATGGGGTGCCTAGTGTGTGGGCTGA
CTCACCTCACTTGAGTTGCGCTGAGTGCACGCTCTGCTGTCGGTCACCAGT
CTGTCATCTGACTGATGAATGGATCACGCAACGGGCGAGAGAGTGCGTAG
CGGATGGCTCTGTTCCCTTCTCCCTACTGATCGCTGCGTCGTCGCTGGGCG
AGCGGATCGGCTCAGCNACGCAGGATGCAGTATCTATCCATCAGGCATGA
GCNNNAAGAACTGTGAGTAACGTCAGGCAGGGCNGNCGTACATGACGTG
CTGCTGTTCTTGNTCGCCCCCGNNNCTACAAACGATCTCAGTCA

VITAE

Miss Alice Rau was born on February 16, 1988 in Guben, Germany. High school A-level was achieved in 2007 at the Franz-Groger-Gymnasium, Peitz, Germany. She graduated with a Bachelor degree of Science in Water Science from the Faculty of Chemistry of University of Duisburg-Essen, Germany. Bachelor Thesis was accomplished at the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB) Berlin, Germany from May to September, 2011.

Later, she pursued her master's degree study in the International Program in Environmental Management, National Center of Excellence for Environmental and Hazardous Waste Management (NCE-EHWM), Graduate School, Chulalongkorn University, Bangkok, Thailand from 2011-2013.

Rau, A., Phuwapraisirirsan, P., Luepromchai, E., and **Pinyakong**, **O.** (2013) Characterization of biosurfactants produced by *Bacillus* sp. GY19 and potential applications in soil washing. Proceeding. International Conference on Environmental and Hazardous Substance Management. Bangkok, Thailand, 21-23 May