Downstream purification of surfactin produced by *Bacillus subtilis* ATCC 21332

by

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Declaration

I By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Abstract

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is the most fatal disease from a single infectious agent. In 2014, 9.6 million people fell ill with TB and 1.5 million died from the disease. Surfactin offers a promise as an alternative antimicrobial agent against the organisms causing TB, as it possesses the ability to lyse cell membranes as well as the ability to alter membrane permeability. The use of surfactin as a medical drug is limited by its haemolytic activity, thus it can be used in other applications in the fight against TB such as hand sanitizers or in equipment and surface sterilisers. The antimicrobial activity of surfactin is limited in environments with high protein and lipid impurities thus it is necessary to purify surfactin from the medium in which it is produced. The aim of this study is therefore to propose a suitable strategy with operating conditions for surfactin purification. Since the study of surfactin purification is dependent on the analysis of surfactin concentration, an additional aim of this study is to develop and validate an analytical technique for surfactin concentration.

The proposed unit operations for surfactin purification were acid precipitation, solvent extraction and adsorption. Prior to optimisation of these unit operations, surfactin was produced batch-wise from *Bacillus subtilis* ATCC 21332 with a maximum surfactin concentration of 1109 mg/L and selectivity (surfactin to antifungal ratio) of 5.5 g_s/g_a. Thin layer chromatography (TLC) was developed for quantifying surfactin concentration, and . was further validated using the linearity and working range, limit of detection, limit of quantification, instrument limit of detection, accuracy, precision, specificity, selectivity and robustness under diverse solvent and sampling conditions.

After surfactin production and development of the TLC analytical technique, surfactin was isolated from cell-free supernatants of *B. subtilis* into a precipitate by acid precipitation. Acid precipitation was carried out by adding hydrochloric acid to cell-free supernatants to lower the pH, and recovering surfactin with the precipitate by centrifugation. Acid precipitation was optimised by studying the effect of the operating pH on surfactin purity, surfactin recovery, surfactin selectivity and relative concentrations of protein and lipid impurities. The recommended operating pH for acid precipitation was pH 4, where the surfactin purity, recovery and selectivity were 97%, 43% and 5.7 g_s/g_a respectively.

The precipitate from acid precipitation was further purified by solvent extraction. Solvent extraction was carried out by selectively dissolving surfactin in dry acid precipitates using various organic solvents of differing polarity. These solvents, in order of decreasing polarity, were: methanol, *i*-propanol, chloroform:methanol (1:1 v/v), acetonitrile, chloroform:methanol (2:1 v/v), acetone, chloroform, ethyl

acetate, methyl *tert*-butyl ether (MTBE), petroleum ether and *n*-hexane. Solvent extraction was optimised by studying the effect of solvent polarity on surfactin purity, surfactin recovery and relative concentration of protein and lipid impurities in solvents after extraction. Polar solvents had better surfactin recoveries and purities in comparison to non-polar solvents. MTBE gave both the highest recovery (100%) and highest purity (80%), thus was the best solvent for extraction. This recovery and purity was also higher than the recoveries and purities achieved by adsorption and acid precipitation, thus solvent extraction was the best purification technique in this study.

The precipitate from acid precipitation was also purified by adsorption. Adsorption was carried out using HP-20 non-polar resins on adsorption liquids formed by solubilising the precipitates in alkaline water. The solubilised precipitates were further mixed with water or methanol to form the adsorption liquid. Adsorption was optimised by studying the effect of initial pH, operating temperature, resin concentration to surfactin concentration (RC/SC) ratio and methanol concentration on the percentage of surfactin in the adsorption liquid adsorbed onto resins (% SA) and the factor by which surfactin selectivity improved after the adsorption (IS) using surface designs. The study of surfactin adsorption using surface designs or using methanol based adsorption liquid were new experimental approaches, not having being previously reported.

% SA improved with initial pH, operating temperature, RC/SC ratio and methanol concentration. IS independent of operating temperature, decreased with increase in RC/SC ratio and increased with pH and methanol concentration. The recommended initial pH, operating temperature, RC/SC ratio and methanol concentration was 11.5, 45 °C, 5 g_r/g_s and 30 % (v/v) respectively. The surfactin recovery and purity after purification by adsorption at the selected operating conditions were found to be 91% and 58% respectively. The presence of methanol in adsorption liquid was seen to improve surfactin adsorption rates. Equilibrium was reached 5 times faster in adsorption liquids with methanol compared to adsorption liquids without methanol. Surfactin adsorption can be defined as multilayer adsorption as it fitted the Freundlich model.

Acid precipitation, solvent extraction and adsorption were successful in purifying surfactin from *B. subtilis* cultures. This is of major significance as surfactin can be used in the fight against TB, which is the major cause of death from a single infectious agent globally.

Abstract (Afrikaans)

Onder siektes wat deur 'n aansteeklike agense veroorsaak word, is tuberkulose (TB), wat deur *Mycobacterium tuberculosis* veroorsaak word, die dodelikste. In 2014 het 9.6 miljoen mense siek geword van TB en 1.5 miljoen het van die siekte gesterf. Surfactin blyk 'n belowende alternatiewe antimikrobiese agens te wees teen organismes wat TB veroorsaak, soos dit die vermoë het om selmembrane te vernietig, asook om membrane se deurlaatbaarheid te verander; dus is dit in staat om M. tuberculosis te denatureer. Die gebruik van surfactin as mediese middel is beperk deur sy hemolitiese aktiwiteit. Dit kan dus gebruik word in ander toepassings in die stryd teen TB, soos handverzorgers of in toerusting en oppervlaksterilisators. Die antimikrobiese werking van surfactin is beperk in omgewings met hoë proteïen- en lipoïedonsuiwerhede, en dus is dit nodig om surfactin te suiwer van die medium waarin dit vervaardig word. Die doel van hierdie studie is dus om 'n gepaste strategie vir die suiwering van surfactin voor te stel, met bedryfstoestande. Aangesien die studie van surfactinsuiwering is afhanklik van die analise van surfactinkonsentrasie; is 'n verdere doel van hierdie studie om 'n tegniek te ontwikkel en te valideer vir die analise van surfactinkonsentrasie.

Die voorgestelde stappe vir surfactinsuiwering was suurpresipitasie, oplosmiddelonttrekking en adsorpsie. Voor optimalisering van hierdie stappe, is surfactin bondelgewys uit *Bacillus subtilis* ATCC 21332, met 'n maksiumum surfactinkonsentrasie van 1109 mg/L en selektiwiteit (verhouding surfactin tot antifungale middel) van 5.5 g_s/g_a, vervaardig .Dunlaag-chromatografie (DLC) is ontwikkel vir surfactin konsentrasie analise, was verder gevalideer deur na lineariteit en werksomvang, perke van opsporing, perke van kwantifisering, instrumentperke van opsporing, akkuraatheid, presisie, spesifisiteit, en selektiwiteit en robuustheid onder verskillende oplosmiddel- en monsternemingstoestande te verwys.

Na die surfactin produksie en ontwikkeling van die DLC analitiese tegniek, is surfactin deur suurpresipitasie tot 'n presipitaat geïsoleer van selvrye supernatante van *B. subtilis*. Suurpresipitasie is uitgevoer deur chloorwaterstof by selvrye supernatante te voeg om die pH, en verhaal surfactin in die presipitaat deur sentrifugasie. Suurpresipitasie is geoptimaliseer deur die effek van die werkende pH op suiwerheid, herstel, en selektiwiteit van surfactin, en relatiewe konsentrasies van proteïen- en lipoïedonsuiwerhede te ondersoek. Die aanbevole werkende pH vir suurpresipitasie was pH 4, en die suiwerheid, herstel, en selektiwiteit van surfactin was onderskeidelik 97%, 43% en 5.7 g_s/g_a.

Die presipitaat van die suurpresipitasie is verder gesuiwer deur oplosmiddelekstraksie. Oplosmiddelekstraksie is uitgevoer deur surfactin selektief in die presipitaat op te los deur verskeie

organiese oplosmiddels met verskillende polariteite te gebruik. Hierdie oplosmiddels, in volgorde van afnemende polariteit, was metanol, i-propanol, chloroform:metanol (1:1 v/v), asetonitriel, chloroform:metanol (2:1 v/v), asetoon, chloroform, etielasetaat, metiel *tert*-butieleter(MTBE), petroleum-eter en n-heksaan. Oplosmiddelekstraksie is geoptimaliseer deur ondersoek in te stel na die uitwerking van die polariteit van die oplosmiddel op suiwerheid, herstel, en selektiwiteit van surfactin, en relatiewe konsentrasies van proteïen- en lipoïedonsuiwerhede in oplosmiddels na ekstraksie. Daar is bevind dat polêre oplosmiddels beter surfactinherstel en surfactinsuiwerheid toon in vergelyking met nie-polêre oplosmiddels. MTBE het die hoogste surfactinsuiwerheid (80%) en surfactinherstel (100%) gelewer, en was dus die beste oplosmiddel vir surfactinsuiwering deur ekstraksie van oplosmiddels. Hierdie herstel en suiwerheid was ook hoër as die herwinnings en suiwerhede wat deur adsorpsie en suur neerslag verkry is, dus was oplosmiddelwinning die beste suiweringstegniek in hierdie studie.

Die presipitaat van suurpresipitasie is ook deur adsorpsie gesuiwer. Adsorpsie is uitgevoer deur die gebruik van HP-20 nie-polêre hars op adsorpsievloeistowwe wat gevorm is deur die presipitate in alkaliese water meer oplosbaar te maak. Die meer oplosbare presipitaat is, verder, met water of metanol gemeng om die adsorpsievloeistof te vorm. Adsorpsie is geoptimaliseer deur ondersoek in te stel na die effek van aanvanklike pH, bedryfstemperatuur, die verhouding van harskonsentrasie tot surfactinkonsentrasie (HK/SK) op die persentasie surfactin in die adsorpsievloeistof wat deur die hars geadsorbeer is (%SA), en die faktor waarteen die surfactinselektiwiteit verbeter het na die adsorpsie (VS) deur aanwending van oppervlakontwerpe. Die bestudering van surfactinadsorbsie deur oppervlakontwerpe is 'n nuwe eksperimentele benadering, nie voorheen geraporteer nie.

% SA verbeter met aanvanklike pH, bedryfstemperatuur, HK/SK-verhouding en metanol konsentrasie.. VS was onafhanklik van bedryfstemperatuur, het afgeneem met toenames in HK/SK-verhouding en verhoog met pH en metanol konsentrasie. Die aanbevole aanvanklike pH, bedryfstemperatuur, HK/SK-verhouding en metanol konsentrasie was onderskeidelik 11.5, 45°C, 31 g_r/g_s en 30% (v/v). Die surfactin herstel en suiwerheid na suiwering deur adsorpsie by die geselekteerde bedryfsomstandighede was onderskeidelik 91% en 58%. Die waarneming was dat die teenwoordigheid van metanol in die adsorpsievloeistof die koers waarteen surfactin geabsorbeer is, verbeter het. Ekwilibrium is vyf keer vinniger bereik in adsorpsievloeistowwe met metanol as in dié sonder metanol. Adsorpsie van surfactin kan gedefinieer word as multilaagadsorpsie met die feit dat dit geskik was vir die Freundlich-model.

Ekstraksie en adsorpsie was suksesvol in die versywering van surfactin B.subtillis cultures. Dit is van groot relevansie siendat surfactin gebruik kan word in die geveg teen TB, wat die grootste oorsaak van sterftes van ‘n enkele agent global is.

Journal paper

B Dlamini, V Rangarajan and K G Clarke (under review) Thin layer chromatography for the quantification of the surfactin lipopeptide – a manual approach, J Analytical Chemistry

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Glossary and nomenclature

Acronyms

ATCC 21332	Strain of *Bacillus subtilis*
C/M (1:1)	Mixture of chloroform and methanol in the volume ratio 1:1
C/M (2:1)	mixture of chloroform and methanol in the volume ratio 2:1
CCC	Circumscribed central composite design
CDW	Cell dry weight
CMC	Critical micelle concentration
FCC	Face-centred central composite design
h/d ratio	Column height to diameter ratio
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
ICC	Inscribed central composite design
ILOD	Instrument limit of detection
LOD	Limit of detection
LOQ	Limit of Quantification
MTBE	Methyl tert-butyl ether
MWCO	Molecular weight cut-off
PVDF	Polyvinylidene fluoride
RC/SC ratio	Resin concentration to surfactin concentration ratio
RP-HPLC	Reversed phase- high performance liquid chromatography
TB	Tuberculosis

Units

% (m/m)	Mass percentage
% (v/v) or vol%	Volume percentage
% (w/v)	Percentage weight per unit volume
°C	Degrees Celsius
g	Gram
g_a	Grams of antifungals
g_r	Grams of resins
g_s	Grams of surfactin
g_{tl}	Grams of total lipopeptides
h	Hours
KDa	Kilodaltons
L	Litre
mg	Milligrams
mm	Millimetres
$mm^2/g/L$	Square millimetres per grams per litre
nm	Nanometres
rpm	Revolutions per minute

Symbols

% $A_{antifungals}$	Percentage of antifungals adsorbed during surfactin adsorption
% SA	Percentage of surfactin adsorbed during surfactin adsorption
μ	True values of measured surfactin concentrations (g_s/L)
1/n	Empirical constant
c	Intraparticle diffusion constant (g_s/g_r)
$C_{a,e}$	Equilibrium antifungals concentration after adsorption (g_s/L)
$C_{a,i}$	Antifungals concentration in adsorption liquid (g_a/L)
C_{Ai}	Antifungals concentration in supernatants (g_a/L)
C_d	Surfactin concentration in desorption liquid (g_s/L)
C_e	Equilibrium surfactin concentration after adsorption (g_s/L)
C_i	Surfactin concentration in adsorption liquid (g_s/L)
C_{Si}	Surfactin concentration in supernatants (g_s/L)
C_t	Surfactin concentration at a particular time during adsorption (g_s/L)
IS	Factor by which surfactin selectivity over antifungals improved after acid precipitation and adsorption
k_1	First order kinetics model rate constant (1/h)
k_2	Second order kinetics model rate constant [$(g_s/g_r)^2/h$]
K_F	Freundlich constant [$(g_s/g_r)(L/g_s)^{1/n}$]
k_i	Intraparticle diffusion rate constant [$(g_s/g_r)(1/h^{0.5})$]
K_L	Langmuir constant (L/g_s)
m	First order kinetics model constant
M	Mass of residue after adsorption (g)

M_{Ai}	Mass of antifungals in supernatants (g_a)
M_{Ap}	Mass of antifungals in precipitate after acid precipitation (g_a)
M_{DS}	Mass of total solids extracted into solvents during solvent extraction (g)
M_P	Mass of dry precipitate after acid precipitation (g)
M_S	Mass of surfactin extracted into solvents during solvent extraction (g_s)
M_{Si}	Mass of surfactin in supernatants (g_s)
M_{SP}	Mass of surfactin in precipitate (g_s)
P_S	Surfactin purity
q_e	Surfactin adsorption capacity at equilibrium (g_s/g_r)
q_m	Theoretical maximum surfactin adsorption capacity (g_s/g_r)
q_t (g_s/g_r)	surfactin adsorption capacity at a particular time during adsorption
R^2	Regression coefficient
R_A	Antifungals recovery
R_b	Detergent to-lipid ratio in biological membrane
R_S	Surfactin recovery
S	TLC standard curve slope ($mm^2/g/L$)
S_S	Surfactin selectivity (g_s/g_a)
t	Time (h)
V_d	Volume of desorption liquid (L)
Vi	Volume of adsorption liquid (L)
W	Resin mass (g)
α	Accuracy

β	Precision
χ	Average values of measured surfactin concentrations (g_s/L)

Glossary

Integrated bioreactor	Bioreactor with *in situ* recovery
Multiple spotting	Adding a sample more than once on a particular spot on a TLC plate with intermediate drying
Simulated supernatant	Imitation of the media with used for surfactin production spiked with bovine serum albumin and surfactin.
Surfactin selectivity	Ratio of surfactin concentration to antifungals concentration in a sample

1 Introduction

This thesis provides detail on the purification of surfactin, produced by *Bacillus subtilis* ATCC 21332, which can be used in the fight against tuberculosis (TB). TB, caused by *Mycobacterium tuberculosis,* is an airborne disease that most often affects the lungs. According to World Health Organisation (WHO) data reviewed in March 2016, TB is a major infectious killer disease globally. In 2014, TB resulted in 9 million infections and 1.5 million deaths. TB is a global disease, but over 95% of cases and deaths are in developing countries. The TB mortality in South Africa is 44 per one hundred thousand people in the year 2014. Approximately a third of the world population has latent TB, and people with immune systems compromised by factors such as HIV, malnutrition or diabetes and tobacco use have a much higher risk of falling ill. HIV positive people are 20 to 30 times more likely to develop active TB disease, and approximately 0.4 million people died of HIV-associated TB in 2014, while a third of HIV deaths in 2015 were due to TB (WHO, 2016).

TB is preventable and curable using standard anti-TB drugs, which have been around for decades, provided these drugs are available and taken appropriately. However, some bacterial strains causing TB have developed resistance to standard anti-TB drugs. TB caused by bacteria which is resistant to isoniazid and rifampicin, which are the two most powerful standard anti-TB drugs, is called multidrug-resistant tuberculosis (MDR-TB). The rise and proliferation of MDR-TB is primarily caused by inappropriate use of anti-TB drugs, or use of poor quality medicines. There was a threefold increase new cases of MDR-TB between 2009 and 2013, and approximately 480 000 new cases were reported in 2013. MDR-TB can be treated with second-line drugs. Second-line treatment is however unreliable as it may have limited treatment options and limited access to recommended medication (WHO, 2016).

Surfactin offers promise as an alternative antimicrobial agent against the organisms causing TB, with potential effectiveness against MDR-TB (Das, et al., 2008). According to Heerklotz et al. (2004), surfactin possesses the ability to lyse cell membranes as well as the ability to alter membrane permeability. This can result in denaturing of *M. tuberculosis*, which causes TB. The use of surfactin as a drug is, however, limited by its haemolytic activity. Surfactin can therefore be used for functions such as surface and equipment sterilisation or in hand sanitizers. In this case, surfactin would be used to denature TB in phlegm in order to reduce the TB causing bacteria released to the air. The use of surfactin in anti-bacterial soaps can also be investigated as surfactin activity against cells is selective, and surfactin has a low toxicity for mammalian cells thus may not be harmful to the skin (Vollenbroich, et al., 1997). In

addition to antibacterial properties, surfactin has other properties such as antiviral (Vollenbroich, et al., 1997) and antitumor properties (Kameda, et al., 1974).

Surfactin is a biosurfactant, and biosurfactants have both lipophilic and hydrophilic moieties. The lipophilic group consists of a hydrocarbon chain of a fatty acid or sterol ring. The hydrophilic part contains a carboxyl group (composed of fatty acids or amino acids), a phosphoryl group (composed of phospholipids), and a hydroxyl group (composed of saccharides and peptides) (Kim, et al., 2004). Surfactin is classified as a lipopeptide biosurfactant, and is the most powerful biosurfactant synthesized by a linear, non-ribosomal peptide synthase to form a mixture of hepapeptides with a fatty acid chain consisting of 13-16 carbon atoms (Jauregi, et al*.,* 2013). The hepapeptide (L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu) part of surfactin has variable amino acids at positions 2, 4, and 7, where Glu is glutamic acid, Leu is leucine, Val is valine, and Asp is aspartic acid (Yang, et al., 2015). Surfactin therefore has numerous homologues. A typical structure of surfactin is shown in Figure 1-1.

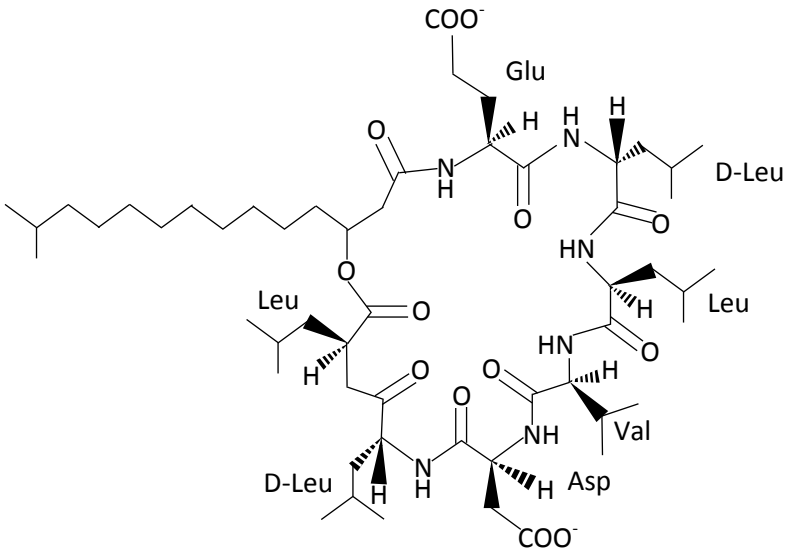

Figure 1-1: Example of a surfactin structure (redrawn from Kosaric & Sukan (2014))

To use surfactin in its potential applications in the fight against TB, a cost-effective purification technique for surfactin purification from *B. subtilis* cultures needs to be developed. The optimization of surfactin purification techniques is highly dependent on surfactin concentration analysis. Reversed phase-high performance liquid chromatography (RP-HPLC) has proven to be effective for surfactin analysis, but it is not readily available due to its expensive nature. A cheaper but accurate method for surfactin quantification is therefore needed. The first aim of this study was therefore to develop and

validate an analytical technique for analysis of surfactin concentration. This would allow the second, more significant aim, to be pursed. The second aim of this study was to propose a surfactin purification strategy with operating conditions. The operating conditions would be obtained through optimization of the proposed purification strategy.

This thesis first presents a literature review (chapter 2). The literature review consists of four parts. The first and second parts provide a review on surfactin antibacterial properties and production strategies. Production strategies were evaluated to determine an efficient strategy to produce surfactin for later use in surfactin purification and analysis studies. The literature review then provides and evaluates surfactin purification strategies, which was done in order to propose a suitable strategy for surfactin purification. Since it was realized that the analysis of surfactin concentration is of high importance in surfactin purification studies, the last part of the literature review provides an evaluation of strategies for analysis of surfactin concentration in order to develop an appropriate method for surfactin analysis.

The hypotheses and specific objectives of this study are then presented in chapter 3, based on the literature review. Methodologies for testing the hypotheses and carrying out the specific objectives are then presented in chapter 4. The methodology section begins by detailing how surfactin was produced, and how parameters for determining if surfactin production was efficient were obtained. The methodology section then details how a thin layer chromatography (TLC) was developed for surfactin concentration analysis. The methodology section then provides procedures for carrying out and optimising the various proposed surfactin purification techniques (acid precipitation, solvent extraction and adsorption) in order to recommend operating conditions for theses purification techniques. Lastly, the methodology section provides procedures for analysis of various parameters (such as glucose concentration and nitrate concentration).

The results as well as a discussion of the results obtained from the methodology section are then provided in chapter 5. Finally, conclusions and recommendations based on the results and discussion are provided in chapter 6. The conclusions evaluate whether the specific objectives were met and where the hypotheses were validated. Recommendations provide suggestions on improvements that can be done in future experiments in order to extend this study.

2 Literature review

2.1 Antibacterial activity of surfactin

Surfactin has biological properties such as antiviral (Vollenbroich, et al., 1997), antibacterial (Heerklotz & Seelig, 2007) and antitumor properties (Kameda, et al., 1974). Additionally, lipopeptide biosurfactants with surfactin characteristics have antibacterial activity against multidrug-resistant bacteria (Das, et al., 2008). Bence (2011) showed that surfactin has potential effectiveness against *M. tuberculosis*, where *Mycobacterium aurum* was used as a surrogate of *M. tuberculosis.* The biological properties of surfactin therefore hold promise for effectiveness against TB and MDR-TB causing organisms

Surfactin has a potential use in medical applications as it has selective antibacterial activity. Eeman et al. (2006) suggested that surfactin activity is selective depending on the composition of the lipid matrix of the target cell. Grau et al. (1999) found that surfactin perturbation was stronger in membranes which have phospholipids with a shorter chain length. Snook et al. (2009) stated that surfactin is mainly effective against Gram-negative bacteria although it is also effective against some Gram-positive bacteria. A drawback against the use of surfactin in medical applications is that it has haemolytic activity (Dufour, et al., 2005).

Various authors (Shen, et al., 2010; Deleu, et al., 2003; and Eeman, et al., 2006) have suggested that surfactin biological activities were likely due to the ability of surfactin to interact with biological membranes. According to Shen et al. (2010), surfactin was able to strongly interact with membranes due to its high amphiphilic character. Surfactin (in solution) has peptide ring which has a horse-saddle structure and a fatty acyl chain on the opposite end (Bonmatin, et al., 1992). The fatty acyl chain is capable of extending into lipid bilayers resulting in interaction of surfactin with biological membranes through hydrophobic interactions (Heerklotz & Seelig, 2001 and Maget-Dana & Ptak, 1995). Homologues containing long chain fatty acid were seen to have stronger antimicrobial activities in comparison to those with shorter chains (Dhanarajan, et al., 2016). Additionally, conic molecules with large head groups have relatively higher constraints in the lipid packing thus more potent compared to rod like molecules (Dufour, et al., 2005). Surfactin structure and hydrophobicity thus affect the antibacterial activity of surfactin, hence different surfactin isoforms could have different antibacterial activities.

The interaction of surfactin with biological membranes results in membrane permeability changes and/or membrane disruption (Heerklotz, et al., 2004). The molecular mechanism of surfactin activity

against biological membranes is not fully understood, but numerous suggestions have been made. One suggestion was that surfactin results in permeability changes in lipid bilayers in membranes by inducing cationic pores in the membranes (Sheppard, et al., 1991), which results from the ability of surfactin to bind with monovalent and divalent cations (Eeman, et al., 2006). The channel-forming activity can be increased by the membrane dipole potential, which results in an increase in both the number of open channels and their conductance (Ostroumova, et al., 2010). Surfactin membrane activity therefore partly depends on presence of monovalent and divalent ions. It has also been suggested that the denaturing of membranes by formation of ion-conducting pores is prominent at moderate surfactin concentrations, while the detergent effect prevails at high concentrations (Bonmatin, et al., 2003).

It has been suggested that surfactin results in permeability changes and/or membrane disruption through the detergent effect. Surfactin inserts into membranes, but has a preference for micelle formation over membrane insertion (Heerklotz & Seelig, 2001). Surfactin thus forms micelles in the membranes which result in pore formation in the membranes as well as leakages of the target cell membranes (Heerklotz & Seelig, 2007 and Shen, et al., 2010). It was also proposed that surfactin interacts with membranes by binding with the outer monolayer, before slowly moving to the inner monolayer. The binding of surfactin with the outer monolayer therefore results in an asymmetric increase in lateral pressure on the membrane. This causes the bilayer to bend, and may lead to membrane disruption at a certain surfactin threshold concentration (Heerklotz & Seelig, 2001 and Heerklotz & Seelig, 2007).

The solubilisation of target cell membranes by the detergent effect is induced by the detergent to-lipid ratio in the membrane (R_b). Membrane leakage begins when R_b is approximately 0.05 and membrane lysis or solubilisation begins when R_b is 0.22 and is completed when R_b is 0.43 (Heerklotz & Seelig, 2007). The aqueous surfactin concentration thus determines the antibacterial activity of surfactin.

In addition to dependence on surfactin concentration, the antibacterial activity of surfactin is also dependent on surfactin purity. Snook et al. (2009) noted that the antimicrobial activity of surfactin is limited in environments with high protein and lipid concentrations. Mukherjee et al. (2009) found that purified surfactin had a higher antimicrobial activity compared to impure surfactin at the same concentration.

2.2 Surfactin production

To take advantage of the uses and potential uses of surfactin, surfactin can be produced batch wise, fed-batch wise or continuously in submerged bioreactor systems (Isa, et al., 2008). An ideal production process is one that results in high surfactin quantity and selectivity. High surfactin selectivity is necessary as it significantly reduces the cost and complexity of the downstream purification process (Rangarajan & Clarke, 2016). Surfactin selectivity is the ratio of surfactin concentration to antifungals concentration in supernatants of *B. subtilis*. *B. subtilis* produces two other lipopeptides (iturin and fengycin) in addition to surfactin, which are also known as antifungals. These antifungals result in complexity of downstream purification as they have an amphiphilic nature like surfactin thus present difficulty in separating from surfactin (Dhanarajan, et al., 2015).

Studies of surfactin production from various *Bacillus* spp. (*Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus spizizenii*) in literature are summarised in Table 2-1. The studies in Table 2-1 were compared to determine optimal conditions for surfactin production. Ideal production is one with relatively high final surfactin concentration in cultures after production and a relatively low harvesting time. The maximum amount of surfactin was obtained in the study by Wei et al. (2007) at some 3340 mg/L, although the harvesting time was not specified. The study by Chen et al. (2006) produced surfactin in the shortest amount of time (24 h).

Table 2-1: Summary of surfactin production studies in literature

Bacteria species	Carbon and nitrogen source (g/L)	Trace elements and other elements (g/L)	Reactor type	Surfactin (mg/L)	Production duration (h)	Source
***B. subtilis* BS5**	Molasses: 160 (mL/L), $NaNO_3$: 5	$ZnSO_4 \cdot 7H_2O$: 0.16, $FeCl_3 \cdot 6H_2O$: 0.27, $MnSO_4 \cdot H_2O$: 0.017	Shake flasks	1120	72	Abdel-Mawgoud, et al., (2008b)
***B. subtilis* BBK006**	0.2% $C_6H_{12}O_6$, NH_4Cl: 1	NaCl: 5, $MgSO_4$: 0.12, $CaCl_2$: 0.11	Integrated bioreactor	136		Chen, et al., (2006)
***B. subtilis* BBK006**	0.2% $C_6H_{12}O_6$, NH_4Cl: 1	NaCl: 5, $MgSO_4$: 0.12, $CaCl_2$: 0.11	Shake flasks	92	24	Chen, et al., (2006)
***B. subtilis* ATCC 21332**	$C_6H_{12}O_6$: 40, NH_4NO_3: 4	$MgSO_4$: 0.096, $CaCl_2$: 0.00078, $FeSO_4$: 0.00061, Na_2EDTA: 0.0015	Bioreactor	800		Cooper, et al., (1981)
***B. subtilis* ATCC 21332**	$C_6H_{12}O_6$: 10 - 40, NH_4NO_3- 4	Not specified	Bioreactor	439	48	Davis, et al., (1999)
***B. subtilis* LSFM-05**	Glycerol: 50, $NaNO_3$: 3	NaCl: 0.1, $MgSO_4.7H_2O$: 0.5, Vitamin stock solution	Bioreactor	930	60	De Faria, et al., (2011)
***B. subtilis* ATCC 21332**	$C_6H_{12}O_6$: 40, NH_4NO_3: 4	$CaCl_2$: 0.7, Na_2EDTA: 0.0015	Shake flasks	1500	72	Huang, et al., (2015)
***B. amyloliquefaciens* MB199**	Sucrose: 21, NH_4NO_3: 2.5	$MgSO_4.7H_2O$: 0.2, $MnCl_2.4H_2O$: 0.002, yeast extract: 0.2	Shake flasks	134	48	Liu, et al., (2012)

***B. amyloliquefaciens* DSM 23117**	$C_6H_{12}O_6$: 40; NH_4NO_3: 4	$MgSO_4.H_2O$: 0.332, $MnSO_4.H_2O$: 0.0017, $FeSO_4.7H_2O$: 0.002, $CaCl_2.4H_2O$: 0.001, yeast extract: 0.5	Bioreactor	249	32	Pretorius, et al., (2015)
***B. licheniformis* DSM 13**	$C_6H_{12}O_6$: 40; NH_4NO_3-: 4	$MgSO_4.H_2O$: 0.332, $MnSO_4.H_2O$: 0.0017, $FeSO_4.7H_2O$: 0.002, $CaCl_2.4H_2O$: 0.001, yeast extract: 0.5	Bioreactor	0		Pretorius, et al., (2015)
***B. subtilis* ATCC 21332**	$C_6H_{12}O_6$: 40; NH_4NO_3-: 4	$MgSO_4.H_2O$: 0.332, $MnSO_4.H_2O$: 0.0017, $FeSO_4.7H_2O$: 0.002, $CaCl_2.4H_2O$: 0.001, yeast extract: 0.5	Bioreactor	882		Pretorius, et al., (2015)
B. subtilis* subs. *spizizenii	$C_6H_{12}O_6$: 40; NH_4NO_3: 4	$MgSO_4.H_2O$: 0.332, $MnSO_4.H_2O$: 0.0017, $FeSO_4.7H_2O$: 0.002, $CaCl_2.4H_2O$: 0.001, yeast extract: 0.5	Bioreactor	36.5		Pretorius, et al., (2015)
***B. subtilis* ATCC 21332**	$C_6H_{12}O_6$: 40 NH_4NO_3: 4	$CaCl_2$- 0.00078; $FeSO_4$- 0.00061; Na_2EDTA- 0.0015 $MnSO_4$- 0.0015	Shake flasks	2600	120	Wei & Chu, (2002)
***B. subtilis* ATCC 21332**	$C_6H_{12}O_6$: 40; NH_4NO_3: 4	$CaCl_2$-0.00078 $FeSO_4$-0.61 Na_2EDTA-0.0015 $MnSO_4$- 0.0015	Shake flasks	3000		Wei, et al., (2004)
***B. subtilis* ATCC 21332**	$C_6H_{12}O_6$: 40; NH_4NO_3: 4	$CaCl_2$- 0.00078 Na2EDTA- 0.0015 $MgSO_4.7H_2O$- 0.2 $FeSO_4.7H_2O$- 0.083 $MnSO_4.H_2O$- 0.034	Shake flasks	3340		Wei, et al., (2007)

The quantity of and selectivity to surfactin achieved during production as well as the maximum production time depends on various factors, such as the bacteria species used for production, nutrient media, environmental conditions in which the bacteria species is grown and production mode. These factors are discussed in section 2.2.1 to section 2.2.4.

2.2.1 Assessment of surfactin production by different bacterial species

Surfactin is produced biologically by several *Bacillus* spp. under certain types of media and process conditions (Kosaric & Sukan, 2014), discussed in section 2.2.2 and 2.2.3. In a study by Pretorius et al. (2015), the surfactin production efficiency of four *Bacillus* spp. (*B. amyloliquefaciens* DSM 23117, *B. licheniformis* DSM 13, *B. subtilis* ATCC 21332 and *B. subtilis* subs. *spizizenii*) was determined by comparing the surfactin quantity, yield and productivity by these different *Bacillus* spp. *B. subtilis* ATCC 21332 excelled in surfactin production efficiency as it gave better surfactin quantity and selectivity. *B. subtilis* ATCC 21332 produced approximately 3.5 times more surfactin than *B. amyloliquefaciens*, while it produced approximately 24 times more surfactin than *B. licheniformis* and *B. subtilis* subs. *spizizenii* did not produce surfactin at all (Table 2-1). *B. subtilis* also produced the least antifungal concentration among these species, hence relatively higher surfactin selectivity. *B. subtilis* produced approximately 5 times, 2 times and 1.5 times less antifungals compared to *B. amyloliquefaciens*, *B. licheniformis* and *B. spizizenii* respectively (Pretorius, et al., 2015). *B. subtilis* ATCC 21332 is also commonly used for surfactin production in literature (Table 2-1).

2.2.2 Effect of nutrient media on surfactin production

Surfactin production is also influenced by the nutrient composition of the production medium. Surfactin can be produced from complex media, including those of agro-industrial wastes. Abdel-Mawgoud et al. (2008a) produced surfactin at concentrations of 1.12 g/L using molasses as a carbon source (Table 2-1). Complex media are relatively cheap, but may have high impurity loads. To avoid these high impurity loads, chemically defined media are used. The use of chemically defined media allows effective process modification, product consistency, ease of scale-up, lower impurity concentrations and better adaptability of the system to different types and modes of bioreactor operation (Rangarajan & Clarke, 2016).

Chemically defined media mainly consist of a carbon source, nitrogen source and trace metals. Surfactin is mainly produced from glucose and ammonium nitrate at concentrations of 40 g/L and 4 g/L as a carbon and nitrogen sources respectively. Sucrose and glycerol have also been used as carbon sources in

surfactin production (Table 2-1). The concentration of carbon and nitrogen sources affects surfactin production. For example, an excess initial glucose concentration in the nutrient media can lead to production of undesirable fermentative by-products such as acetates, lactates and 2,3-butanediol (Rangarajan & Clarke, 2016).

The concentration of trace elements in nutrient media has a significant influence on surfactin production. Metal cations (manganese (II), iron (II), magnesium and potassium ions) enhance surfactin production. Manganese (II) ions promote the synthesis of nitrate reductase, hence the channelling of more nitrate ions towards anaerobic metabolism during the oxygen-depleted product formation phase. Iron (II) ions are growth stimulants and a cofactor for various enzymes involved in the formation of lipopeptides. Addition of trace elements also has some drawbacks. The addition of iron (II) ions results in the induction of the acid metabolite phthalic anhydride, which reduces the lipopeptide yield. Some metal ions, such as zinc (II) ions, supress the growth of *B. subtilis*, while other metal ions (copper (II), cobalt (II), nickel (II) and aluminium ions) can hinder *B. subtilis* growth. Metal ions of heavy metals are likely to result in growth inhibition due to their toxic properties (Rangarajan & Clarke, 2016).

Surfactin production has been carried out using different media (Table 2-1). However, there is likely to be an optimal medium specifically for surfactin production. There is currently more research being done on the optimisation of nutrient media for surfactin production.

2.2.3 Effect of environmental conditions on surfactin production

Surfactin production is also affected by environmental conditions. These include temperature, pH, agitation, dissolved oxygen concentration and the presence of particles (activated carbon and polymers) in cultures during production (Ohno, et al., 1995; Makkar & Cameotra, 1997; Yeh, et al., 2006; Davis, et al., 1999; Kim, et al., 1997; Chtioui, et al., 2010; Gancel, et al., 2009; Chen, et al., 2006 and Davis, et al., 2001. Temperature affects the selectivity of the lipopeptide produced (Ohno, et al., 1995), and possibly the rate at which surfactin is produced as an increase in temperature improves bacteria growth (Ratkowsky, et al., 1982). *B. subtilis* strains produce more than one lipopeptide and Ohno et al. (1995) found that iturin A was optimally produced at 25 °C while surfactin was optimally produced at 37 °C. Although the optimum temperature for surfactin production is 37 °C, most studies on surfactin production were carried out at 30°C (including Cooper, et al., 1981; Davis, et al., 1999 and Pretorius, et al., 2015).

For surfactin production using *B. subtilis* ATCC 21332, the pH of the cultures should be maintained between 6 and 8.5. Surfactin precipitates at pH lower than 6, while no fermentation occurs at pH greater than 8.5 (Liu, et al., 2007). Makkar & Cameotra (1997) found that the optimum pH for surfactin production was pH 7, while Abushady et al. (2005) found that pH between 6.5 and 7 are optimum for surfactin production. Abushady et al. (2005) also found that surfactin production continuously declined when pH was decreased below 6.5 or increased above 7. The pH is expected to drop during production, partly because of acidification resulting from the addition of iron (II) ions (Wei, et al., 2004). Most studies use disodium hydrogen phosphate and potassium dihydrogen phosphate as buffers for controlling pH during production, but sodium hydroxide has also been used. Cooper et al. (1981) proposed 30mM KH_2PO_4, 40mM Na_2HPO_4, for controlling pH during surfactin production.

Agitation is necessary to ensure mixing during production. Additionally, an increase in agitation speed results in an increase in the overall volumetric oxygen transfer coefficient, hence improveing surfactin production (Yeh, et al., 2006). However, high agitation speeds result in increased shear on the organisms, and may increase foam formation (Yeh, et al., 2006). According to Abushady et al. (2005), the optimum agitation speed for surfactin production in shake flasks is 150 rpm.

The quantity of dissolved oxygen influences the selectivity of surfactin production. High oxygen transfer conditions favour surfactin production while low to medium oxygen transfer conditions favour fengycin production (Rangarajan & Clarke, 2016). Davis et al. (1999) and Kim et al. (1997) found that surfactin was favoured in oxygen limited conditions. On the other hand, Pretorius et al. (2015) found that an increase in dissolved oxygen enhanced surfactin production.

Particles such as activated carbon and polymers were found to improve lipopeptide production (Chtioui, et al., 2010; Gancel, et al., 2009; and Yeh, et al., 2006). Yeh et al. (2006) reported an enhanced yield (36-fold) of surfactin using *B. subtilis* through the incorporation of activated carbon in the growth medium as activated carbon improved cell dry weight (CDW), hence surfactin yield. Chen et al. (2006) found that the addition of activated carbon into *B. subtilis* cultures during production improved surfactin production by *in situ* recovery (surfactin recovery during production). According to Drouin & Cooper (1991), surfactin inhibits its own production by *B. subtilis*, resulting in poor yield. *In situ* recovery has also been done by solvent extraction (Chtioui, et al., 2010) and foam fractionation (Chen, et al., 2006), and these studies have also shown that *in situ* recovery improves surfactin production.

Environmental factors (such as aeration, agitation and addition of particles in cultures during production) also indirectly affect surfactin production as they can determine foam formation during production. Foam formation negatively affects surfactin production (Chen, et al., 2006 and Davis, et al., 2001). Foaming can result in undesirable stripping of product, nutrients, and cells into the foam. Foaming may result in the need for antifoams or mechanical foam breakers, which would increase the overall cost of the process. Chemical antifoams also reduce the oxygen transfer rate, affect cell physiology and are potential impurities that may have to be removed in downstream separation (Davis, et al., 2001).

2.2.4 Effect of mode of operation on surfactin production

Lipopeptide production can be carried out both in submerged and solid-state bioreactor systems. The choice of a suitable strategy is influenced by numerous factors such as substrate availability, process economics and the desired end application of the product. Submerged production systems have a comparatively higher yield, easier to scale up and allow easier monitoring and control of process parameters (Rangarajan & Clarke, 2016). Submerged cultivation can be carried out in batch, fed-batch and continuous systems (Kosaric & Sukan, 2014).

Surfactin production studies are commonly carried out batch-wise in shake flasks or bioreactors (Table 2-1), where a bioreactor is defined as a controlled stirred fermenter. Bioreactors are more effective for surfactin production in comparison to shake flasks as they produce more surfactin under similar conditions. In a study by Chen et al. (2006), surfactin production was improved by approximately 1.5-fold through the use of a bioreactor compared to using shake flasks (Table 2-1). Shake flasks, however, have a major advantage in that they are relatively easier and cheaper to operate than bioreactors. Batch production also has an advantage over fed-batch and continuous processes as they are relatively easy to setup as there are no additional considerations such as feeding rates. Batch cultivation in shake-flasks is therefore suitable for laboratory testing of production methodologies.

2.3 Surfactin purification from *Bacillus* cultures

During production, surfactin is secreted into the culture medium by the ubiquitous gram-positive rod-shaped *Bacillus* spp. (Kosaric & Sukan, 2014). The fact that surfactin is an extracellular product is critical in determining the concentration and purification techniques of surfactin. The concentration and purification of surfactin from *Bacillus* cultures may be necessary depending on the intended surfactin application. For example, applications such as treatment of heavy metals wastewater do not require a

high biosurfactant purity (Das, et al., 2009) while applications such as medical and cosmetic industries require high surfactin purity and conformity requirements are higher (Dhanarajan, et al., 2015). Surfactin recovery can be achieved through downstream purification, and a single downstream processing unit operation is often not sufficient for bio-product recovery with a high purity. A sequence of downstream unit operations may therefore be required (Chen, et al., 2008c).

The mechanism of downstream separation techniques depends on surfactin properties such as ionic charge, solubility in water, hydrophilicity, size, micelle forming ability, foam forming ability, polarity, molecular weight, and molecular shape (Kosaric & Sukan, 2014; Chen & Juang, 2008b and Wang, et al., 2010). These properties mainly arise due to the amphiphilic nature of surfactin. Surfactin is an amphiphilic molecule as it consists of two parts, which are a polar (hydrophilic) group and a non-polar (hydrophobic) group (Kuyukina & Ivshina, 2010).

The mechanism of downstream techniques also depends on the type and concentration of impurities in cultures after production. During surfactin production from *B. subtilis*, it has been reported that supernatants (before acid precipitation) contain macromolecules (surfactin micelles, polysaccharides, peptides, and proteins), midmolecules and small molecules (mineral salts medium, alcohols, phthalic acid, amino acids, glycine, serine, threonine, and alanine) (Chen & Juang, 2008a). *B. subtilis* also produces iturin and fengycin, which present a difficulty in surfactin purification as they have an amphiphilic nature like surfactin (Dhanarajan, et al., 2015).

Downstream purification techniques for lipopeptide purification include phase separation, extraction, foam fractionation, precipitation, adsorption, chromatography as well as ultrafiltration and nanofiltration (Chen, et al., 2008c). The method or combination of methods for surfactin recovery must be cautiously selected as they constitute a significant portion (approximately 60%) of the economics of the whole process of biosurfactant production (Chen & Juang, 2008b). Lastly, the applicability to large scale recovery should be considered in selection of downstream processes (Chen & Juang, 2008b). It may be necessary to use a combination of downstream separation techniques for surfactin purification. More separation steps, however, could mean more equipment hence high capital and production costs and reduced surfactin recovery.

2.3.1 Phase separation

The first step in the downstream purification of a bioproduct excreted into the growth medium is usually the removal of insoluble impurities such as cells from cultures. Phase separation in bioprocess cultures

depends on liquid viscosity, density differences as well as cell size and shape. Bioprocess cultures may have properties which may render phase separation difficult, such as high viscosities, gelatinous materials, compressible filter cakes, low density differences compared to water and a high degree of initial dispersion and diluteness of particulate suspension. Pre-treatment may be necessary prior to solid-liquid phase separation. Such pre-treatment may include aging, which acts to cluster cells in later stationary phase where their density increases relative to water, heat treatment, pH treatments and the addition of chemicals which aid flocculation (Dutta, 2008). Palme et al. (2010) showed that ultrasonic waves can be used to agglomerate cells, such that they can be recovered by sedimentation. Phase separation can also be, and is most commonly, carried out by centrifugation and filtration.

Filtration separates solid particles from cultures by forcing the fluid through a filtering medium which allows the liquid to pass while solids are retained (Dutta, 2008). Filtration can be divided into cross-flow and dead-end filtration. Cross-flow filtration is recommended in bioprocesses as the tangential flow along the filter medium minimizes and controls filter cake formation, which can limit liquid flux when such small particles as cells are being filtered out. The efficiency of filtration depends on cell shape and size as well as the fluid viscosity (Kosaric & Sukan, 2014). Filtration in bioprocessing is however affected by the presence of proteins in solution. Although filter pores can be an order of magnitude larger than the solubilised proteins, severe pore plugging by proteins often still occurs during filtration (Marshall & Munro, 1993).

Centrifugation can be used in situations where filtration is ineffective, such as in the case of small particles. Centrifugation however requires more expensive equipment and greater operating costs than filtration and typically cannot be scaled up to achieve the capacity of filtration equipment. Centrifugation separates solids by enhancing settling velocities of particles by centrifugal forces, and depends on density differences of the phases being separated (Dutta, 2008). Centrifugation is commonly used for removal of biomass after surfactin production, and is affected by rotation speed. A centrifugation speed of 10000 rpm is commonly used for biomass removal (Chen & Juang, 2008b and Mubarak, et al., 2014) in *B. subtilis* ATCC 21332 cultures. Centrifugation is also affected by temperature. Low temperatures cause cells to agglomerate, hence more easily removed by centrifugation (Kosaric & Sukan, 2014).

2.3.2 Precipitation

The solubility of organic solutes depends on the solution properties such as temperature, pH, composition, ionic strength and dielectric constant. Any alteration of these properties may result in

phase change of the organic solutes (Dutta, 2008). Insoluble solutes can then be separated by phase separation techniques. The addition of the divalent cations (such as ions of cadmium, copper, zinc, iron, cobalt, calcium, manganese, magnesium or mercury) can result in surfactin precipitation (Arima, et al., 1972). This can allow the removal of impurities from cultures during biosurfactant purification. Additionally, precipitation can be achieved by low pH or presence of ammonium sulphate as discussed in section 2.3.2.1 and section 2.3.2.2.

2.3.2.1 *Acid precipitation*

Acid precipitation is a one of the most commonly used and relatively cheap methods for recovering biosurfactants from fermentation cultures. The low pH causes the negative charges of biosurfactant molecules to be neutralised, thus reducing their solubility in water (Kosaric & Sukan, 2014). When the pH of the culture is decreased below pH 6.5, macromolecular impurities and surfactin start to precipitate (Liu, et al., 2007). The precipitate is often stored at 4 °C after precipitation to minimise the co-aggregation of other macromolecules (Rangarajan & Clarke, 2016).

Acid precipitation has a high recovery factor (>97%), but usually leads to relatively low surfactin purity of <60% (Chen & Juang, 2008b). Acid precipitation has been studied at pH 4 and pH 2 (Chen, et al., 2007; Chen & Juang, 2008a and Wang, et al., 2010) (Table 2-2). From Table 2-2, it was seen that relatively high surfactin recovery and purity can be achieved at pH 4. Acid precipitation has been carried out at pH 2 in various studies such as those by Sivapathasekaran et al. (2009) and Dhanarajan et al. (2015), although no data on surfactin recovery and purity was provided.

Table 2-2: Summary of studies on surfactin recovery by acid precipitation in literature, where % surfactin recovery refers to the percentage of surfactin supernatants recovered in the precipitate and % surfactin purity as well as % lipopeptide purity refers to the mass fraction of surfactin and lipopeptides in the precipitate respectively.

Operating pH	% Surfactin recovery	% Surfactin purity	% Lipopeptide purity	Reference
2		33.2	56.6	Wang, et al., 2010
4	>97	55		Chen, et al., 2007
4		53		Chen & Juang, 2008a

Acid precipitation has a disadvantage as it may result in reactions such as hydrolysis of the lipopeptide functional groups. In a study by Besson & Michel (1987), lipopeptide hydrolysis was observed when

lipopeptides were exposed to HCl at temperatures greater than 105 °C. There is therefore a possibility that no hydrolysis occurs acid precipitation is carried out at room temperature. Acid precipitation can also result in esterification of lipopeptides (Desjardine, et al., 2007).

2.3.2.2 *Salting out*

Ammonium sulphate salting out is mainly used for protein purification, but has also been used for surfactin purification. The addition of ammonium sulphate to bioprocess cultures causes water molecules to bind to sulphate ions. This reduces the amount of water molecules available to bind with protein molecules, resulting in precipitation of the proteins which are not hydrated by binding to water molecules. Ammonium sulphate is a common salt for salting out since it is highly soluble in water, relatively cheap, and does not denature proteins (Chen, et al., 2008b). A disadvantage of salting out is that the salts added act as an impurity in the surfactin produced. Subsequent purification may therefore be required after salting out to remove the salts resulting in increased overall purification costs. Additionally, is not attractive as it results in co-precipitation of proteins (Rangarajan & Clarke, 2016).

Chen et al. (2008b) studied surfactin recovery from a *B. subtilis* culture by ammonium sulphate salting out. The surfactin was co-precipitated with protein molecules in the bioprocess cultures, and a maximum purity and recovery of 61% and 80% were obtained respectively. Chen et al. (2008b) also studied ammonium sulphate salting out after destabilising surfactin micelles using ethanol at a concentration of 33% (v/v). This method improved the purity and recovery to 69% and 92% respectively. The optimum ammonium sulphate concentration was 23% w/v.

2.3.3 Foam fractionation

The foam foaming ability of surfactin can be useful for surfactin recovery by foam fractionation. Foaming occurs more readily at surfactin concentrations exceeding the critical micelle concentration (CMC) (Chen, et al. 2006), When gas bubbles are passed through bioprocess cultures, surfactin binds to the surface of the gas bubbles due to its surface activity. The bubbles are then removed from the system as foam to recover the surfactin (Stevenson & Li, 2014). Foaming is particularly attractive as it is most efficient when dilute solutions are used, cheap to operate and can be used in the presence of biomass thus does not need an extra centrifugation step for biomass removal (Davis, et al., 2001). Since surfactin can be recovered in the presence of biomass, foam fractionation can be used to recover surfactin during production (*in situ* recovery). Surfactin can also be recovered after production (*ex situ* recovery).

In situ recovery of surfactin by foam fractionation is capable of achieving high recoveries (≥90%) (Chen, et al., 2006 and Noah, et al., 2002). However, no data on purity has been provided for surfactin recovery by foam fractionation. Chen et al. (2006) found that *in-situ* foam fractionation has a surfactin enrichment (the ratio of surfactin concentration in the foam to the surfactin concentration in the remaining liquid after foam fractionation) of approximately 50.

Davis et al. (2001) studied *ex-situ* recovery of surfactin from *Bacillus* cultures by foam fractionation, and high recoveries (up to 95%) were obtained. In the study by Davis et al. (2001), it was noticed that the presence of cells in the cultures increased surfactin recovery since cells improved the foam forming ability of the solution, thus more liquid was carried up into the foam. Maximum surfactin enrichments of 8.4 and 51.6 were obtained from cell-free and cell containing systems respectively.

A disadvantage of foam fractionation is that it is not possible to simultaneously attain the maximum surfactin recovery and maximum surfactin enrichment. Davis et al. (2001) found that the maximum surfactin enrichment was obtained in the latter stages of foaming, where a low volume of liquid was collected in the foam per unit time hence the recovery was relatively low. At the initial stages of foam fractionation, a high surfactin recovery was attained. The high surfactin recovery was, however, accompanied by relatively high impurity content in the foam resulting in low surfactin enrichment (Davis, et al., 2001). According to Chen & Juang (2008b), the major problem of this separation technique is that it leads to relatively low biosurfactant purity.

2.3.4 Solvent extraction

Solvent extraction purifies surfactin by selectively extracting surfactin in impure mixtures into solvents, and then recovering the purified surfactin from solvents. The selectivity of the extraction solvent toward surfactin and impurities is therefore critical for purification by solvent extraction. Surfactin can dissolve in organic solvents as it has a hydrophobic end (Chen & Juang, 2008b). Organic solvents have selective extraction of surfactin from culture supernatants or acid precipitates as they improve the purity of surfactin after extraction. Selective extraction is due to different solubilities of surfactin and impurities in different organic solvents. (Chen & Juang, 2008a; Chen & Juang, 2008b and Juang, et al., 2012).

Surfactin recovery by solvent extraction has been carried out by various strategies (solid-liquid extraction, liquid-liquid extraction, chemical assisted extraction, pertraction and non-dispersive extraction) (Table 2-3). Solid-liquid extraction refers to mixing impure surfactin in solid form with an organic solvent to dissolve the surfactin, while dissolving relatively fewer impurities (Chen & Juang,

2008b). Liquid-liquid extraction refers to mixing impure surfactin in aqueous form with organic solvents. The surfactin is then extracted into the organic solvents while impurities are left in the aqueous phase. The organic solvent is usually insoluble in water to allow easy separation of the organic and aqueous phases. If an organic solvent soluble in water is used, it is then separated from the aqueous phase by centrifugation (Geisslera, et al., 2017 and Chen & Juang, 2008a).

Liquid-liquid extraction has been improved by addition of chemicals in the aqueous phase to assist extraction of surfactin into the organic solvents (Chen & Juang, 2008b and Juang, et al., 2012). This is referred to as chemical assisted extraction. Liquid-liquid extraction can also be carried out by pertraction, where surfactin is extracted from the aqueous surfactin mixture into an organic solvent before being transferred to a receiving aqueous phase (section 2.3.4.4). Lastly, liquid-liquid extraction can be done by non-dispersive extraction, where extraction is carried out in microporous membrane (section 2.3.4.5).

Solvent extraction has some draw backs. This process may result in excessive usage and waste of solvents resulting in increased operating costs, and organic solvents have environmental impacts which need to be considered. Additional equipment is usually needed for solvent regeneration, and high temperature recovery may be necessary which consumes energy (Kosaric & Sukan, 2014). Solvent extractions are slow processes as a low agitation speed (<250 rpm) may be required during extraction to avoid excessive foam formation (Chen & Juang, 2008a). Additionally, solvent extraction can result in esterification of lipopeptide functional groups (Besson & Michel, 1987). According to Zhao et al. (2013), surfactin is spontaneously esterified in methanol solutions with acids due to the presence of carboxyl groups in the surfactin structure. Desjardine et al. (2007) studied the efficacy of a lipopeptide (tauramamide) after estirification. They found that estirification does not affect the minimum inhibitory concentration of the lipopeptide, but the potency of the lipopeptide was reduced.

Solvent extraction has advantages as it is relatively less complex compared to ultrafiltration and adsorption. Solvent extraction does not require high pressure operation and is not affected by plugging of filter membranes like ultrafiltration. Additionally, it is relatively cheaper than chromatography techniques (Kosaric & Sukan, 2014).

Surfactin recovery by solvent extraction has been studied extensively in literature (Table 2-3). The ideal process and solvents for solvent extraction are those that simultaneously achieve a high surfactin recovery and purity. From Table 2-3, it can be seen that chemical assisted extraction using *n*-hexane as a

solvent is the best process for solvent extraction as it achieved the highest surfactin purity (91%), while the recovery it achieved (>96%) is comparable to the maximum recovery in solvent studies in literature (99.6%). The surfactin purity and recovery achieved by solvent extraction is influenced by various factors (e.g. solvent type, chemical assistance), which are discussed in section 2.3.4.1 to section 2.3.4.5.

Table 2-3: Summary of studies on surfactin recovery by solvent extraction in literature, where % purity refers to the mass percentage of surfactin in the purified surfactin while % recovery refers to the percenage of surfactin recovered into solvents during extraction

Solvent Extraction type	Solvent	Pre-purification stages	% Purity	% Recovery	Reference
Non-dispersive solvent extraction	*n*-hexane	Acid precipitation	78	60	Chen & Juang, 2008a
Liquid-liquid extraction	Chloroform:methanol (2:1, v/v)	None		99.6	Geisslera, et al., 2017
Liquid-liquid extraction	Ethyl acetate	Acid precipitation	58-60	99	Chen & Juang, 2008b
Liquid-liquid extraction	*n*-hexane	Acid precipitation	58-60	21	Chen & Juang, 2008b
Solid-liquid extraction	Ethyl acetate	Acid precipitation	84	78	Chen & Juang, 2008b
Solid-liquid extraction	*n*-hexane	Acid precipitation	60	62	Chen & Juang, 2008b
Chemical assisted extraction	*n*-hexane	Acid precipitation	70	95	Chen & Juang, 2008b and Juang, et al., 2012
Chemical assisted extraction	*n*-hexane	Acid precipitation	70	98	Chen & Juang, 2008b and Juang, et al., 2012
Pertraction	*n*-heptane			97	Dimitrov, et al., 2008
Chemical assisted extraction (TOA)	*n*-hexane	Acid precipitation	85	>96	Juang, et al., 2012
Chemical assisted extraction (TOA, NaCl)	*n*-hexane	Acid precipitation	91	>96	Juang, et al., 2012

2.3.4.1 *Effect of solvent and surfactin polarity on solvent extraction*

Surfactin purification by solvent extraction is dependent on solvent and surfactin polarity. Surfactin is an amphiphilic molecule, and the polar and non-polar groups of the molecule interact very differently with polar solvents and non-polar solvents (Alexandridis & Lindman, 2000). The non-polar lipid part contributes to high solubility in organic solvents (Yang, et al., 2015). The polarity of surfactin is dependent on pH. According to Liu et al. (2015), the micropolarity of surfacitn increases with a decrease in pH.

Surfactin is mainly soluble in polar solvents, but it is partially soluble in some non-polar solvents. Surfactin is soluble in alkaline water, acetone, methanol, ethanol, *n*-propanol, *i*-propanol, *n*-butanol, *i*-butanol, *t*-butanol, ethyl acetate, chloroform, methylene chloride, dioxane, benzene, tetrahydrofuran, dimethylformamide and glacial acetic acid (Arima, et al., 1972). Surfactin is insoluble or sparingly soluble in carbon tetrachloride, petroleum ether, ligroin, petroleum benzene, hexane and cyclohexane. Surfactin is also soluble in ether but gradually precipitates, sometimes becoming a gel (Arima, et al., 1972).

Organic solvents such as methanol, ethanol, butanol, diethyl ether, *n*-pentane, *n*-hexane, ethyl acetate, acetone, acetic acid, chloroform, and dichloromethane have been used, either single or in combination for biosurfactant purification by solvent extraction. The most effective solvent for surfactin extraction would be the mixture of chloroform and methanol in various ratios, which allows the polarity of the extraction to be varied to target the extractable material (Kosaric & Sukan, 2014). Due to environmental and safety considerations, chloroform is not attractive due to its high toxicity (Chen & Juang, 2008b). Ethyl acetate and methyl tert-butyl ether (MTBE) are better alternatives for chloroform as polar solvents and *n*-heptane may be safer than *n*-hexane as a nonpolar solvent (Kosaric & Sukan, 2014).

Chen & Juang (2008b) studied liquid-liquid extraction of surfactin from cultures pre-treated by acid precipitation using equal volumes of the organic solutions *n*-hexane and ethyl acetate (Table 2-3). It should be noted that *n*-hexane is non-polar, while ethyl acetate is polar. From this study, the solvent polarity had a significant effect on surfactin recovery as the recovery was higher when ethyl acetate was used as a solvent (≥95%) compared to when *n*-hexane was used (21%). Polar solvents therefore recover more surfactin than non-polar solvents. Solvent polarity did not have a significant effect on surfactin purity after solvent extraction, as the final purity was approximately 60% when either ethyl acetate or n-hexane was used as a solvent. The similar purities mean that polar solvents recover more surfactin and

more impurities, while non-polar solvents recover less surfactin and less impurities. Polar solvents are therefore preferable for solvent extraction due to their high recoveries.

The low recovery in non-polar solvents can be improved by replacing the organic solvent used for extraction with a fresh one during extraction. This is referred to as repeated extraction. Chen & Juang (2008b) did repeated extraction to improve the surfactin recovery from *n*-hexane by solid liquid extraction, and the surfactn recovery improved from 59% and a purity of 62%. Repeated extraction, however, did not improve surfactin recovery when ethyl acetate was used as a solvent (Geisslera, et al., 2017). The actual values of the recovery were, however, not reported in the study by Geisslera et al. (2017).

2.3.4.2 *Effect of the nature of mixture being purified on solvent extraction*

'Nature of mixture being purified' refers to the surfactin and impurity composition in the mixture, whether the mixture is in solid or liquid state, as well as the presence of bacterial cells in the mixture being purified. The surfactin and impurity composition can depend on the bacteria species and strain used for production and the pre-purification the mixture has already undergone. Geisslera et al. (2017) studied surfactin recovery from cultures of *B. amyloliquefaciens* and *B. methylotrophicus* by solvent extraction using chloroform:methanol (2:1, v/v) as a solvent. The recovery when surfactin was produced from *B. amyloliquefaciens* (96.5%) was different to that obtained when surfactin was produced from *B. methylotrophicus* (99.6%), although it cannot be determined if these values are significantly different at a 95% confidence interval based on the given data.

Solvent extraction can be used to recover surfactin from a solid (precipitate) or liquid (solubilised precipitate and cell-free supernatants after production). Purification of surfactin in liquid mixtures by solvent extraction is referred to as liquid-liquid extraction, and solid-liquid extraction if surfactin is in solid mixtures. Solid-liquid extraction has an advantage over liquid-liquid extraction as it does not result in foam formation, which results in an emulsion after liquid-liquid extraction (Chen & Juang, 2008b). Geisslera et al. (2017) found, through preliminary studies, that higher overall surfactin recoveries are achievable when extraction is done directly on cell-free supernatant rather than after acid precipitation.

Chen & Juang (2008b) studied both liquid-liquid extraction and solid-liquid extraction under the same conditions, using *n*-hexane and ethyl acetate as solvents (Table 2-3). When ethyl acetate was used as a solvent, a higher surfactin recovery was achieved in liquid-liquid extraction (99%) than in solid-liquid extraction (78%). A lower purity (60%) was achieved for liquid-liquid extraction compared to that

achieved by solid-liquid extraction (84%). When *n*-hexane was used as a solvent, the maximum recovery achieved in liquid-liquid extraction (21%) was lower than that in solid-liquid extraction (62%), while the purities were comparable at approximately 60% (Table 2-3). Solid-liquid extraction is therefore more efficient in terms of recovery for polar solvents, while liquid-liquid extraction is more efficient for non-polar solvents (Chen & Juang, 2008b).

Liquid-liquid extraction can be used for surfactin recovery in the presence of cells in the aqueous phase. Liquid-liquid extraction can thus be used to recover surfactin during surfactin production. Drouin & Cooper (1991) attempted to extract surfactin during batch production using an aqueous two-phase system based on polyethylene-glycol (PEG) and dextran in the presence of sodium phosphate. The cells were seen to partition to the lower dextran rich-phase while surfactin dissolved in preferably in the upper PEG-rich phase during cultivation. The presence of sodium phosphate causes surfactants to distribute according to charge, thus the anionic surfactin was partitioned to the top phase. The study by Drouin & Cooper (1991) improved surfactin production, but no data on purity and recovery of the recovered surfactin was provided.

2.3.4.3 *Effect of ultrasound and chemical assistance on solvent extraction*

Lipopeptide extraction can also be assisted using ultrasonic waves (Yuan, et al., 2012), but this has not yet been studied for surfactin purification. Surfactin extraction can also be assisted by the addition of the chemicals (tri-n-octylmethylammonium chloride (Aliquat 336) and tri-n-octylamine (TOA)) in the aqueous phase. This is known as chemical assisted extraction or reverse miceller extraction as the added chemicals cause surfactin to form reverse micelles in the aqueous phase. Chemical assisted extraction consists of an extraction step (where surfactin is extracted from supernatants into an organic phase) and a stripping step (where surfactin is stripped from the organic phase into water).

Chen & Juang (2008b) and Juang et al. (2012) studied surfactin purification by chemical assisted extraction using *n*-hexane as the solvent. Chemical assisted extraction achieved surfactin recoveries of approximately 95% and 98% when Aliquat 336 and TOA were used respectively (Chen & Juang, 2008b). This is greater than the extraction achieved when hexane was used as a solvent in conventional liquid-liquid extraction (21%) (Table 2-3). The extraction step was seen to be positively influenced by the presence of methanol (which results in disruption of micelles) and the increase in pH in the aqueous phase (which affects surfactin charge). The presence of 10% (v/v) methanol in the aqueous phase improved surfactin recoveries improved from 73% to 95%, while an increase in pH from 7 to 10 increased recoveries from 87% to 92% (Chen & Juang, 2008b).

The stripping step recovers surfactin from the organic phase, and can be promoted by the presence of alcohols (as they disrupt micelles) and salts (as increased ionic strength results in thinner double-electric layers and smaller inner diameters of the reverse micelles) in the stripping solution. The surfactin purity after purification by chemical assisted extraction can reach up to about 70% (Chen & Juang, 2008b and Juang, et al., 2012). This is greater than that obtained through acid precipitation (53%) and conventional solvent extraction with *n*-hexane and ethyl acetate (58–60%). In studies by Chen & Juang (2008b) and Juang et al. (2012) sodium chloride and ammonium sulphate salts were studied for surfactin stripping in the presence of methanol, and ammonium sulphate had similar surfactin stripping (88%) compared to sodium chloride (approximately 90%).

Although chemical assistance improves surfactin recovery and purity, it has two major drawbacks. Firstly, the additional step of extracting surfactin from the organic solvent results in increased equipment and operating costs. Secondly, the chemicals added may be toxic, and they may not be fully removed from the surfactin after the stripping step (Chen & Juang, 2008b and Dimitrov, et al., 2008). To avoid these drawbacks, extraction can be carried out in liquid membrane extraction which combines both extraction and stripping steps and does not require chemical assistance.

2.3.4.4 *Effect of liquid membranes on solvent extraction*

Liquid membrane extraction (also known as pertraction) consists of two aqueous phases separated by an organic phase which is insoluble in both aqueous phases (Figure 2-1). Surfactin is extracted from the aqueous feed solution into the organic phase. This results in a surfactin concentration gradient between the organic phase and the receiving solution, hence surfactin is stripped from the organic phase into the receiving aqueous phase.

Dimitrov et al. (2008) studied surfactin recovery by pertraction using *n*-heptane as the organic phase, and found a surfactin recovery of 97%. This is higher than recoveries obtained in conventional liquid-liquid extraction or solid liquid extraction when a non-polar solvent was used, hence pertraction improved surfactin recovery. In addition to improving surfactin recovery, pertraction allows continuous regeneration of the organic solvent thus less use of organic solvents (Dimitrov, et al., 2008).

Unlike the study by Chen et al. (2008b), the study by Dimitrov et al. (2008) showed that an increase in pH reduced surfactin recovery. The recovery improved from 83% to 97% when the pH was reduced from 6.05 to 5.65. The low pH in the feed solution favours the formation of β-sheet micelles, which allow surfactin transfer and accumulation into the organic phase (Dimitrov, et al., 2008).

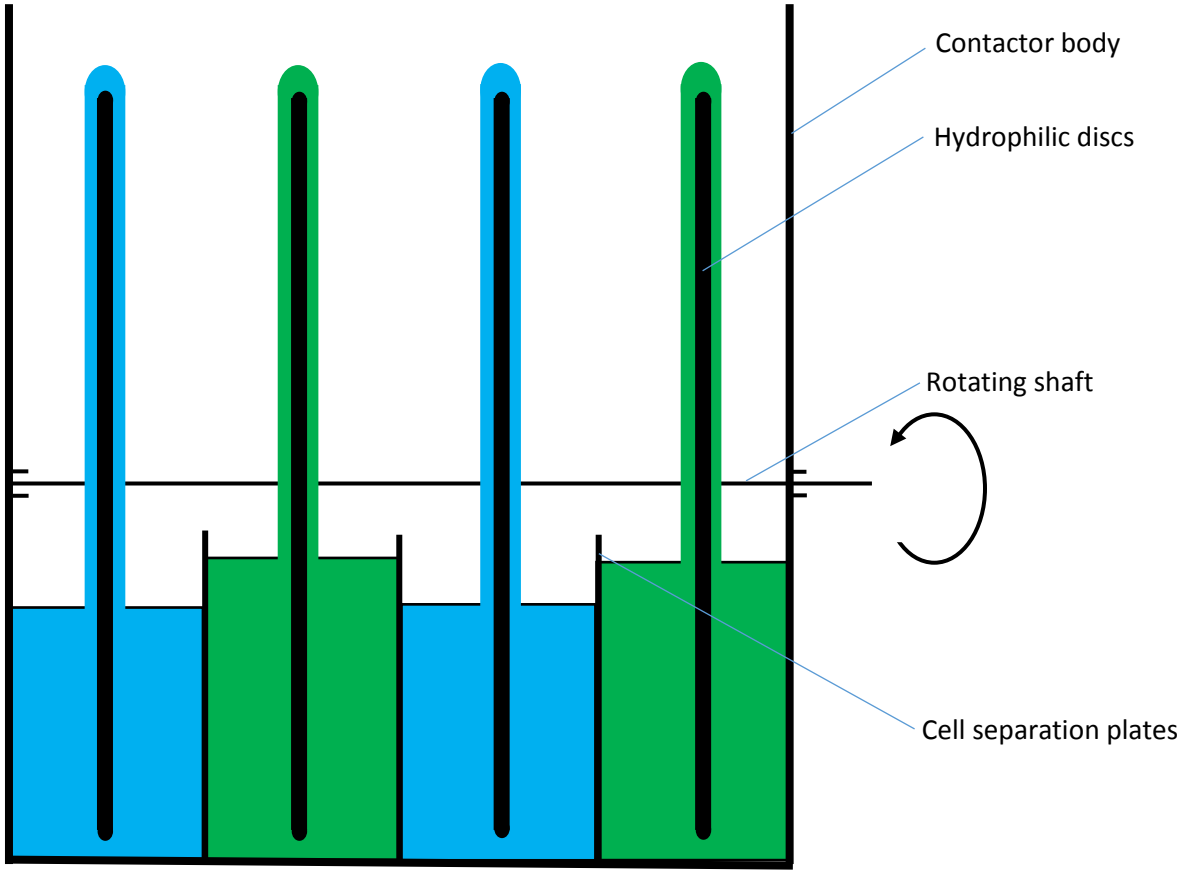

Figure 2-1: Rotating disc contactor used for pertraction where the blue, green and light pink areas show the feed solution, receiving solution and the organic phase respectively (adapted from Dimitrov et al. (2008))

2.3.4.5 *Effect of microporous membranes on solvent extraction*

Solvent extraction can be done in microporous membranes, and this is referred to as non-dispersive solvent extraction. A fluid is contacted with microporous membranes and fills the membrane pores. If an immiscible liquid is brought to contact with the wet membrane, an interfacial contact area is established between the two liquids. This method has advantages as it avoids foam formation, and is simpler to operate than traditional solvent extraction units and allows for lower equipment and material costs and significant energy savings (Chen & Juang, 2008a).

Chen & Juang (2008a) studied the nondispersive extraction of surfactin from pre-treated cultures of *B. subtilis* using *n*-hexane as a solvent and polyvinylidene fluoride (PVDF) hollow fibres as the microporous membrane. This method was 1.5 times faster, and extracted was 2 times more surfactin compared to batch liquid-liquid extraction. This is because surfactin was adsorbed onto the surface of the PVDF

hollow fibres, and then transferred to the organic phase. This process achieved a surfactin purity and recovery of 78% and 60% respectively (Table 2-3).

2.3.5 Ultrafiltration and nanofiltration

Ultrafiltration has various advantages over other surfactin purification techniques. Ultrafiltration has lower mass transfer limitations, does not require the use of large volumes of organic solvents which may be toxic, and can be easily scaled up. Ultrafiltration minimizes denaturation and physical damage of biomolecules from shear effects. Ultrafiltration does not have problems of solute re-solubilisation as solutes are retained in the solution phase (Chen, et al., 2007). Lastly, ultrafiltration is a very simple process which requires no phase change. However, it has a major drawback as it requires high pressure operation which result in increased equipment and operation costs (Sen & Swaminathan, 2005).

Surfactin recovery by ultrafiltration and nanofiltration has been studied in literature (Table 2-4). Ultrafiltration works by permeation of relatively small molecules through a membrane under pressure while larger molecules are retained (Clarke, 2013). Surfactin recovery has been studied by ultrafiltration carried out using polyethersulfone (PES), cellulose ester (CE), hydrosart (HT) and regenerated cellulose (RC and YM) membranes with various molecular weight cut-offs (MWCO). The MWCO is shown is shown in the membrane name e.g. cellulose ester membranes with MWCO of 50 KDa are named as CE 50.

Ultrafiltration and nanofilltration were compared to determine a process which simultaneously achieves high recoveries and purities. The study by Isa et al. (2007), where a two-stage ultrafiltration was used, is the only study that was able to achieve surfactin purity greater than 90% by ultrafiltration. This study achieved the highest surfactin recovery (96±5%) in comparison to other studies in literature. Purification by ultrafiltration is affected by various factors (e.g. membrane properties and filter cake formation), and these are discussed in sections 2.3.5.1 and 2.3.5.2.

Table 2-4: Summary of studies on surfactin recovery by ultrafilatratioin and nanofiltration in literature, where % purity refers to the mass percentage of surfactin in the purified surfactin while % recovery refers to the percenage of surfactin recovered into the final surfactin product after membrane filtration

Filtration process	Membrane material	Pre-purification stages	% Recovery	% Purity	Reference
two stage ultrafiltration	PES100	Acid precipitation	87	85	Chen, et al., 2007
two stage ultrafiltration	PES100, YM1	acid precipitation	79	86	Chen, *et al*., 2007
One stage cross-flow filtration	PES 100	Acid precipitation	83	79	Chen, et al., 2008a
Two stage Dead end ultrafiltration	PES100	Acid precipitation	85	75	Chen, et al., 2008a
One stage ultrafiltration	PES100	Acid precipitation	68	83	Chen, et al., 2008b
One stage ultrafiltration	PES100	Salting out	81	78	Chen, et al., 2008b
One stage nanofiltration	YM1	Salting out	81	79	Chen, et al., 2008b
Two stage ultrafiltration (centrifugal device)	RC 10	None	91±7	94±1	Isa, et al., 2007
Two stage ultrafiltration (stirred cell)	RC 10	None	96±5	93±1	Isa, et al., 2007
Two stage ultrafiltration	PES 10	None	96±5	94±2	Isa, et al., 2007
Two stage ultrafiltration	CE 30	None	95		Lin & Jiang, 1997
One stage crossflow filtration	PES10	None	94.4	87.2	Mubarak, et al., 2014
One stage crossflow filtration	HT10	None	96.3	88.2	Mubarak, et al., 2014
One stage dead end filtration	Amicon XM-50	Foam fractionation	98.5	70	Sen & Swaminathan, 2005

2.3.5.1 *Effect of surfactin and membrane properties on ultrafiltration and nanofiltration*

Ultrafiltration and nanofiltration are capable of separating dissolved and suspended molecular species through a filter membrane under pressure based on size differences between surfactin and impurities. Surfactin forms micelles of about 50-100 molecules at the CMC with nominal diameters ranging from two to three orders of magnitude larger than the single surfactin molecules (Sen & Swaminathan, 2005). Lin & Jiang (1997) found that the size of surfactin micelles range between 30 KDa to 50 KDa, while Chen et al. (2007) reported that they range between 50 KDa and 100 KDa.

Surfactin micelles in culture supernatants can therefore be separated from smaller molecules such as salts, glucose and amino acids through ultrafiltration using membranes with pore diameters of approximately 30 KDa. To separate surfactin from macromolecules such as proteins, pore diameters of approximately 100 KDa would be appropriate as these would allow surfactin micelles to pass through while inhibiting the passage of protein macromolecules. Chen et al. (2008b) studied surfactin recovery by single stage ultrafiltration using PES 100 membranes. In this study, a surfactin recovery and purity of 63% and 83% was achieved (Table 2-4).

Surfactin can also be purified by ultrafiltration and nanofiltration in monomer form, by dispersing the micelles using low molecular weight alcohols. This allows surfactin to pass through membranes below 30 KDa, hence separate surfactin from macromolecules. This property has been used by Lin & Jiang (1997) and Chen et al. (2007) who studied surfactin recovery from *B. subtilis* cultures by two stage ultrafiltration using CE 30 and PES 100 membranes respectively. Surfactin micelles were retained in the first ultrafiltration stage. The retained micelles were then dispersed by using methanol and collected in the permeate of the second ultrafiltration stage. Lin & Jiang (1997) and Chen et al. (2007) obtained recoveries of 95% and 87% respectively, while Chen et al. (2007) further got purities of 87%. In a study by (Chen, et al., 2008c), a surfactin purity of 76% was obtained from two-stage ultrafiltration (Table 2-4).

Surfactin monomers can be further purified from relatively smaller molecules such as salts by using membranes with smaller pores in nanofiltration. Chen et al. (2008b) studied nanofiltration using YM 1 membranes. Surfactin recoveries and purities of 79% and 81% were obtained. Chen et al. (2007) used nanofiltration after a single stage ultrafiltration step using YM 1 membranes, and achieved a recovery and purity of 79% and 86% respectively (Table 2-4). However, the relatively low flux of the nanofiltration process makes this process unattractive (Chen, et al., 2008b and Chen et al. 2007).

Membrane material is expected to affect surfactin recovery by ultrafiltration. Surfactin monomers adsorb stronger on more hydrophobic membranes reducing the pore radius, and this results in a flux decline (Chen, et al., 2008a). Less negatively charged membranes also allow surfactin to easily pass through as surfactin has an overall negative charge (Isa, et al., 2007). Although it was expected that membrane material affects surfactin recovery by ultrafiltration, Isa et al. (2007) found that this is not always true. Isa et al. (2007) studied surfactin recovery by ultrafiltration using different membranes with similar MWCO (RC 10 and PES 10) and found that surfactin recoveries and purities of approximately 95% (Table 2-4) for both membranes.

2.3.5.2 *Effect of filter cake formation on ultrafiltration and nanofiltration*

During ultrafiltration and nanofiltration, contaminants deposit onto the filter membrane to form a filter cake. The presence of protein contaminants in filter cakes results in an establishment of a concentration polarization layer, which block membrane pores hence reduce the efficiency of ultrafiltration and nanofiltration. Concentration polarization can be avoided by using stirred cell devices in dead end filtration, which cause turbulence near the membrane surface. Isa et al. (2007) studied surfactin recovery by a two stage membrane filtration processes using centrifugal and stirred cell devices using 30 and 10 KDa MWCO regenerated cellulose membranes. A similar recovery and purity of approximately 95% was obtained in both studies (Table 2-4). Sen & Swaminathan (2005) studied surfactin recovery by a single-step ultrafiltration process in a magnetically stirred cell system using Amicon XM-50 filtration membranes. A surfactin purity and recovery of about 70% and 98.5% respectively was obtained in this study (Table 2-4).

Cake formation can also be reduced using cross cross-flow filtration. In cross-flow filtration, the retentate flows parallel to the membrane surface unlike dead-end filtration where the retentate flows perpendicular to the membrane surface (Chen, et al., 2008a). Mubarak et al. (2014) studied surfactin recovery from *B. subtilis* cultures by one-step cross-flow ultrafiltration using HT10 and PES10 membranes. Recoveries of 94.4% and 96.3% were achieved for the PES10 and HT10 membranes respectively, while purities of 87.2% and 88.9% were achieved respectively (Table 2-4). The flux was seen to increase with increasing cross-flow velocity, but decrease with increasing the initial surfactin concentration and TMP. A higher cross-flow velocity removes cake formed on membrane resulting in slow formation of the concentration polarization layer.

In addition to cross-flow ultrafiltration, fouling can also be reduced by membrane cleaning. Chen et al. (2008a) studied surfactin recovery from pre-treated *B. subtilis* cultures by cross-flow ultrafiltration with

in situ cleaning. The cleaning solutions were deionized water, NaOH solution (pH 11 and 13) and 1 wt% Terg-a-zyme solution, and PES 100 membranes were used. A surfactin recovery and purity of 83% and 79% were obtained respectively. This was higher compared to the recovery and purity of 85% and 75% obtained through two-stage dead end filtration under similar conditions (Chen, et al., 2008a) (Table 2-4).

Fouling can also be reduced by pre-purification of *B. subtilis* cultures prior to ultrafiltration. Chen et al. (2008b) studied surfactin recovery from *B. subtilis* cultures by pre-treating the culture through ammonium sulphate salting-out using PES 100 and YM 1 membranes. Purities of 78% and 79% were obtained for the PES 100 and YM 1 membranes respectively, while recoveries were 81% in both cases (Table 2-4). Fouling can also be reduced by changing the hydrophilicity of the membrane and periodic flushing or back-flushing. Back-flushing allows ultrafiltration to be carried out for longer before it is stopped for intensive physical or chemical cleaning (Chen, et al., 2008a).

2.3.6 Chromatography

Chromatography purifies surfactin based on its degree of interaction with stationary and mobile phases. Chromatography depends on factors such as hydrophobicity, molecular size and molecular charge (Kosaric & Sukan, 2014). Chromatography consists of a solid adsorbent (stationary phase) and a solvent containing the solutes to be separated (mobile phase) in a column. The solutes travel through the stationary phase at different rates in the column depending on their specific equilibrium characteristics. The least adsorbed solute moves more rapidly through the column than more adsorbed solutes. The rate at which solutes move through the column can be modelled using adsorption isotherms which relate the solute concentration on the adsorbent's surface (Clarke, 2013).

Chromatography has some drawbacks. It is prone to clogging in the presence of solid particles. These processes may require the usage of solvents and buffers for equilibration, which may lead to increased operating costs as well as generation and release of hazardous waste. Additionally, costs may arise from washing, elution, and regeneration of the resins. Due to the high operating costs, chromatography may only be suitable for analytical purposes or purification of valuable pharmaceuticals or cosmetics products on a small scale (Kosaric & Sukan, 2014). These methods include TLC, RP- and the cheaper alternatives: ion-exchange chromatography, adsorption chromatography and size exclusion chromatography (SEC). Purification by ion-exchange chromatography as well as adsorption chromatography will be discussed in section 2.3.7. RP-HPLC, SEC and TLC are discussed in section 2.3.6.1 to section 2.3.6.3.

2.3.6.1 *Purification by RP-HPLC*

RP-HPLC is widely used for high quality purification for research in the fields of cosmetics and medicine (Yang, et al., 2015). RP-HPLC relies on pumps to pass the sample mixture being purified through a column filled with adsorbent material. The different components of the sample mixture pass through the column at different flow rates as they interact differently with the adsorbent material resulting in separation of the components as they flow out of the column (Yang, et al., 2015).

RP-HPLC purifies molecules based on their hydrophilicity, and is thus capable of separating surfactin homologues. Homologues may differ in composition by only a single amino acid and/or the fatty acid residue. These different amino acids have diverse hydrophilic characteristics and thus interact differently with the mobile phase (methanol or acetonitrile), and the fraction with higher hydrophilicity in the peptide ring is easily eluted in a C18 column. The fraction containing the longer fatty acid, on the other hand, is harder to elute since the hydrophobic fatty acid chains combine with the modified filler of the C18 column. The fraction containing the longer fatty acid therefore requires a relatively higher proportion of methanol/acetonitrile (Yang, et al., 2015).

Razafindralambo et al. (1993) studied purification of antifungal lipopeptides from *B. subtilis* cultures by adsorption into C18 gel followed by RP-HPLC. This method was effective for isolating and fractionating iturin A and surfactin, which were co-produced by *B. subtilis*. The biocompatibility of this method allows maintenance of the structure and function of lipopeptides during purification (Razafindralambo, et al., 1993). Kim et al. (2004), Sivapathasekaran et al. (2009), Chen et al. (2008d) and Liu et al. (2008) also used RP-HPLC for the purification of surfactin and other biosurfactants.

Dhanarajan et al. (2016) studied purification of three lipopeptide families (surfactin, fengycin and iturin) produced by *Bacillus megaterium* by analytical HPLC, before HPLC was scaled up to semipreparative HPLC. Lipopeptides were pre-purified by in situ foam fractionation and filtration to remove solid particles before injection into the HPLC system. This method purified surfactin to a purity of 97% with a recovery of 96%. Although further scale-up to larger columns may lead to higher lipopeptide throughput, it would also lead to a higher column particle size. This would result in difficulty in separating the lipopeptide homologues.

2.3.6.2 *Surfactin purification by SEC*

Size exclusion, or gel, chromatography separates solutes based on their size differences. A sample solution is passed through porous gel beads with controlled pore sizes. Smaller molecules are allowed to pass through the bead pores, while most large molecules cannot penetrate the pores. The larger molecules therefore move more rapidly than smaller molecules, since the smaller molecules have a longer effective path length. This creates a time dependant size distribution in the eluent (Clarke, 2013).

A study by Mukherjee et al. (2009) showed that size exclusion chromatography can be used to purify biosurfactants after acid precipitation. In the study by Mukherjee et al. (2009), a lipopeptide biosurfactant produced by *B. circulans* was purified by gel filtration chromatography with a Sephadex G-50 matrix. UV–visible spectroscopy and TLC analysis of the biosurfactants before and after purification was used to show that the biosurfactant was significantly purified.

According to (Mukherjee, et al., 2009), the micelle forming ability of biosurfactants is the major property of biosurfactants affecting their purification by SEC. Vanittanakom et al. (1986) used Sephadex LH-20 for biosurfactant purification. No data on purity and recovery was, however, given in this study.

2.3.6.3 *Surfactin purification by TLC*

Lipopeptide purification by thin layer chromatography depends on lipopeptide polarity. Standard TLC uses a highly polar stationary phase, and a mobile phase. The distance components in the mixture being separated travel depend on polarity of the components (Geisslera, et al., 2017). Lipopeptide purification by TLC also depends on the polarity of the mobile phase. Geisslera et al. (2017) found that polar solvents such as methanol have a high affinity to lipopeptides, thus polar solvents result in greater migration of lipopeptides on TLC plates compared to non-polar solvents.

Mukherjee et al. (2008) used high performance thin layer chromatography (HPTLC) to purify lipopeptide biosurfactants produced by *B. subtilis*. In their study, a chloroform:methanol:water mixture in the ratio 65:25:4 was used to as a mobile phase. UV light was used for lipopeptide detection, and the detected lipopeptides were scraped off from the plates. The lipopeptide fractions in the scraped silica were dissolved in a mixture of chloroform and methanol in the ratio of 65:35, and the silica was removed by centrifugation. The solvent was then vaporised to recover the pure lipopeptide.

Sivapathasekaran et al. (2010) used TLC for surfactin purification after pre-treatment by acid precipitation and methanol extraction. Korenblum et al. (2012) purified a surfactin-like molecule in a silica gel 60 column after pre-treatment by acid precipitation and solvent extraction using chloroform-

methanol (2:1 v/v). The lipopeptides were then eluted with chloroform-methanol (9:1 v/v) and methanol. It should be noted that surfactin purification by TLC can only be used in analytical scale. Mukherjee et al. (2008) and Sivapathasekaran et al. (2010) purified surfactin for small scale analysis such as FTIR analysis

2.3.7 Adsorption and ion-exchange

Adsorption and ion-exchange have been used to purify surfactin in *B. subtilis* cultures by either adsorption of surfactin from cultures or adsorption of impurities from cultures (Wang, et al., 2010; Dhanarajan, et al., 2015; Liu, et al., 2007; Montastruc, et al., 2008 and Chen, et al., 2008c). According to Dhanarajan et al. (2015), surfactin adsorption can become the most superior method for lipopeptide concentration for correctly selected resins and optimal working conditions.

Surfactin purification by adsorption has several advantages. The resins can be regenerated and reused. Adsorption is capable of achieving purities and separating different lipopeptide families present (Dhanarajan, et al., 2015). Moreover, adsorption is capable of recovering lipopeptides from dilute aqueous solutions, has low toxicity and can be easily scaled up (Chen, et al., 2008c).

Few studies (Table 2-5) have attempted to purify surfactin by adsorption. These studies were compared to determine in which study surfactin purification was done optimally. Optimal adsorption can be defined as one where a high surfactin recovery and purity are simultaneously achieved. Based on these studies, the study by Dhanarajan et al. (2015) had the highest purity (91.6%), while studies by Chen et al. (2008c) gave the highest recoveries (95%). Factors affecting adsorption (e.g. resin properties) are discussed in section 2.3.7.1 to section 2.3.7.4.

Table 2-5: Summary of studies on surfactin recovery by adsorption in literature, where % purity is the mass fraction of surfactin in the product after purification while % recovery is the fraction of surfactin in adsorption liquid recovered into the surfactin product after purification by adsorption

Purification strategy	Pre-purification stage	Resin type	% Recovery	% Purity	Reference
Surfactin adsorption	None	X-5 resin	78.4	48.3	Wang, et al., 2010
Surfactin adsorption	Acid precipitation	HP-20	88	91.6	Dhanarajan, et al., 2015
Ion exchange	Pure surfactin	Activated carbon			Liu, et al., 2007
Ion exchange	None	Activated carbon			Montastruc, et al., 2008
Adsorption (removal of impurities)	Two-stage ultrafiltration	XAD-7	95	88	Chen, et al., 2008c
Ion exchange (removal of impurities)	Two-stage ultrafiltration	AG1-X4	94	80	Chen, et al., 2008c

Optimal surfactin purification by adsorption depends on various factors, which include properties of adsorption resins, resin concentration and surfactin concentration, properties of liquid being purified and mode of operation (static or dynamic).

2.3.7.1 *Effect of resin properties on surfactin adsorption*

Resins can be screened by comparing the adsorption capacities, desorption capacities, percentage of surfactin adsorbed into resins during adsorption (% SA), percentage of surfactin desorbed from resins during desorption as well as surfactin adsorption rate (Jia & Lu, 2008; Jin, et al., 2008; Zhang, et al., 2008; Wang, et al., 2010 and Dhanarajan, et al., 2015). Resin properties include pore diameters, particle size and polarity. High pore diameters improve desorption capacities, but reduce adsorption capacities (Jia & Lu, 2008).

Smaller resin particle sizes or higher resin surface areas improve the surfactin adsorption capacity and adsorption rates as it increases the surface available for surfactin adsorption on the resins at a constant resin mass. This observation is only expected in a well agitated system, where the external limitations to mass transfer have been reduced and internal transport of surfactin onto the adsorption surface is the

rate limiting step. Liu et al. (2007) studied the effect of particle size on surfactin adsorption and found that initial adsorption rates increased from approximately 4 to 24 mg/L/h when adsorbent particle size was decreased from 1.4 to 0.4 g/L. A drawback with smaller particle sizes is however the difficulty in collecting the resins (Liu, et al., 2007).

Surfactin adsorption is also dependent on resin polarity. Adsorption resins can be classified as non-polar, neutral or polar. Wang et al. (2010) and Dhanarajan et al. (2015) studied surfactin adsorption by twelve resins of different polarity, and found that non-polar resins gave high surfactin adsorption (approximately 95%). Non-polar resins are therefore more suitable for surfactin adsorption in comparison to polar and neutral resins. Polar and neutral resins are therefore more suitable for surfactin purification by adsorptive removal of impurities. Neutral resins (XAD-7) have been used for surfactin purification in pre-treated *B. subtilis* cultures by adsorptive removal of impurities in a study by Chen et al. (2008c). In addition to neutral resins, Chen et al. (2008c) also used charged ion-exchange resins (AG1-X4). These resins adsorbed impurities in *B. subtilis* cultures more quickly and preferably than surfactin. A recovery and purity of 95% and 88% was achieved using XAD-resins, while a recovery and purity of 94% and 80% was achieved by the AG1-X4 resins (Table 2-5).

2.3.7.2 *Effect of resin concentration and initial surfactin concentration on surfactin adsorption*

An increase in resin concentration increases the surface area available to adsorb surfactin hence % SA increases until approximately all of the surfactin is adsorbed. Thereafter, an increase in resin concentration results in a decrease in adsorption capacity since the resin mass increases while there is no more surfactin to adsorb (Dhanarajan, et al., 2015 and Wang, et al., 2010). Dhanarajan et al. (2015) and Wang et al. (2010) studied the effect of increase in resin concentration while keeping total lipopeptide concentration constant at 1 g/L and 0.372 g/L respectively. In the study by Dhanarajan et al. (2015), the adsorption ratio increased with increasing resin concentration until 10 g/L, after which the amount adsorbed then stayed constant. In the study by Wang et al. (2010) the adsorption ratio of surfactin increased rapidly with increase in resin concentration from 0 g/L t o10 g/L, before increasing slowly to saturation at 25 g/L.

An increase in surfactin concentration, at a constant resin concentration, increases the surfactin adsorption capacity until the resin becomes saturated. This increase is due to an increase in driving force to overcome mass transfer limitations between aqueous and solid phases, hence improving surfactin diffusion through the liquid film surrounding the adsorbent (Dhanarajan, et al., 2015). Wang et al. (2010), however, found that the kinetics of adsorption or ion exchange are not dependent on surfactin

concentration as larger micelles exist at higher surfactin concentrations. Large micelles make pore or intraparticle diffusion and internal adsorption within the resin particles difficult, causing surfactin to primarily adsorb on outer surfaces of the resin (Wang, et al., 2010). After resin saturation, an increase in surfactin concentration would result in reduced recoveries of the surfactin.

Dhanarajan et al. (2015) and Chen et al. (2008c) studied the effect of initial surfactin concentration on adsorption capacity. In the study by Dhanarajan et al. (2015), where HP-20 non-polar resins were used, an increase in initial surfactin concentratioin improved surfacitn adsorption capacity, and an optimum surfactin concentration of 3 g/L was obtained at a resin concentration of 10 g/L. Chen et al. (2008c) found that an increase in surfactin concentration improved surfactin adsorption, when neutral XAD-7 resins were used, and equilibrium is attained more quickly at low initial surfactin concentration (<500 mg/L). This behaviour is likely due to the fact that surfactin forms large micelles (size 108–142 nm) at high concentrations, resulting in poor intra-particle diffusion and internal adsorption within the resins.

Unlike the study by Dhanarajan et al. (2015) and the study by Chen et al. (2008c) when XAD-7 resins were used, Chen et al. (2008c) found that the surfactin adsorption capacity decreases with increasing initial surfactin concentration when AG1-X4 ion exchange resins were used. This was possibly due to the increase in surfactin micelle size and impurity concentration with increase in initial surfactin concentration. Increase in impurities result in competition for adsorption sites on resins (Chen, et al., 2008c).

Liu et al. (2007) suggested that surfactin adsorption is actually dependent on the surfactin concentration to resin concentration ratio (RC/SC ratio) rather than resin concentration and surfactin concentration independently. Liu et al. (2007) studied the effect of the increase in surfactin concentration and resin concentration on surfactin adsorption while maintaining the RC/SC ratio (7.6 g_r/g_s). It was seen that the % SA at equilibrium and the overall rate of adsorption was similar despite the different the different initial surfactin concentration, which ranged from 8.5 g_s/L to 38 g_s/L, and resin concentration if the RC/SC ratio is constant. Equilibrium was achieved after approximately 33 h of adsorption at different resin concentrations when the RC/SC ratio was held constant.

2.3.7.3 *Effect of environmental factors on surfactin adsorption*

The effect of temperature on surfactin adsorption is unclear. Liu et al. (2007) found that an increase in temperature negatively affects surfactin adsorption (surfactin adsorption capacity decreased by approximately two times when the temperature was increased from 20 °C and 40 °C). This suggests that

surfactin adsorption is an exothermic process. Dhanarajan et al. (2015), on the other hand, found that high temperatures enhance surfactin adsorption (an increase in temperature from 25 °C to 45 °C resulted in an increase in the adsorption ratio form approximately 78% to approximately 97%). This suggests that surfactin adsorption is an endothermic process. The study by Liu et al. (2007) is expected to be more accurate as pure surfactin was used, while the study by Dhanarajan et al. (2015) is more relevant as surfactin was purified from culture supernatants.

Although the effect of operating temperature on surfactin adsorption capacities was unclear, it was found that an increase in tempeature increases surfactin adsorption rates. This is because higher temperatures increase molecular movement during adsorption (Dhanarajan, et al., 2015). In a study by Liu et al. (2007), the adsorption rate was up to three times faster when the temperature was increased from 20 °C to 40 °C.

Surfactin adsorption is also affected by the pH of cultures being purified. As noted by Wiczling & Kaliszan (2010), an acidic group, such as those found in surfactin, is generally ionized (deprotonated) at a pH above its pKa, while a basic group is ionized (protonated) at a pH below its pKa. A change in pH can thus result in ionization of the side chains of amino acids present in the peptide moiety of lipopeptides thus varying their adsorption properties (Dhanarajan, et al., 2015). A change in pH also affects adsorption by influencing the charge of surfactin. Surfactin micelles have a negative charge, and they have a more negative charge at the pH extremes (pH 6 and pH 11). In addition to the increased negative charge at the pH 6 and pH 11, surfactin also forms smaller micelles (108 and 142 nm respectively) at these pHs. This could be another factor causing increased adsorption at higher pH extremes (Chen, et al., 2008c).

Liu et al. (2007), however, found that pH does not affect surfactin adsorption. This is possibly because a small pH range (6.5 to 8.5) was used to study the effect of pH on surfactin adsorption. The acidic groups in surfactin were not ionized. Wang et al. (2010) proposed that pH 7 is suitable for surfactin adsorption studies as surfactin and fengycin have the best stability at this pH. Chen et al. (2008c) found that increased surfactin adsorption occurs at extremes of the pH range studied (6 and 11). In the study by Chen et al. (2008c), there was an interaction between surfactin concentration and pH. Surfactin concentration and pH were both seen to influence the zeta potential of surfactin, and the zeta potential decreased with increasing surfactin concentration. Chen et al. (2008c) found that the pH changes during adsorption, but the effect of the change in pH is unknown. Chen et al. (2008c) suggested that a buffer be used for surfactin adsorption studies, but surfactin was seen not to affect adsorption in the presence of buffers in the study by Liu et al. (2007).

Surfactin adsorption is also affected by ionic strength. Liu et al. (2007) studied the effect of ionic strength on surfactin adsorption by using K_2SO_4 to vary the ionic strength. It was seen that an increase in ionic strength reduces the % SA as well as the surfactin adsorption rate (an increase in K_2SO_4 concentration from 0.05 g/L to 0.1 g/L reduced % SA by approximately two-fold). Buffer addition may increase the ionic strength (Breuer & Jeffrey, 2004), thus may potentially reduce the efficiency of surfactin adsorption.

The quantity of impurities also affect surfactin adsorpton. Liu et al. (2007) and Montastruc et al. (2008) studied surfactin adsorption onto activated carbon from pure surfactin and *B. subtilis* cultures respectively. A lower adsorption capacity (26% lower) was obtained in study by Montastruc et al. (2008) compared to that by Liu et al. (2007) under similar conditions. This was probably due to adsorption of other impurities, such as proteins, in the study by Montastruc et al. (2008).

Impurities can be reduced by resin washing after adsorption. After adsorption, the resins are wet with adsorption liquid which has impurities. The adsorption liquid together with some water-soluble impurities can be washed off with deionized water (Wang, et al., 2010 and Dhanarajan, et al., 2015). Razafindralambo et al. (1993) found that methanol solutions of up to 50% methanol can be used for washing resins, while achieving a 100% surfactin recovery. Wang et al. (2010) studied surfactin purification in *B. subtilis* supernatants by adsorption using X-5 resins, and washed the resins using deionized water. A surfactin adsorption and desorption ratio of 90% and 94% were obtained respectively. The surfactin purity was improved from 24.5% to 48.3% (Table 2-5)

It can also be postulated that surfactin adsorption can be affected by methanol addition in *B. subtilis* cultures. Methanol increases the negative charge of surfactin molecules (Isa, et al., 2007) and disrupts surfactin micelles in adsorption solutions (Chen, et al., 2008b). Surfactin exists as micelles in dynamic equilibrium with monomers in aqueous solution, and only surfactin monomers, rather than micelles, are adsorbed during adsorption. The surfactin micelles then release more monomers to restore dynamic equilibrium between monomers and micelles (Danov, et al., 1996). The addition of methanol is thus expected to improve surfactin adoption rate as it disrupts micelles to form monomers. The presence of methanol in the adsorption liquid also affects the polarity of the solution. It is expected that for less polar solvents, more surfactin will be adsorbed compared to other lipopeptides and impurities (Dhanarajan, et al., 2015). The addition of methanol is therefore also expected to improve the surfactin selectiity.

Liu et al. (2007), however, studied surfactin adsorption rates at surfactin concentrations above CMC (where surfactin exists as micelles) and concentrations below CMC (where surfactin does not exist as micelles) at a constant resin concentration. Although higher adsorption rates were expected at surfactin concentrations below CMC, the adsorption rates were found to be similar. This observation was possibly due to the fact that low surfactin concentrations have a low driving force for adsorption (Liu, et al., 2007), hence low adsorption rates at concentrations below CMC.

2.3.7.4 *Effect of static and dynamic modes on surfactin adsorption*

Surfactin adsorption is also dependent on whether adsorption is carried in a static mode (in shake flasks) or dynamic mode (in columns). Static adsorption studies are commonly used for optimising factors affecting adsorption (e.g. temperature) due to its simplicity, while actual surfactin adsorption is commonly done by dynamic adsorption (Liu, et al., 2007; Wang, et al., 2010 and Dhanarajan, et al., 2015). Dynamic surfactin adsorption improves the adsorption efficiency compared to static adsorption. Wang et al. (2010) tested the surfactin adsorption using different resins (DM130, X-5, HPD-300 and D101) by both static and dynamic adsorption. For the X-5 resin, the % SA was 86.1% and 95% in static mode and dynamic mode respectively. Chen et al. (2008c) found that the surfactin adsorption capacities achieved in static and dynamic mode under similar conditions were 0.16 g_s/g_r and 0.26 g_s/g_r respectively.

Static adsorption is affected by agitation rates, while dynamic adsorption is affected by the height to diameter (h/d) ratio of columns. Agitation is necessary to ensure external transport is not rate limiting. Liu et al. (2007) studied the effect of agitation rate between 80 and 180 rpm, and that the residual surfactin was constant at approximately 10.7 mg/L at the different agitation rates. Agitation speed therefore does not improve surfactin adsorption at agitation speed greater than 80 rpm (Liu, et al., 2007). Column studies are affected by the height/diameter (h/d) ratio. A high h/d ratio may lead to insufficient use of the bed, while a low h/d ratio could result in short retention time of solute and reduce the mass transfer rate (Dhanarajan, et al., 2015).

2.4 Surfactin analytical strategies

The analysis of surfactin concentration is crucial for surfactin purification studies. Surfactin concentration has been analysed by direct and indirect methods. Indirect methods determine surfactin concentration based on physical properties of surfactin (ability to alter surface tension, ability to cause haemolysis and antibacterial activity) (Bence, 2011; Moran, et al., 2002 and Sen & Swaminathan, 2005). Indirect methods have an advantage in that they require less expensive equipment and reagents (Heyd,

et al., 2008 and Moran, et al., 2002). The major disadvantage of indirect methods is that they are susceptible to the presence of other surface-active compounds (such as iturin and fengycin) in the solution (Heyd, et al., 2008).

Direct methods quantify surfactin based on its absolute concentration in cultures. These include the dry weights method, RP-HPLC and HPTLC. In the dry weights method, surfactin is purified from cultures and the dry weight of the purified surfactin per unit volume of culture is defined as the surfactin concentration. Biosurfactant purification for quantification by dry weights has been done by solvent extraction, ultrafiltration and TLC (Moran, et al., 2002 and Das, et al., 2014). The major disadvantage of this method is that it is affected by losses of some surfactin during the series of purification steps (Moran, et al., 2002).

RP-HPLC, which is able to separate surfactin into its isoforms when the gradient method is used, is the most commonly used direct quantification of surfactin concentration (Dhanarajan, et al., 2015; Chen, et al., 2008c; Wang, et al., 2010; Juang, et al., 2012 and Chen & Juang, 2008). This technique, however, has limitations. Firstly, it is limited by the unavailability of individual standards of surfactin isoforms. The surfactin standards currently available in the market contain more than one isoform with and overall surfactin purity of less than 98%, may result in poor estimations of individual isoform concentrations, hence poor accuracies of the HPLC technique. To prevent the poor accuracies which may arise for availability of isoforms, the isocratic method can be used in RP-HPLC. In the isocratic mode, surfactin is quantified as a lipopeptide family rather than individual isoforms. This method is however still limited by high equipment costs and high analysis time (Geisslera, et al., 2017). Deng et al. (2017) developed a method for surfactin analysis, liquid chromatography-tandem mass spectrometry, which reduces the analysis time compared to RP-HPLC. This method is, however, also limited by high equipment costs.

A method has been recently developed for surfactin analysis by high performance thin layer chromatography (HPTLC) (Geisslera, et al., 2017). This method has a poor resolution compared to high performance liquid chromatography (HPLC), but it is suitable for general quantification of lipopeptide families. HPTLC, unlike HPLC, has no peak interference in between the three lipopeptide groups. HPTLC also reduces the chances of cross-contaminations since a new plate is used for each analysis. This method also allows for the use of surfactin analysis when surfactin is dissolved in a wide range of solvents (Geisslera, et al., 2017). This method has also been used for quantification of a biosurfactant lipopeptide by Mukherjee et al. (2008) and Sivapathasekaran et al. (2010). Although HPTLC is cheaper than HPLC, it still has high equipment costs.

A cheaper surfactin analysis technique would be TLC. TLC would be relatively cheaper than HPTLC as it is not automated (e.g. it does not require an automatic spotter or a densiometric scanner). Like HPLTC, TLC would quantify surfactin concentration by relating the area of bands formed by surfactin on TLC plates after development to the surfactin concentration of the sample spotted on the plate. It is expected that there is a linear relationship between the area of bands formed by surfactin on TLC plates and the surfactin concentration on the sample spotted on the TLC plates for a particular concentration range (Geisslera, et al., 2017).

TLC is has not been used for quantification of surfactin concentration in literature, but this method has been used for identifying lipopeptides as well as other impurities in a culture samples. To identify lipopeptides by TLC, retardation factors (R_f) of lipopeptides from culture supernatants are compared those of the pure lipopeptides. In addition to R_f values, the spots can be tested to see if they are lipopeptide spots by testing for the lipid moiety (using a detector such as UV, iodine vapours or primuline) as well as the peptide moiety (using ninhydrin) (Sivapathasekaran, et al., 2010 and Mukherjee, et al., 2008).

To identify impurities by TLC, lipopeptides are identified using R_f values. Any components on the TLC plates which do not have R_f values similar to those of lipopeptides can be referred to as impurities. These impurities can be identified if they are lipids or protein impurities depending on the detector (e.g. ninhydrin or primuline) they respond to. Mukherjee et al. (2009) used impurity assays for determining the degree of biosurfactant purification after purification by gel filtration chromatography. In this study the relative quantity of impurities was identified based on smearing caused by impurities on TLC plates after development, identified by UV light. Mukherjee et al. (2008) used primuline and ninhydrin to identify lipopeptides in developed TLC plates. Although the methodology in the study by Mukherjee et al. (2008) was not used to identify impurities, impurities with lipid parts and protein parts were seen on the TLC plates. Razafindralambo et al. (1993) identified an unknown compund in *B. subtilis* cultures after TLC development, which can be defined as an impurity.

3 Hypotheses and objectives

The hypotheses of this study, which were drawn from critical analysis of the literature, are presented in section 3.1, while the specific objectives required to achieve these hypotheses are given in section 3.2.

3.1 Hypotheses

3.1.1 Surfactin analyses by TLC

- TLC analysis is capable of quantifying surfactin concentration
- TLC analysis can be used to qualitatively analyse impurities with peptide and lipid parts in *B. subtilis* cultures

3.1.2 Surfactin purification

3.1.2.1 *Acid precipitation*

- surfactin can be recovered from cell-free supernatants of *B. subtilis* cultures by acid precipitation to a high recovery and purity
- maximum surfactin recovery and purity achieved after surfactin purification by acid precipitation can be obtained when precipitation is carried out in the range between pH 2 and pH 4
- surfactin purification by acid precipitation does not influence the selectivity of surfactin in the final product

3.1.2.2 *Solvent extraction*

- precipitates obtained after surfactin purification by acid precipitation can be further purified by solvent extraction to a high purity and recovery
- polar solvents are capable of producing higher recoveries compared to non-polar solvents when used for surfactin purification by solvent extraction
- non-polar solvents are capable of producing higher surfactin purities compared to polar solvents when used for surfactin purification by solvent extraction

3.1.2.3 *Surfactin adsorption*

- precipitates obtained after surfactin purification by acid precipitation can be further purified by adsorption to a high purity and recovery

- surfactin purification by adsorption is capable of improving the selectivity of surfactin in the final product
- the fraction of surfactin absorbed as well as the factor by which the selectivity improves during surfactin purification by adsorption is influenced by the temperature at which adsorption is carried out as well as the initial pH, RC/SC ratio and methanol concentration in the adsorption liquid
- Surfactin adsorption can be modelled by the Langmuir or Freundlich model

3.2 Objectives

The main objective of this study was to propose a suitable surfactin purification procedure or procedures, and to provide optimal operating conditions for the proposed purification procedure(s). However, it was also necessary to produce surfactin and develop an analytical technique for surfactin concentration for use in the surfactin purification studies so these became further objectives. Specific objectives are listed below.

3.2.1 Surfactin production

- produce surfactin using *B. subtilis* ATCC 21332

3.2.2 Surfactin analyses by TLC

- develop a methodology for the analysis of surfactin concentration by TLC, and further validate this methodology
- investigate if the TLC analytical technique can be used for qualitatively determining the impurities in *B. subtilis* cultures

3.2.3 Surfactin purification

3.2.3.1 *Acid precipitation*

- investigate the effect of operating pH on surfactin purification by acid precipitation, and recommend an operating pH for acid precipitation

3.2.3.2 *Solvent extraction*

- investigate the effect of solvent polarity on surfactin purification by solvent extraction, and recommend the best solvent for solvent extraction

3.2.3.3 *Adsorption*

- Use a surface design to investigate the effect of the operating temperature as well as the effect of the initial pH, RC/SC ratio and methanol concentration in the adsorption liquid on surfactin purification by adsorption
- provide suitable operating conditions for surfactin purification by adsorption
- propose a suitable for model for modelling surfactin adsorption

4 Methodology

This section outlines how the objectives of this study (discussed in section 3.2) were achieved. Surfactin production and purification were carried out according to the flow diagram shown in Figure 4-1. The black line in Figure 4-1 shows surfactin production and pre-purification by acid precipitation, while the red and blue lines show surfactin purification by solvent extraction and adsorption respectively. In addition to surfactin production and purification, this section provides detail on the TLC analytical technique was developed for surfactin analysis.

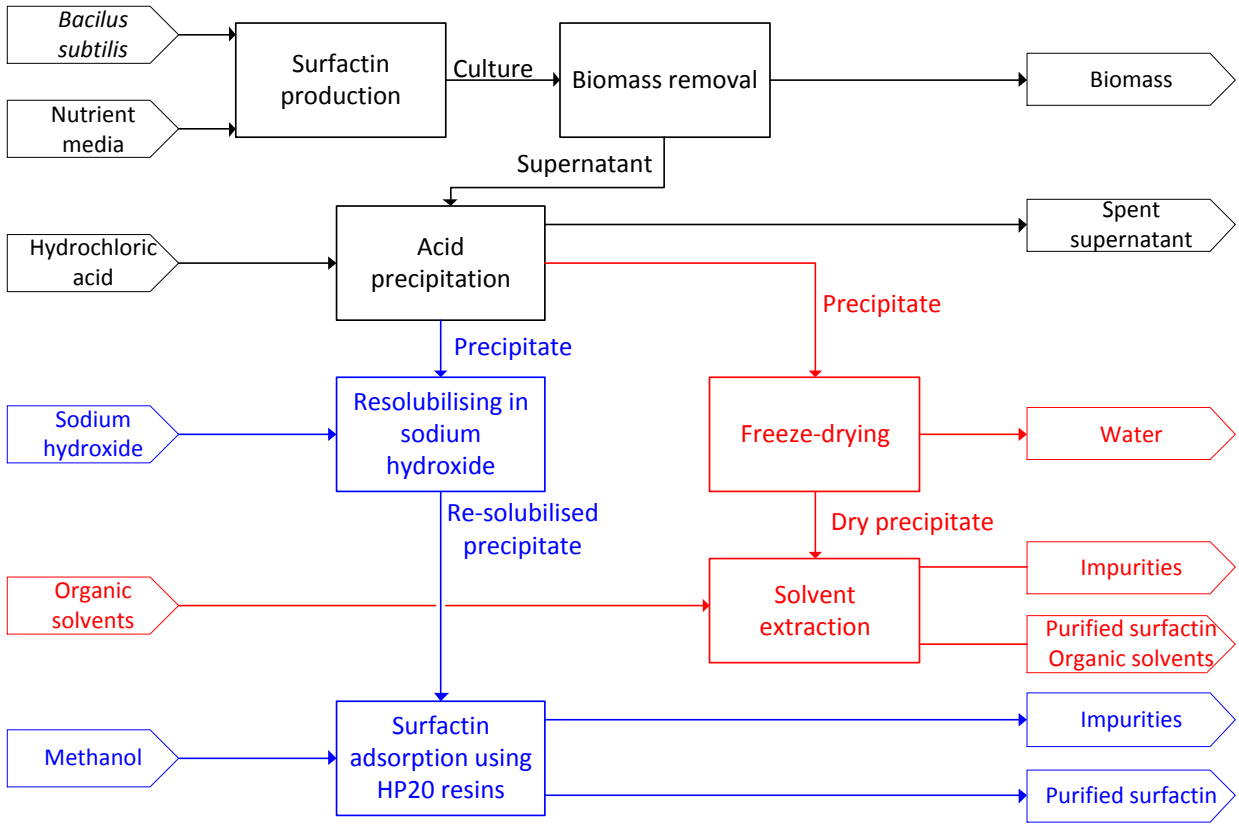

Figure 4-1: Block flow diagram for the proposed surfactin recovery method where the black line show surfactin production and pre-purification by acid precipitation, while the red and blue lines show surfactin purification by solvent extraction and adsorption respectively

4.1 Surfactin production

This section outlines how surfactin was produced from B. subtilis ATCC 21332 as well as how the product and nutrient changed during production.

4.1.1 Microorganisms and culture maintenance

B. subtilis ATCC 21332, which was obtained from the American Type Culture Collection (ATCC), was used in surfactin production experiments. Cultures were stored in 30% (v/v) glycerol mixtures at -18 °C. To recover the cultures, one loopful was transferred aseptically into 25 mL sterile nutrient broth and incubated in an orbital shaker incubator (Labcon®, 30°C, 150 rpm, 24 h). The culture was then aseptically streaked onto nutrient agar plates, and incubated (Labcon, 30°C, 24 h) to grow the bacteria. The streaked plates with *B. subtilis* were kept at 4 °C for a maximum of 3 months.

4.1.2 Growth media

The growth media in surfactin production studies, adapted from Pretorius et al. (2015), is shown in Table 4-1.

Table 4-1: Growth medium for Surfactin production from B. Subtilis (adapted from Pretorius et al. (2015))

Component	**Concentration (g/L)**
$C_6H_{12}O_6.H_2O$	44
NH_4NO_3	4
Na_2HPO_4	7.098
KH_2PO_4	6.805
$MgSO_4.7H_2O$	0.332
$MnSO_4.H_2O$	0.0017
$FeSO_4.7H_2O$	0.002
$CaCl_2.2H_2O$	0.001
Yeast extract	0.5

4.1.3 Surfactin production experiments

4.1.3.1 *Inoculum development*

Prior to bacterial growth, a two-stage inoculation procedure was followed. In the first inoculation stage, two loops of *B. subtilis* were aseptically transferred from nutrient agar plates, which were prepared as discussed in section 4.1.1, to 100 mL of sterile growth media and incubated (Labcon, 30 °C, 150 rpm, 18 h). After incubation, 10 mL of the first inoculum was aseptically transferred to 90 mL growth media in 500 mL baffled shake flasks. The second inoculum was incubated (Labcon, 30 °C, 150 rpm, 18 h).

4.1.3.2 *Surfactin production procedure*

To grow bacteria and produce surfactin, 15 mL culture from the second inoculum was transferred to 135 mL of sterile growth media in 500 mL baffled shake flasks. The shake flasks were then incubated (Labcon, 30 °C, 150 rpm, 60 h).

For analysis of products and depletion of nutrients during surfactin production, experiments were performed in duplicate. 2 mL samples were taken at approximately 12 h intervals during surfactin production for 72 h. These samples were analysed for cell dry weight (CDW), glucose concentration, ammonium concentration, nitrate concentration as well as surfactin and antifungal (fengycin and iturin) concentrations using analytical techniques discussed in section 4.6. The surfactin selectivity (S_S) was defined as the ratio between the surfactin concentration (C_S) and antifungal concentration (C_A) in a particular sample as shown in Equation 4-1.

$$S_S = \frac{C_S}{C_A} \qquad \textit{Equation 4-1}$$

4.2 Development of the TLC analytical technique

Prior to surfactin purification studies, the TLC analytical technique was developed. This section discusses the development of the TLC analytical technique. The validation of the TLC analytical technique is discussed in section 5.2, but the chemicals and materials used for both the development and validation are given in section 4.2.1.

4.2.1 Chemicals and materials

Surfactin (≥ 98%) (Sigma-Aldrich) was used in the preparation of the calibration curve; this surfactin was assumed to be 100% pure in the calculations. Iturin (≥95%) and fengycin (≥90%), also purchased from Sigma-Aldrich, were used in selectivity studies. Commercial surfactin (≥90%) kindly provided by Kaneka Corporation (Japan), was used in validation studies. Where commercial surfactin was used, the

concentrations were converted to pure surfactin by multiplying by 92% and the corresponding pure surfactin concentration was reported in the text. 92% was chosen as a multiplier as it is close to the lower rage of the commercial surfactin purity (≥90%).

The TLC chamber used in this study was a Latch-lid chromatotank fitted with a Teflon coated TLC plate rack which hold six 100 mm by 100 mm TLC plates. Silica gel 60 F_{254} TLC plates (Merck Millipore) were used as the stationary phase. The TLC plates and TLC chamber were both obtained from Sigma-Aldrich. The TLC plates come in square shape of 200 mm length. Each TLC plate was cut into square TLC plates of 100 mm length. The spots where samples were to be spotted on the plates were marked in pencil 15 mm from the lower edge and 10 mm from the left edge and right edges. The distance between these spots was 10 mm, hence the plate has a capacity of 9 samples (Figure 4-2).

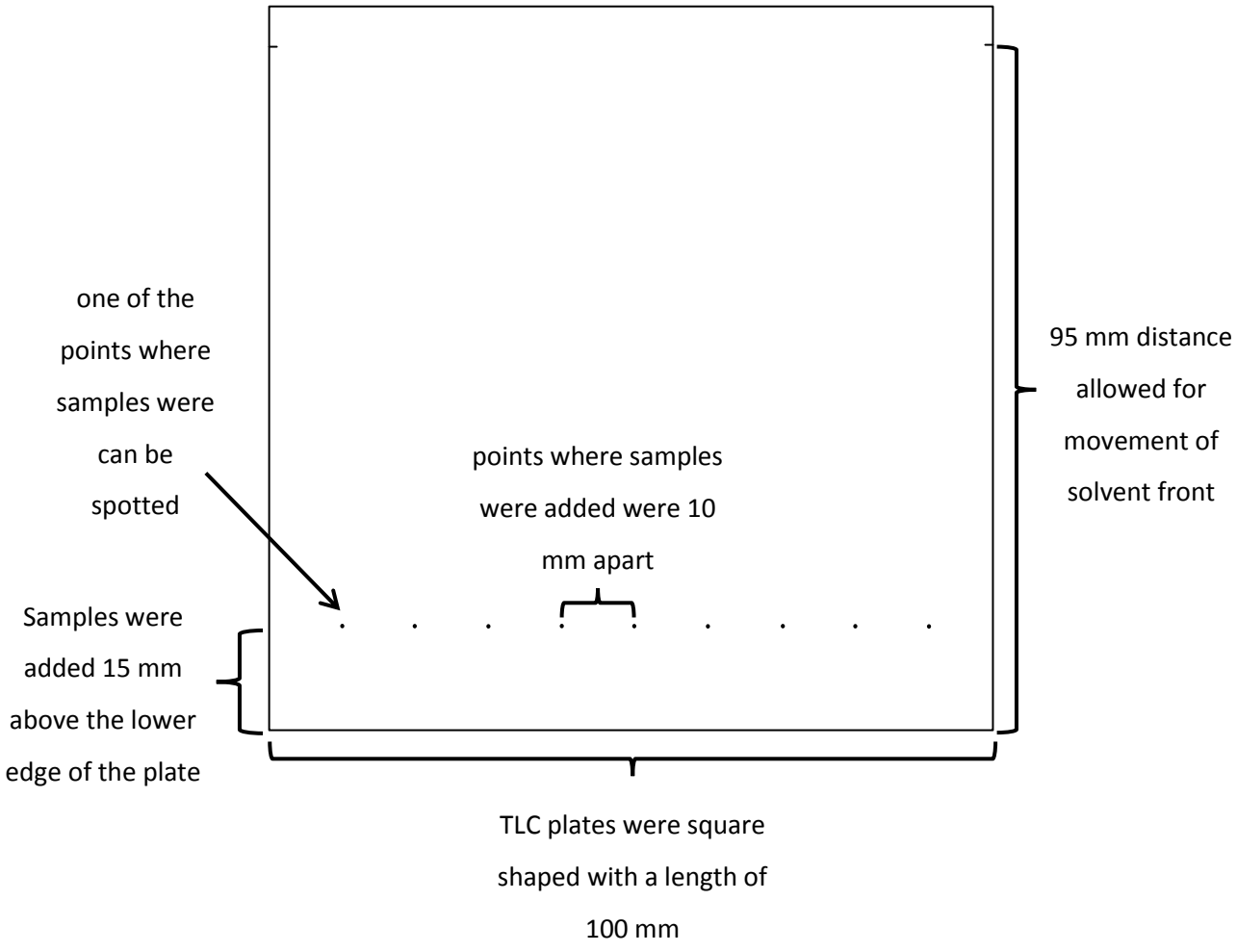

Figure 4-2: TLC plate after it was prepared for sample spotting

Analytical grade methanol and chloroform (Sigma-Aldrich) were used for preparation of the mobile phase. A mixture of chloroform, methanol and water in the volume ratio of 65:25:4 was used as the mobile phase. Other solvents (ethanol and *i*-propanol) used for validation were also obtained from Sigma-Aldrich. For specificity and selectivity studies, a simulated supernatant of *B. subtilis* was prepared by adding 2 g/L bovine serum albumin (Sigma-Aldrich) and 2 g/L surfactin to the medium used in surfactin production (Table 4-1). This simulated supernatant was also used to assess the sensitivity of the technique to multiple spotting (discussed in section 5.2.2.4.2). To study impurity assays, the concentrated supernatant containing 2.48 g/L surfactin, prepared as discussed in section 4.5.1 was used.

Primuline and ninhydrin, used for identifying lipid and protein components on the TLC plates after development were obtained from Sigma-Aldrich. Solid primuline was dissolved in 80% (v/v) acetone in water to form the liquid primuline reagent of 0.005% w/v.

4.2.2 TLC procedure for determining surfactin concentrations and component R_f values

The TLC chamber was initially equilibrated by adding 60 mL of the mobile phase into the chamber and sealing it for 30 minutes. Samples (or standards) were spotted onto TLC plates using a 5 µL Wheaton capillary tube, into which a 1 µL sample had been transferred. The spotted TLC plate was placed into the mobile phase in the equilibrated TLC chamber, ensuring no splashing and that the solvent forms a straight front. This was done to ensure symmetric bands with no peak tailing. A freshly prepared solvent was used after development of every plate to ensure a constant polarity of the mobile phase. After the mobile phase had migrated a distance of 95 mm up the TLC plate, the plates were taken out of the chamber and dried in a fume cupboard. The TLC plates were then sprayed with primuline reagent, ensuring the entire plate is uniformly soaked with primuline, to show the area of the bands formed by surfactin present on the TLC plates. The TLC plates were then scanned (minimum 600 dpi, Canon Canoscan LIDE 220).

Image J (open source image processing and analysis in Java (https://imagej.nih.gov)), was used to calculate the area of surfactin bands on the scanned TLC plates as well as the distance travelled by the surfactin or other components on the TLC plate. The retardation factor (R_f) of surfactin or other components showing on the TLC plates was determined by dividing the distance travelled by surfactin or a particular component by the distance travelled by the solvent front. Surfactin concentration was determined by converting the area obtained by Image J into concentration through a standard curve.

The standard curve was prepared using surfactin (≥98%) from Sigma-Aldrich. A standard surfactin solution of 4 g/L was made in methanol. This standard solution was diluted to form 3, 2, 1 and 0.5 g/L samples. These standards were used to spot samples of 0.5 g/L to 4.5 g/L (with increments 0.5 g/L) on TLC plates. To spot the 4.5 g/L standard, the 4 g/L standard was spotted and dried, before the 0.5 g/L standard was spotted. This was repeated for spotting the 3.5, 2.5 and 1.5 g/L standards. The spotted plates were then developed, and the areas of the standards were determined by image J. The areas obtained were plotted against the concentration spotted at each point to determine the standard curve. The standard curve is shown in Figure 5-4. Experiments for standard curve development were repeated eight times.

4.2.3 TLC procedure for identifying impurities

To identify co-existing impurities, samples were spotted on the TLC, and the plate was developed and dried as discussed in section 4.2.2. For assay of lipid impurities, the developed TLC plates were sprayed with primuline to identify any lipid components on the TLC plate. Components with lipid components are visible as white bands on the TLC plate.

To identify proteins or components with peptide parts, the ninhydrin was applied on the developed TLC plate by means of a cotton wool. The cotton wool was dampened with ninhydrin, and the damp cotton wool was used to gently apply ninhydrin across the developed TLC plate. After ninhydrin was applied, the plate was then dried the plates with a domestic hairdryer until the purple spots became visible on the places where peptide parts were present.

4.3 Surfactin purification by acid precipitation

This section details how surfactin was recovered from cell free supernatants by acid precipitation, as well as the quantification of recovery, purity and selectivity after acid precipitation.

4.3.1 Procedure for surfactin purification by acid precipitation

Acid precipitation was used to purify cell-free supernatants of *B. subtilis*. Cell-free supernatants were prepared by removing biomass from *B. subtilis* cultures prepared as discussed in section 4.1.3.2. Biomass was removed by slow centrifugation (Eppendorf 5702 R, 2900×*g*, 30 minutes) followed by fast centrifugation (Eppendorf Minispin, 14000×*g*, 5 minutes). The supernatant was decanted into beakers while the biomass was removed from the Falcon tubes and autoclaved (121 °C, 15 minutes) before being discarded. 16% (m/m) HCl was used for acid precipitation of surfactin from the cell free supernatant,

while 2.5 M NaOH was used to control the pH of the cell free supernatants to the desired pH. The precipitate was then placed at 4 °C for a minimum of 2 h, before removal of the spent supernatant by centrifugation (Eppendorf 5702 R, 2900×*g*, 10 minutes).

4.3.2 Calculation of surfactin recovery, purity and selectivity after acid precipitation

Acid precipitation was optimised by comparing the surfactin purity (P_S), surfactin recovery (R_S) and improvement in surfactin selectivity (IS) at pH 2, pH 3 and pH 4. P_S was calculated by dividing the mass of surfactin in the precipitate after acid precipitation (M_{SP}) by the mass of the dry precipitate (M_p) as shown in Equation 4-2. R_S was calculated by dividing M_{SP} by the mass of surfactin in the cell-free supernatant (M_{si}) as shown in Equation 4-3.

$$P_S = 100\frac{M_{SP}}{M_P} \qquad \textit{Equation 4-2}$$

$$R_S = 100\frac{M_{Sf}}{M_{Si}} \qquad \textit{Equation 4-3}$$

IS was determined by dividing R_S by the antifungals recovery (R_A) as shown in Equation 4-4. The antifungals recovery was calculated by dividing the mass of antifungals in the dry precipitate after acid precipitation (M_{AP}) by the mass of antifungals in the cell free supernatant (M_{Ai}) as shown in Equation 4-5. The selectivity of surfactin in the precipitate was also determined by dividing the surfactin concentration in the dry precipitate by the antifungals concentration in the dry precipitate according to Equation 4-1.

$$IS = \frac{R_S}{R_A} \qquad \textit{Equation 4-4}$$

$$R_A = 100\frac{M_{AP}}{M_{Ai}} \qquad \textit{Equation 4-5}$$

To determine M_{Si} and M_{Ai} experiments were done using 30 mL cell free supernatant ($V_{supernatant}$) in 50 mL Falcon tubes. The surfactin and antifungals concentration in the cell free supernatant (C_{Si} and C_{Ai}) was determined by HPLC analysis as discussed in section 4.6.3. M_{Si} and M_{Ai} were then determined by multiplying the $V_{supernatant}$ by C_{Si} and C_{Ai} respectively.

To determine M_P, acid precipitation was carried out at pH 2, pH 3 and pH 4 as discussed in section 4.3.1 in pre-weighed Falcon tubes. The Falcon tubes were pre-weighed by initially drying (Memmert, 60 °C, 2 h) and cooling in a desiccator before weighing to four decimal places (Sauter AR 100) to determine the initial Falcon tube mass (m_{ti}). The precipitate after acid precipitation was dried (Memmert, 37 °C, 48 h) and cooled in a desiccator. The mass of the Falcon tube with the dry precipitate (m_{tf}) was measured (Sauter AR 100) to four decimal places, and M_p was then obtained by subtracting m_{ti} from $m_{tf.}$

To determine M_{Sf} and M_{Af}, the dry precipitate in the Falcon tubes was crushed using a spatula. 25 mL deionized water was added into the dry precipitate. 1.25 M NaOH was added into the precipitate water mixture to solubilise the dry precipitate. NaOH was added at time intervals during solubilisation to ensure that the pH of the precipitate-water mixture was always greater than 7. The sum of the volume of deionized water and the volume of the total NaOH added (V_f) was noted. Solubilisation was done in an orbital shaker (25 °C, 90 rpm, 24 h). The surfactin and antifungals concentration in the solubilised precipitate (C_{Sf} and C_{Af} respectively) was determined by HPLC analysis as discussed in section 4.6.3. M_{Sf} and M_{Af} were then determined by multiplying C_{Sf} and C_{Af} by V_f respectively.

4.4 Surfactin purification by solvent extraction

This section details how surfactin purification by solvent extraction was carried out and the quantification of the recovery and purity after extraction.

4.4.1 Procedure for surfactin purification by solvent extraction

Solvent extraction was used to purify dry precipitates after surfactin purification by acid precipitation. Dry surfactin precipitates were prepared by first producing surfactin as discussed in section 4.1.3.2, and recovering the supernatant by centrifugation (Eppendorf 5702 R, 2900×*g*, 30 minutes). Surfactin was recovered from the supernatant by acid precipitation at pH 2 as discussed in section 4.3.1, and the precipitate was frozen at -18 °C before freeze drying (VirTis sentry 2.0, -40 °C, 100 Torr, 48 h). The precipitate was further dried (Memmert, 37 °C, 24 h) and stored in a desiccator.

Organic solvents of differing polarity were then used to selectively extract surfactin from the dry precipitates in an incubator (Labcon, 30 °C, 150 rpm, 24 h). The organic solvents used, in order of decreasing polarity, were methanol, *i*-propanol, chloroform:methanol mixture in the volume ratio 1:1 (C/M 1:1), acetonitrile, chloroform:methanol mixture in the volume ratio 2:1 (C/M 2:1), acetone, chloroform, ethyl acetate, MTBE, petroleum ether and *n*-hexane.

Extraction was carried out in 100 mL Erlenmeyer flasks, which were sealed by covering with aluminium foil, and wrapping with Sellotape. During extraction, a fraction of the dry precipitate is dissolved in the organic solvents, while the other fraction remained undissolved. The undissolved fraction is referred to as the undissolved precipitate. After extraction, the undissolved precipitate is separated from organic solvents through centrifugation (Eppendorf 5702R, 2900×*g*, 10 minutes). Prior to centrifugation, the solvents with undissolved precipitate were poured from the 100 mL Erlenmeyer flasks (in which extraction was carried out) into 50 mL Falcon tubes. The flasks were then rinsed with 5 mL of the solvent

used for extraction, and this rinsing solvent was poured into the 50 mL Falcon tubes with solvents and undissolved precipitate before centrifugation.

4.4.2 Calculation of surfactin recovery and purity after solvent extraction

Solvent extraction was optimised by comparing the surfactin recovery (R_S) and purity (P_S) achieved by each solvent after extraction of surfactin from the dry precipitate. R_S was calculated by dividing the mass of surfactin extracted into the solvents (M_S) by the mass of surfactin in the dry precipitate (M_{SP}) as shown in Equation 4-6. P_S was calculated by dividing M_S by the total mass of solids in the dry precipitate which were dissolved in the solvents during extraction (M_{DS}) as shown in Equation 4-7.

$$R_S = 100\frac{M_S}{M_{SP}} \qquad \textit{Equation 4-6}$$

$$P_S = 100\frac{M_S}{M_{DS}} \qquad \textit{Equation 4-7}$$

To determine M_{SP}, 30 mL alkaline water (V_w) was added to 0.1 g of the dry precipitate in a 100 mL Erlenmeyer flasks. The tube was sealed and agitated (Labcon, 150 rpm, 30 °C, 48 h) to solubilise the dry precipitate. The surfactin concentration in alkaline water ($C_{s,w}$) water was determined by the TLC analytical technique discussed in section 4.2.2. M_{SP} was then determined by multiplying V_w by $C_{s,w}$.

To determine M_S, solvent extraction was carried out using 0.1 g of the dry precipitate and 30 mL solvent as discussed in section 4.4.1. Surfactin concentration in the solvents after extraction (C_S) was determined by TLC analysis as discussed in section 4.2.2. The mass of surfactin extracted into the solvents was then determined by multiplying C_S by the sum of the solvent used for extraction and that used for rinsing the flasks after extraction.

M_{DS} was determined by subtracting the sum of the mass of the undissolved precipitate during solvent extraction (M_{US}) and the mass of the undissolved precipitate during solubilisation of the precipitate in alkaline water (M_{UW}) from the mass of the dry precipitate before extraction (M_P), as shown in Equation 4-8.

$$M_{DS} = M_p - (M_{US} + M_{UW}) \qquad \textit{Equation 4-8}$$

To determine M_{US} and M_{UW}, 0.1 g of dry precipitate (M_P) was used for solvent extraction and solubilisation in alkaline water in pre-weighed falcon tubes respectively. The Falcon tubes were pre-weighed by drying (Memmert, 60 °C, 2 h) and cooing in a desiccator before being weighed (Sauter AR 100) to four decimal places. The undissolved precipitate after extraction and solubilisation was then recovered in by centrifugation (Eppendorf 5702 R, 2900×g, 10 min), before drying in a water bath in a

fume cupboard and further drying in an oven (Memmert, 37 °C, 36 h). The Falcon tubes with dry undissolved precipitates were then cooled in a desiccator and weighed (Sauter AR 100) to four decimal places. M_{US} and M_{UW} were then obtained by subtracting the mass of Falcon tubes with dry undissolved precipitate from the mass of the empty Falcon tube. M_{UW} was assumed to be the mass of biomass, since there were no undissolved solids when the dry precipitate was resolubilised in acid precipitation studies, where centrifugation was done at 14000×*g*.

4.5 Surfactin purification by adsorption

This section details how surfactin purification by adsorption was carried and the quantification of the recovery, purity and selectivity after adsorption. Additionally, this section provides detail on how surfactin purification was optimised as well as how adsorption kinetics and isotherms were studied. HP-20 non-polar resins were used to purify resolubilised surfactin precipitates from the acid precipitation purification step. The resin properties, according to manufacturer's specifications, are shown in Table 4-2.

Table 4-2: HP-20 resin properties specified by the manufacturer

Uniformity coefficient	1.5
Water content	59.40%
Particle size distribution through 250 micrometres	2.70%
Effective size	0.35 mm

4.5.1 Procedure for surfactin purification by adsorption

The resins were prepared for adsorption by pre-treatment with 95% (v/v) ethanol in an incubator (Labcon, 30 °C, 150 rpm, 24) to remove any impurities (monomers and porogenic agents) trapped inside the pores during the synthesis process. After pre-treatment, the resins were recovered using a Buchner vacuum filter (Millipore) lined with a 0.2 µm paper disk (Anatech) and washed with deionized water before drying (Memmert, 40 °C, 48 h). The adsorption resins were stored at room temperature, which falls within the recommended temperature range (4 °C to 38 °C) for resin storage, and exposure of the resins to direct sunlight was avoided.

The solubilised surfactin precipitates from the acid precipitation were prepared by initially producing surfactin as discussed in section 4.1.3.2, and recovering the supernatant by slow centrifugation

(Eppendorf 5702 R, 2900×*g*, 30 minutes). Surfactin was recovered from the supernatants by acid precipitation at pH 2 as discussed in section 4.3.1, and the precipitate was dissolved in alkaline water to form a stock solution at pH 8, with a surfactin and antifungal concentration of 2.48 g/L and 1.05 g/L respectively (determined by HPLC analysis). The stock solution was further centrifuged by fast centrifugation (Eppendorf Minispin, 14000×*g*, 5 min) to remove any biomass as slow centrifugation was used before acid precipitation. The stock solution was then kept in 50 mL Falcon tubes and stored at -18 °C.

The stock solution was sterilised by filtration through a syringe filter (Anatech, 0.22 µm) before adsorption studies to avoid the growth of bacteria during adsorption experiments. The stock solution was diluted 5 times using sterile deionized water or deionized water with methanol (where indicated in text) to form the adsorption liquid. The pH of the adsorption liquid was adjusted using 4% (m/m) HCl and/or 2.5 M NaOH to the desired pH. A Metrohm 744 pH meter was used for pH measurements, and the supplier of the pH meter confirmed that it is suitable for use in solutions with less than 30% (v/v) methanol. According to Canals et al. (2001), a pH meter is suitable for measuring the pH even in the presence of methanol provided a correction factor is considered, which was found to be insignificant in this study (section 8.3.1).

25 mL of the adsorption liquid was mixed with a desired resin mass (determined by a Sauter AR 100 balance to four decimal places) in sterile 100 mL baffled shake flasks. Adsorption was then carried out in in an incubator (Labcon, 150 rpm, 24 h) at the desired temperature. Preliminary tests showed that equilibrium was reached after 24 h (section 8.3.2).

4.5.2 Calculation of surfactin purity and recovery after adsorption

The surfactin purity (P_S) and recovery (R_S) after purification by adsorption was determined according to Equation 4-9 and Equation 4-10 respectively. In these equations, V_d, C_d and M are the volume of desorption liquid, surfactin concentration in the desorption liquid and mass of residue left when the desorption liquid was evaporated after surfactin desorption, respectively.

$$R_S = \frac{V_i C_i - V_d C_d}{V_i C_i} \qquad \textit{Equation 4-9}$$

$$P_S = \frac{V_d C_d}{M} \qquad \textit{Equation 4-10}$$

To determine C_d, adsorption was carried out as discussed in section 4.5.1 at an initial pH, operating temperature, RC/SC ratio and methanol concentration of 11.5, 45 °C, 5 g_r/g_s and 30% (v/v). After

adsorption, the resins were recovered by filtration using 150 mm diameter filter papers in filter funnels under gravity to ensure all resins are recovered. Filtration was carried out overnight, and the resins were put into 100 mL baffled shake flasks. 25 mL of methanol (V_d) was added into the shake flasks, and the flasks were sealed and agitated (Labcon, 150 rpm, 30 °C, 24 h). The surfactin concentration in the desorption liquid was then determined by TLC analysis as discussed in section 4.2.2.

To determine M, 20 mL of the methanol with desorbed surfactin was put in pre-weighed Falcon tubes. The methanol was then evaporated in a water bath to recover the solid residue. The residue was further dried (Memmert, 37 °C, 24 h). The mass of the Falcon tube with dry solid residue was determined to four decimal places using a Sauter AR 100 balance. M was then determined by subtracting the initial mass of the falcon tubes from the mass of the Falcon tubes with dry residue. Purity and recovery experiments were repeated four times.

4.5.3 Optimisation of the surfactin adsorption purification technique

Surfactin adsorption was optimised based on the percentage of surfactin adsorbed onto resins during adsorption (% SA) and the improvement in surfactin selectivity (IS). % SA and IS were determined according to Equation 4-11 and Equation 4-12.

$$\%SA = 100\left(\frac{C_i - C_e}{C_i}\right) \qquad \textit{Equation 4-11}$$

$$IS = \frac{PSA}{\%A_{antifungals}} \qquad \textit{Equation 4-12}$$

In Equation 4-11, C_i and C_e are the initial and equilibrium surfactin concentrations in the adsorption liquid respectively. In Equation 4-12, % $A_{antifungals}$ is the percentage of antifungals adsorbed (calculated according to Equation 4-13, where $C_{a,i}$ and $C_{a,e}$ are the initial and equilibrium antifungal concentrations).

$$\%A_{antifungals} = \frac{C_{a,i} - C_{a,e}}{C_{a,i}} \qquad \textit{Equation 4-13}$$

Surfactin adsorption was optimised by determining the effect of operating temperature, initial pH, RC/SC ratio as well as the methanol concentration on % SA and IS in the ranges shown in Table 4-3. The RC/SC ratio was optimised by keeping the surfactin concentration constant (0.5 g/L) while varying the resin concentration between 2 g/L and 20 g/L.

Table 4-3: Range at which independent variables were optimised in surfactin adsorption studies

Independent variable	Minimum value	maximum value
Operating temperature (°C)	25	45
Initial pH	6.5	11.5
RC/SC ratio (g_r/g_s)	4	40
Methanol concentration [%(v/v)]	0	30

A face centred factorial design (Table 4-4) was used to study the effect of operating temperature, initial pH and RC/SC ratio. The effect of methanol on the adsorption was then determined, also using a face centred factorial design (Table 4-5). All experimental runs were done in duplicate.

To determine % SA and IS, adsorption was carried out as discussed in section 4.5.1 for all experimental runs in Table 4-4 and Table 4-5, and C_e as well as $C_{a,e}$ were determined by HPLC analysis as discussed in section 4.6.3. C_i and $C_{a,i}$ were determined from dilutions made on the stock solution (prepared in section 4.5.1) and were used together with the determined C_e and $C_{a,e}$ to determine % SA and IS according to Equation 4-11 and Equation 4-12.

Table 4-4: Face centred central composite design for studying the effects of temperature, pH and RC/SC ratio on surfactin adsorption

Standard Order	Run Order	Temperature (°C)	pH	RC/SC ratio (g_r/g_s)
1	3	25	6.5	4
2	8	45	6.5	4
3	3	25	11.5	4
4	2	45	11.5	4
5	3	25	6.5	40
6	8	45	6.5	40
7	3	25	11.5	40
8	2	45	11.5	40
9	3	25	9	22
10	5	45	9	22
11	1	35	6.5	22
12	1	35	11.5	22
13	6	35	9	4
14	7	35	9	40
15	7	35	9	22
16	4	35	9	22

Table 4-5: face centred central composite design for studying the effects of methanol concentration on surfactin adsorption

Standard order	run order	Temperature (°C)	pH	RC/SC ratio (g_r/g_s)	Methanol concentration (vol%)
1	2	25	6.5	4	0
2	4	45	6.5	4	0
3	2	25	11.5	4	0
4	1	45	11.5	4	0
5	2	25	6.5	40	0
6	4	45	6.5	40	0
7	2	25	11.5	40	0
8	1	45	11.5	40	0
9	7	25	6.5	4	30
10	8	45	6.5	4	30
11	7	25	11.5	4	30
12	8	45	11.5	4	30
13	7	25	6.5	40	30
14	8	45	6.5	40	30
15	7	25	11.5	40	30
16	9	45	11.5	40	30
17	3	35	9.0	22	0
18	6	35	9.0	22	30
19	10	35	9.0	4	15
20	10	35	9.0	40	15
21	5	35	6.5	22	15
22	6	35	11.5	22	15
23	11	25	9.0	22	15
24	8	45	9.0	22	15
25	6	35	9.0	22	15
26	6	35	9.0	22	15

4.5.4 Batch adsorption kinetics

Batch kinetics were studied by determining adsorption capacity (q_t) at different time intervals during adsorption as well as the time taken to reach equilibrium (t_{eq}). The q_t was determined according to Equation 4-14, where V, C_i, C_t and W are the volume of adsorption liquid, initial surfactin concentration, surfactin concentration at different time intervals during adsorption and resin mass respectively.

$$q_t = \frac{V(C_i - C_t)}{W} \qquad \textit{Equation 4-14}$$

To determine q_t and t_{eq} batch kinetics were studied by carrying out adsorption as discussed in section 4.5.1 at 45 °C, pH 11.5, adsorption liquid of 30 mL, RC/SC ratio 22 g_r/g_s (hence resin mass of 0.33 g_r) and at methanol concentrations of 0% (v/v) and 30% (v/v). 400 µL samples were taken at time intervals during adsorption for 30 h. The surfactin concentration (C_t) at the different time intervals was determined by HPLC analysis as discussed in section 4.6.3. Adsorption runs in batch kinetic studies were done in duplicate.

Batch kinetics were also modelled using the pseudo first-order model (Equation 4-15) as well as the pseudo second-order model (Equation 4-16) to determine the best model for surfactin adsorption kinetics. This was done in order to determine surfactin adsorption rates. In these models, m is a constant, t is the time at which sample was taken during adsorption, q_e is the adsorption capacity at equilibrium while k_1 and k_2 rate constants for the pseudo fist-order equation and pseudo second-order rate equation respectively. Symbolic regression using the Microsoft Excel R^2 as a model selector was used to determine which model best fits the kinetics data in this study. Since q_e is integrated in the y-axis of the pseudo first-order model, q_e and k_1 in this model were determined by regression using solver in Microsoft Excel. For the pseudo second-order model, q_e and k_2 were determined by plotting $1/q_t$ against 1/t. q_e and k_2 were then determined using the y-intercept and slope of this plot respectively.

$$ln\left(\frac{q_e}{q_e - q_t}\right) = k_1 t + m \qquad \textit{Equation 4-15}$$

$$\frac{1}{q_t} = \frac{1}{t k_2 q_e^2} + \frac{1}{q_e} \qquad \textit{Equation 4-16}$$

4.5.5 Batch adsorption isotherms

Batch isotherms were studied to determine a suitable model for modelling surfactin adsorption based on equilibrium adsorption capacity (q_e). The q_e was determined according to Equation 4-17, where C_i, C_e, V_i and W are the initial surfactin concentration, equilibrium surfactin concentration, volume of adsorption liquid and resin mass respectively.

$$q_e = \frac{V_i(C_i - C_e)}{W}$$ *Equation 4-17*

The Langmuir model (Equation 4-18) and the Freundlich model (Equation 4-19) were tested in this study. Symbolic regression using the Microsoft Excel function R^2 as a model selector was used to determine which model best fits the experimental data in this study. In Equation 4-18, K_L is the adsorption equilibrium constant and q_m is the theoretical maximum adsorption capacity. In Equation 4-19, K_F is the Freundlich constant and 1/n is an empirical constant.

$$\frac{C_e}{q_e} = \frac{C_e}{q_m} + \frac{1}{K_L q_m}$$ *Equation 4-18*

$$lnq_e = lnK_F + \frac{1}{n} lnC_e$$ *Equation 4-19*

To determine q_e, adsorption isotherms were studied by carrying out adsorption as discussed in section 4.5.1, at a methanol concentration of 0% (v/v) and 30% (v/v) in the adsorption liquid. The resin mass (W) was held constant at 0.1 g_r, while the surfactin concentration was varied between 1.92 and 0.32 g_s/L. The initial pH, operating temperature and methanol concentration in the adsorption liquid were held constant at 11.5, 45 °C and 30% (v/v) respectively. C_e was determined by HPLC analysis as discussed in section 4.6.3. Adsorption runs for batch isotherms studies were done in duplicate.

4.6 Analytical methods

4.6.1 Cell concentration

Cell concentration in *B. subtilis* cultures was determined by obtaining absorbance readings of suitably diluted culture samples and subsequently converting these absorbance readings into CDW through the use of a standard curve. 1 mL culture samples were collected during surfactin production, and their absorbance was determined through a spectrophotometer (Varian, 620 nm) using distilled water as a blank. Culture samples with an optical density greater than 0.8 were diluted with deionized water to get an optical density less than 0.8. The absorbance readings were then calibrated using with a CDW standard curve (Figure 4-3) to obtain cell concentrations.

To obtain the CDW standard curve, *B. subtilis* was grown for 24 h as discussed in section 4.1.3.2. The culture was then diluted to dilutions ranging from ×200 to ×25, each dilution with a volume of 16 mL, using 0.85% w/v NaCl solution. 1 mL of each dilution analysed for absorbance (Varian, 620 nm). The remaining 15 mL of each dilution was filtered using a Buchner vacuum filter (Millipore) lined with a 0.2 µm filter pre-weighed paper disk (Anatech). The filter papers were pre-weighed by drying (Memmert,

60°C, 24 h) and cooling in a desiccator before weighing (Sauter AR 100) to 4 decimal places. After filtration, the filter paper with biomass was dried (Memmert, 60° C, 24 h) and cooled in a desiccator before being weighed (Sauter AR 100) to four decimal places. The CDW was determined as the difference in weight between the filter paper with biomass and the initial mass of the filter paper. The CDW was then plotted against the corresponding absorbance at each dilution to obtain the CDW standard curve.

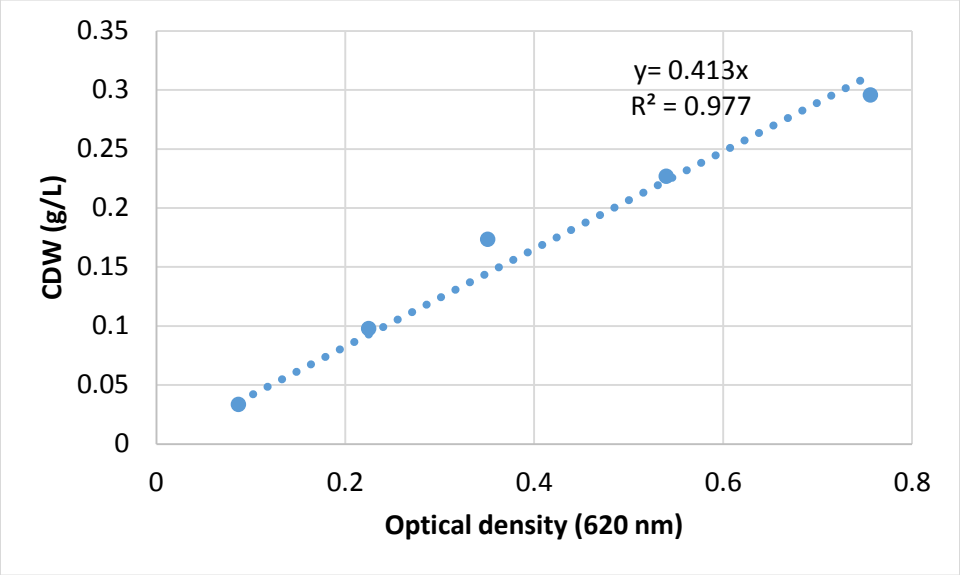

Figure 4-3: Cell dry weigh (CDW) standard curve (experimental repeatability was not studied thus there are no error bars)

4.6.2 Glucose concentration

The glucose concentration in *B. subtilis* cultures was obtained using the dinitrosalicylic acid (DNS) analysis technique (adapted from Miller (1959)). The DNS solution used consisted of 3,5-dinitrosalicylic acid (10 g/L), phenol (2 g/L), sodium metabisulphite (0.5 g/L) and sodium hydroxide (10 g/L).

To carry out DNS analysis, 1 mL culture samples were centrifuged (Eppendorf Minispin, 14000×*g*, 5 minutes) to isolate the supernatant from biomass. Supernatants with a glucose concentration greater than 500 mg/L were diluted with de-ionized water. 1 mL supernatant or diluted supernatant was transferred to test tubes, and 1 mL DNS solution was added into the supernatants in the test tubes. The resulting mixture was then heated in boiling water for 5-6 minutes. 0.33 mL of 40% w/v sodium potassium tartrate solution was then added into the heated mixture, and the mixture was immediately quenched in ice cold water. The absorbance of the quenched mixture was then determined (Varian, 540

nm). The absorbance reading was then calibrated with a glucose standard curve (Figure 4-4) to obtain the glucose concentration in g/L.

To prepare the standard curve, glucose standards with concentrations ranging between 0.05 and 0.5 g/L were prepared. The absorbance readings of the standards were determined in an analogous manner to that of the sample supernatant. These absorbance readings were then related to the glucose concentration of the corresponding standard to obtain the glucose standard curve.

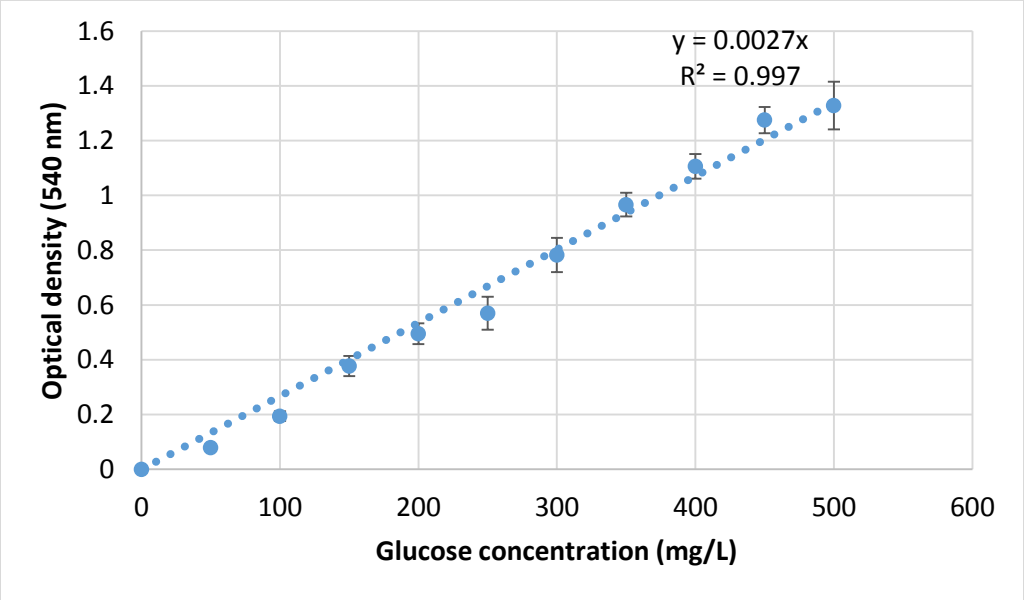

Figure 4-4: Standard curve for glucose quantification by DNS analysis, where the error bars indicate the standard deviation of four replicates

4.6.3 Surfactin, iturin and fengycin concentration

Surfactin concentration in this study was analysed by TLC analysis and HPLC analysis. Surfactin analysis by TLC is detailed in section 4.2.2. This section outlines how the analysis of surfactin and antifungal (iturin and fengycin) concentration determination was carried out by HPLC analysis. Surfactin analysis by HPLC was done for samples from production, acid precipitation and adsorption studies. Samples with a methanol concentration greater than 30% (v/v) were diluted to a methanol concentration ≤30% (v/v) prior to analysis as high methanol concentrations distorts surfactin peak shape and increases elution time.

1 mL samples were centrifuged (Eppendorf Minispin, 14000×*g*, 5 minutes), and 500 µL of the centrifuged samples were then mixed with 500 µL acetonitrile containing 0.05% (v/v) trifluoroacetic acid. This mixture was then filtered using a 0.22 µm syringe filter (Anatech) before injection into the

HPLC column. If the mixture had to be stored to await HPLC analysis, it was stored at -18 ^{o}C and only filtered (0.22 µm) prior to analysis.

50 µL of the mixture was injected in the HPLC column, a Phenomenex Luna 3µm C18 column (250 x 4.6 mm) using a Dionex Ultimate 3000 Diode-array detector. The mobile phase solvent, gradient and flow rate used during analysis are shown in Table 4-6. The eluent of the HPLC column was monitored at 210 nm to obtain sample adsorption peaks. The peak areas obtained were calibrated with the surfactin and antifungal standard curves (Figure 4-5 to Figure 4-7) to obtain the surfactin and antifungal concentrations in g/L, and the corresponding chromatograms are shown in Figure 8-1 to Figure 8-3.

Table 4-6: Mobile phase information for HPLC analysis

Mobile phase A	0.05% (v/v) Trifluoroacetic acid (Fluka®) in water
Mobile phase B	0.05% (v/v) Trifluoroacetic acid in acetonitrile (High purity UV grade, Burdick & Jackson)
Mobile phase gradient	Start at 35% B, increase to 40% B during the next 2 minutes, isocratic at 40% B for the next 5 minutes, increase to 63% B during the next 43 minutes, increase to 80% B during the next 10 minutes, increase to 87% B during the next 35 minutes, return to 35% B during the next 10 minutes and isocratic stabilisation at 35% B for the next 5 minutes.
Flow rate	0.9 mL/min

The surfactin standard curves were prepared using surfactin standards with surfactin concentrations ranging from 0.2 g/L to 2.5 g/L in water, using surfactin (≥98%) (Sigma-Aldrich). The surfactin standards were prepared for HPLC by mixing with acetonitrile and run through the HPLC column, and area of each homologue was determined. The homologue areas were then used to determine the fraction of each homologue in the standard. The concentration of each homologue in the standards was determined by multiplying the surfactin concentration in the standards by the fraction of a particular homologue in the standard. The standard curves for the different homologues was then determined by plotting the homologue area against the homologue concentration. The overall surfactin standard curve is shown in Figure 4-5.

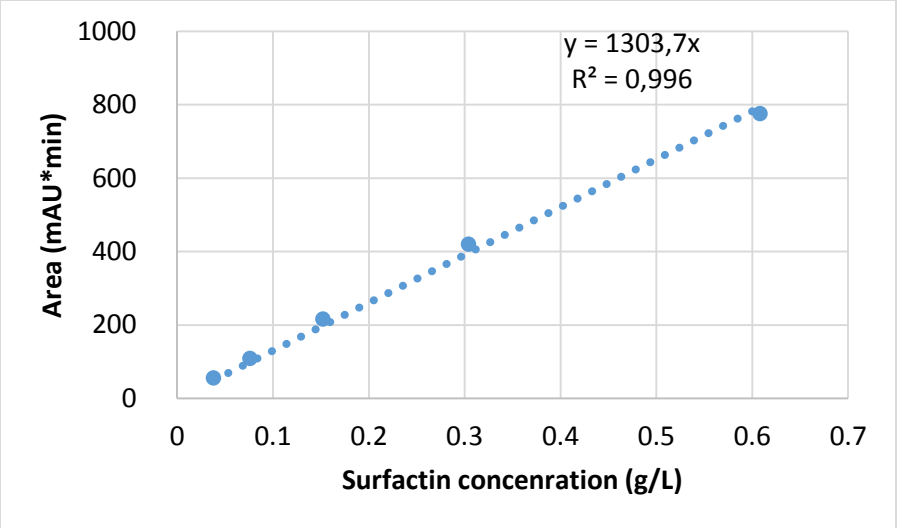

Figure 4-5: Standard curve for surfactin quantification by HPLC analysis (experimental repeatability was not studied thus there are no error bars)

The antifungal standard curves were prepared using fengycin and iturin standards with fengycin and iturin concentrations of 0.1 to 0.5 g/L and 0.05 to 0.2 g/L respectively in methanol. The antifungal standards were prepared using (≥90%) fengycin and (≥95%) iturin, both from Sigma-Aldrich. The standards were run through the HPLC column, and the areas obtained were related to the standard concentrations to obtain the antifungal standard curves shown in Figure 4-6 and Figure 4-7.

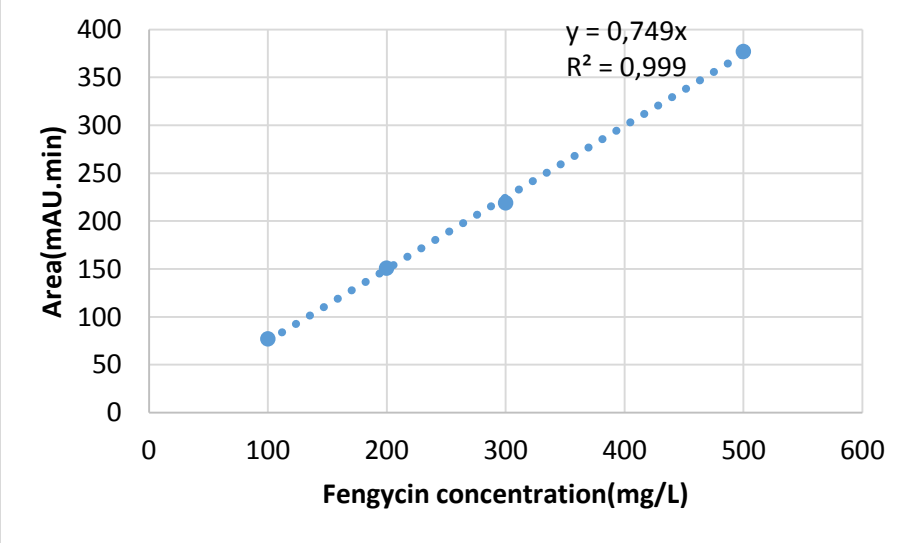

Figure 4-6: Standard curve for fengycin quantification by HPLC analysis (experimental repeatability was not studied thus there are no error bars)

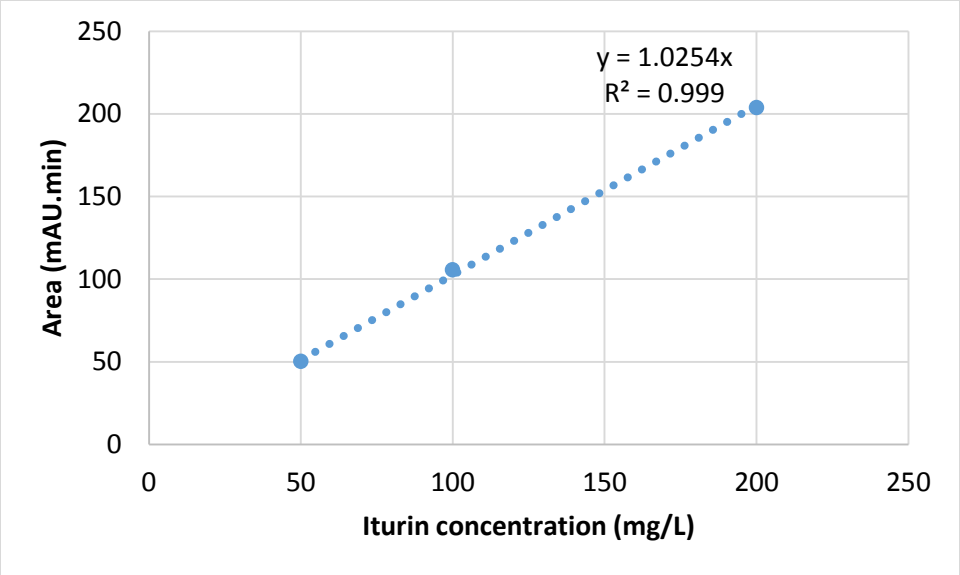

Figure 4-7: Standard curve for Iturin analysis by HPLC analysis (experimental repeatability was not studied thus there are no error bars)

4.6.4 Ammonium concentration

The ammonium concentration in *B. subtilis* cultures was analysed using ammonium test strips (Merck). 1 mL culture samples were centrifuged (Eppendorf Minispin, 14000×*g*, 5 minutes) to isolate the supernatant from biomass. 2 drops of NH_4-1 solution were added to 1 mL of the supernatant. The reaction zone of the test strip was immersed in the supernatant-NaOH mixture for 3 seconds, and suspended in air for 10 seconds. The colour of the reaction zone was then compared to the colour field relating the colour of the reaction zone to the on the ammonium concentration in mg/L.

4.6.5 Nitrate concentration

The nitrate concentration was quantified by ion chromatography. 1 mL culture samples were centrifuged (Eppendorf Minispin, 14000×*g*, 5 minutes) to isolate the supernatant from biomass. 500 µL of the supernatant was mixed with 500 µL of buffer (5 mM sodium carbonate). The supernatant-buffer mixture was then diluted 50 times in deionized water and filtered using a 0.22 µm syringe filter (Anatech).

50 µL of the prepared samples were then injected into the column with specifications described in Table 4-7 to obtain sample peaks. The peak areas were converted into nitrate concentration by means of a standard curve (Figure 4-8). The standard curve was determined by injecting nitrate standards (ranging from 10 to 100 mg/L) into the column and eluted with the mobile phase. Nitrate concentrations were then plotted against the corresponding peak areas to obtain the nitrate standard curve.

Table 4-7: Ion chromatography specifications for nitrate analysis

Separator column	IonPac AS4A-SC 4mm Mobile
Mobile phase	1.8 mM Na_2CO_3/1.7 mM $NaHCO_3$
Flow rate	1 mL/min
Regenerant	25 mM H_2SO_4

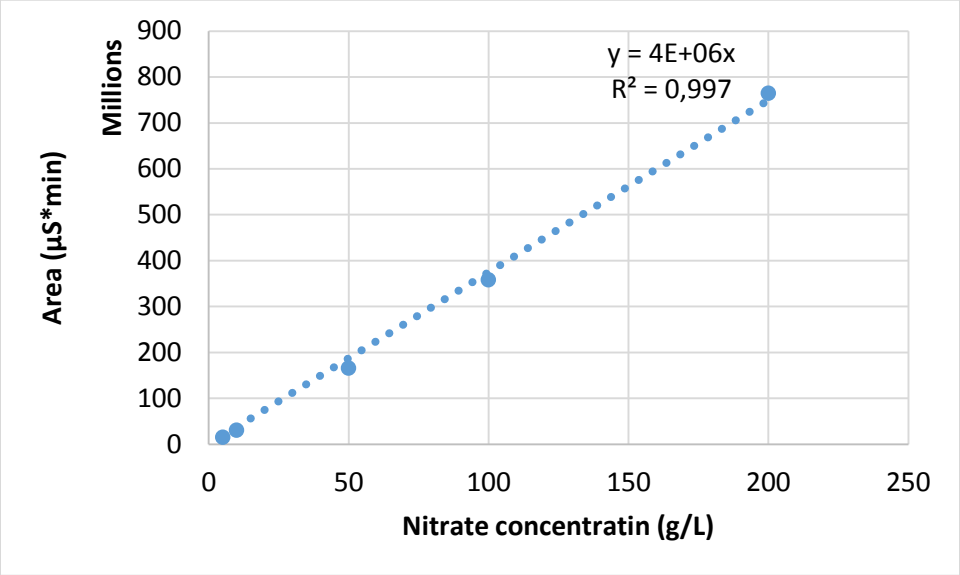

Figure 4-8: Nitrate standard curve (experimental repeatability was not studied thus there are no error bars)

5 Results and discussion

This section presents the results and discussion of the experiments done in order to achieve the specific objectives of this is study. The main aim of this study is to propose surfactin purification techniques with operating conditions. To study surfactin purification, however, the surfactin had to be produced and a surfactin analytical technique had to be developed. This section therefore begins by providing results and discussion of surfactin production studies followed by studies on the development and validation of the TLC analytical technique. Thereafter, this section presents the results and discussion of optimisation experiments on acid precipitation, solvent extraction and adsorption to determine operating conditions of these purification techniques. This section then provides a repeatability analysis of the experiments carried out in this study.

5.1 Surfactin production

To achieve the main aim of this study, surfactin was produced for use in purification studies. It should be noted that the main focus of this study is not on surfactin production. Surfactin was produced from *B. subtilis* ATCC 21332, batch wise in shake flasks. *B. subtilis* was chosen for surfactin production due to its high selectivity towards surfactin production. Additionally, *B. subtilis* is not a human pathogen thus safe to work with (Kosaric & Sukan, 2014). Production was carried out in shake flasks due to the simplicity of shake flasks compared to bioreactors.

During surfactin production, the maximum surfactin concentration in *B. subtilis* cultures was 1109 mg/L. The maximum surfactin concentration in *B. subtilis* cultures during production in literature ranges from 439 mg/L and 3340 mg/L where glucose and ammonium nitrate were used as a carbon and nitrogen respectively (Table 2-1). The maximum surfactin concentration in this study is thus lower than that in literature. This is possibly due to the fact that the nutrient media used in this study was optimised for fengycin production by *B. amyloliquefaciens* DSM23117 (Pretorius, et al., 2015) and not for surfactin production.

The maximum surfactin concentration obtained in this study is greater than that obtained in the study by Pretorius et al. (2015) (882 mg/L), where the same nutrient media and bacteria species were used for surfactin production. Surfactin production in the study by Pretorius et al. (2015) was carried out in a bioreactor rather than shake flasks, thus a relatively higher surfactin concentration was expected (Chen, et al., 2006).

The change in surfactin with time during production is shown in Figure 5-1. Surfactin was seen to increase with time during production, until it reached a maximum (1109 mg/L) at approximately 53 h. According to Figure 5-1, surfactin production in this study was produced in both the exponential phase and stationary phase as in the study by Pretorius et al. (2015). This is in contrast with surfactin production in the study by Davis et al. (1999), although the same bacteria species was used for surfactin production. In the study by Davis et al. (1999), surfactin was mainly produced at the beginning of the stationary phase. This variation is possibly due to the fact that different nutrient media was used in the study by Davis et al. (1999), as Bence (2011) showed that the trend at which surfactin is produced could be influenced by the growth medium.

The suitable harvesting time ranged between 53 and 66 h. After the maximum surfactin concentration had been reached after 53 h, the surfactin concentration remained consistent between 53 h and 66 h. The suitable harvesting time was considered to be the time when surfactin concentration is at its maximum during production. The harvesting time in this study lies within the literature indicated range of harvesting times for surfactin production from *B. subtilis* ATCC 21332 (48- 120 h), shown in Table 2-1. The surfactin concentration is expected to decrease with time if cultivation was continued further than 66 h. Surfactin concentration is expected to decrease as surfactin may inhibit cell growth above a certain concentration and surfactin may be consumed due to substrate scarcity in the growth medium (Mukherjee, et al., 2009 and Wei & Chu, 2002).

The maximum surfactin concentration was seen to coincide with the beginning of the death phase of *B. subtilis* cells during production (Figure 5-1). The cessation in the increase of surfactin concentration might therefore be due to the decrease in metabolising *B. subtilis* cells. Additionally, when comparing Figure 5-1 and Figure 5-2, it can be seen that the death phase coincides with the onset of glucose depletion.

Cell growth was seen to be influenced by ammonium concentration. The exponential phase ended at approximately 29 h (Figure 5-1). From Figure 5-1 and Figure 5-2, it can also be seen that the stationary phase begun just after ammonium has been consumed, even though nitrate was not fully consumed during surfactin production. This is in accordance the study by Davis et al. (1999), who reported that although ammonium nitrate is a dual nitrogen source, the ammonium is consumed prior to nitrate consumption. Since the exponential phase ended before glucose and nitrate were fully consumed, ammonium ions are the overall growth limiting nutrient.

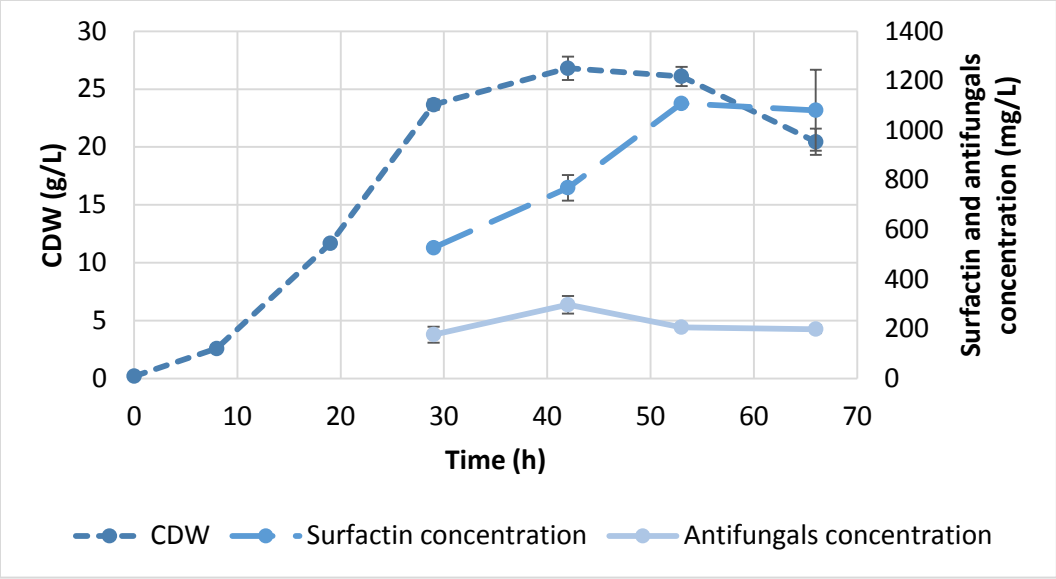

Figure 5-1: Product concentrations in B. subtilis cultures during surfactin production, where CDW is the cell dry weight. Error bars indicate the standard deviation of two replicates

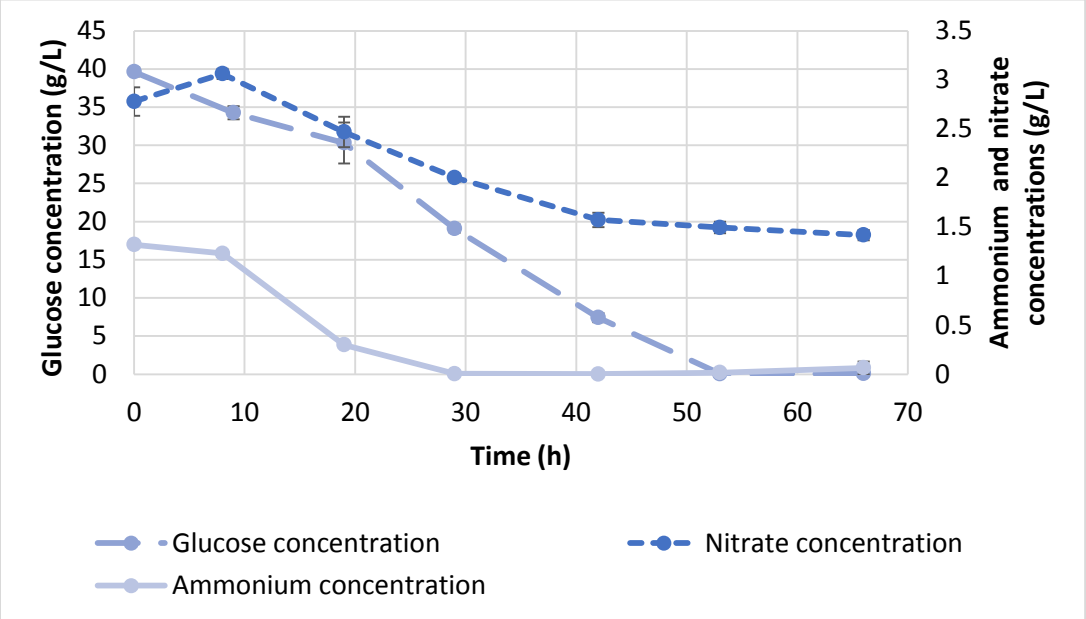

Figure 5-2: Nutrient concentrations in B. subtilis cultures during surfactin production, where error bars indicate the standard deviation of two replicates

Since the surfactin concentration increased with time during production while the antifungals were fairly constant (Figure 5-1), the selectivity of surfactin over antifungals (determined according to Equation 4-1) was seen to increase with production time (Figure 5-3). A single factor ANOVA was used to confirm that there was no difference between the values of antifungal concentration at different times at a 95% confidence interval. The antifungals can be assumed to have been produced between 0 and 24

h into the production process, reaching their maximum in the stationary phase in the production process. This is in contrast with the study by Toure et al. (2004) and Jacques et al. (1999), who stated that surfactin production occurs during the exponential phase, while antifungal production occurs during the stationary phase of bacterial production. The difference is possibly due to the fact that different bacterial species and growth media were used for surfactin and antifungal production in these studies. A comparison of surfactin selectivity in this study with literature data is not possible as selectivity was not studied in previous studies in literature, possibly due to the lack of a fengycin and iturin standards for HPLC analysis.

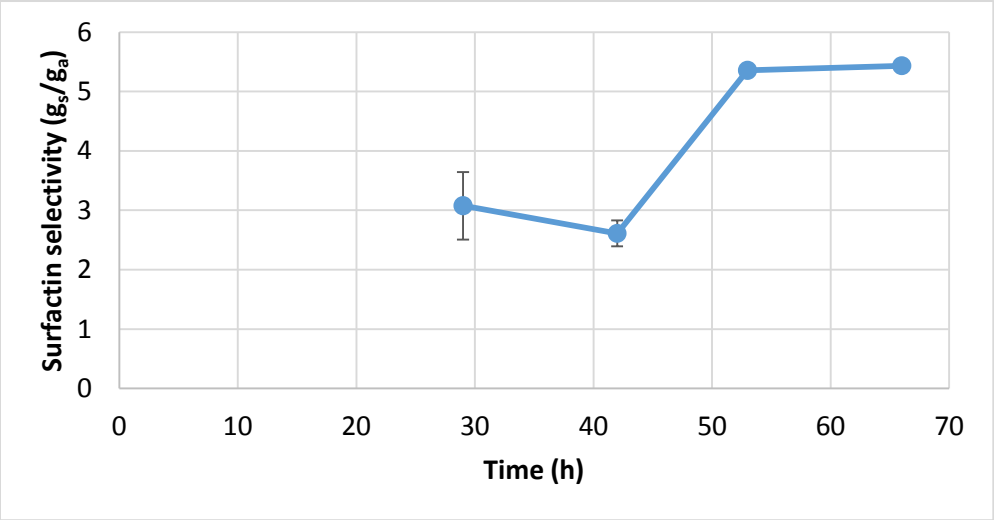

Figure 5-3: Surfactin selectivity in B. subtilis cultures during surfactin production, where error bars indicate the standard deviation of two replicates

5.2 TLC development and validation

To study surfactin purification from *B. subtilis* cultures, it is necessary to determine the surfactin concentration before and after a purification step to allow calculation of the recovery and purity. TLC analysis offers a simple, rapid and cheap quantitative analysis of surfactin compared to other surfactin analysis techniques such as RP-HPLC and HPLTC. In a study by Geisslera et al. (2017), it was seen that the surfactin concentration in a sample is related to the area of the band formed by surfactin on the TLC plate after development. This therefore means that surfactin concentration can be quantified by relating the area of surfactin bands on TLC plates after development to surfactin concentation in samples.

5.2.1 TLC development

The TLC analytical technique was developed by relating the surfactin concentration to the area of bands formed by surfactin on TLC plates after development, as discussed in section 4.2.2, through a standard curve (Figure 5-4). Figure 5-4 shows that there is a linear relationship between surfactin concentration and band area as R^2 is equal to 0.99.

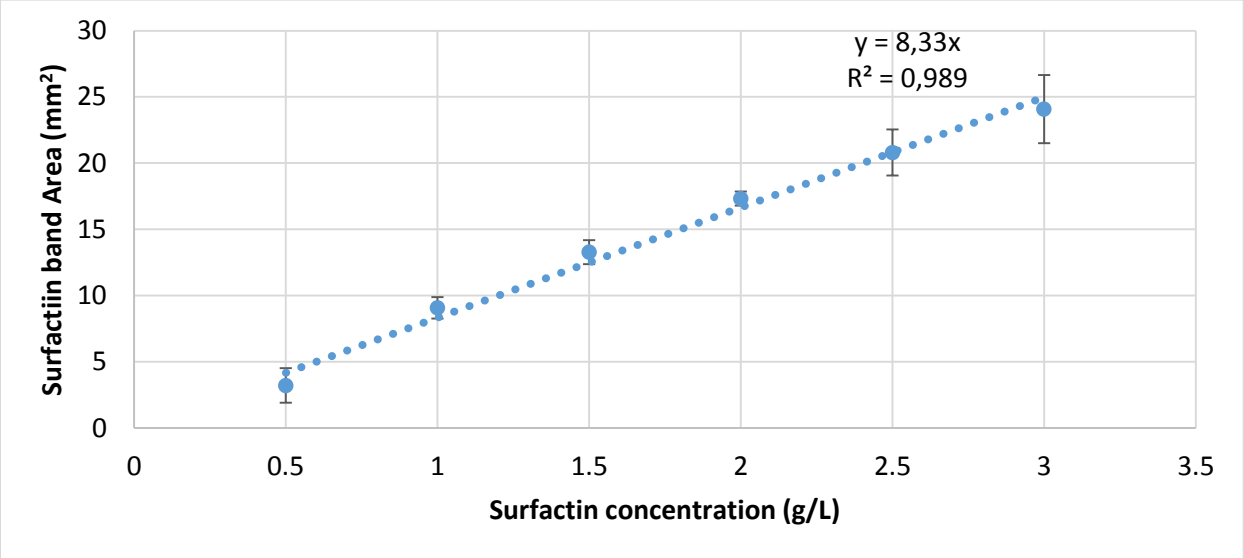

Figure 5-4: Standard curve for surfactin quantification by TLC, where error bars indicate the standard deviation of eight replicates

5.2.2 Validation of the TLC analytical technique for surfactin quantification

The TLC technique was validated to determine its suitability for surfactin analysis. According to UNODC (2009) and Ripp (1996), the linearity, working range, limit of detection (LOD), limit of quantification (LOQ), instrument limit of detection (ILOD), precision, accuracy, specificity, selectivity and robustness are required to validate analytical techniques. These parameters were used to validate the TLC analytical technique as discussed below. Validation was carried out using chemicals and materials provided in section 4.2.1.

5.2.2.1 *Linearity, LOD, LOQ and ILOD*

The linear range was defined as the region where the surfactin band area is directly proportional to surfactin concentration in the calibration curve (Ripp, 1996). The linear range lay between surfactin concentrations of 0.5 and 3 g/L, where the R^2 value was 0.99. The slope of the standard curve was found to be 8.33 mm^2/g/L.

The standard curve was also validated by LOD, LOQ and ILOD. LOD was defined as the lowest surfactin concentration that can be detected and identified with a degree of certainty, and was calculated according to Equation 5-1. The LOQ was defined as the lowest surfactin concentration that can be determined with a good degree of precision and accuracy (Ripp, 1996), and was calculated according to Equation 5-2. In Equation 5-1 and Equation 5-2, σ and S are the standard deviation of the areas of the surfactin bands at the low-level surfactin concentration in the standard curve and the slope of the standard curve respectively. The LOD and LOQ were found to be 0.5 g/L and 1.6 g/L respectively.

$$LOD = \frac{3.3\sigma}{S} \qquad \textit{Equation 5-1}$$

$$LOQ = \frac{10\sigma}{S} \qquad \textit{Equation 5-2}$$

The ILOD was defined as the lowest surfactin concentration that can be detected by the TLC analytical technique (Ripp, 1996). To determine the ILOD, the simulated supernatant (prepared as discussed in section 4.2.1), was diluted to a surfactin concentration of 0.05 g/L. This was used to spot samples ranging from 0.05 g/L to 0.4 g/L (with increments of 0.05 g/L) on a TLC plate by multiple spotting. The plate was developed and primuline reagent was used to detect surfactin. The concentration at which the bands start appearing on the TLC plate was visually determined as the ILOD, and was found to be 0.25 g/L. The linear range, slope, LOD, LOQ and ILOD are summarised in Table 5-1.

Table 5-1:Standard curve slope and validation parameters of the TLC analytial technique

Parameter	**Value**
linear range	0.5-3 g/L
R^2	0.99
Slope	8.33 mm^2/g/L
LOD	0.5 g/L
LOQ	1.6 g/L
ILOD	0.25 g/L

5.2.2.2 *Accuracy and precision*

The TLC technique was also validated by accuracy (a measure of how close the measured surfactin concentration is to the actual concentration) and precision (a measure of random errors in the measured surfactin concentrations under repeatable or reproducible conditions) (Ripp, 1996). Since the accuracy and precision are concentration dependent, they were both analysed at a high concentration (2.27 g/L), medium concentration (1.70 g/L) and low concentration (0.85 g/L) along the linear range of the calibration curve, using samples of commercial surfactin dissolved in methanol (prepared as discussed in section 4.2.1).

The accuracy (α) and precision (β) were calculated according to Equation 5-3 and Equation 5-4 respectively, where μ and χ are the true values and average values of the measured surfactin concentrations respectively. Experiments for accuracy and precision studies were repeated 8 times.

$$\alpha = 100\left(1 - \frac{|\mu - \chi|}{\mu}\right) \qquad \textit{Equation 5-3}$$

$$\beta = 100\left(1 - \frac{\sigma}{\mu}\right) \qquad \textit{Equation 5-4}$$

The accuracy and precision, along the linear range of the calibration curve, are shown in Table 5-2. The precision ranged from 82% to 94%, while the accuracy ranged from 90% to 98%. According to UNODC (2009), a valid analytical technique should have an accuracy greater than 80% at lower concentrations on the calibration, and better than 85% for the other concentrations levels. Additionally, the analytical technique must have a minimum precision of 80% at lower concentrations on the calibration curve. The accuracy and precision determined at various concentrations along the linear range are therefore acceptable. Since the precision could be as low as 82%, this analytical technique can be defined as a semi-quantitative analysis.

Table 5-2: Accuracy and precision determined across the linear range

Surfactin concentration (g/L)	Accuracy	Precision
2.27	93%	94%
1.70	98%	84%
0.85	90%	82%

When considering the concentration of 0.85 g/L, it was seen than high accuracy (90%) can be determined for concentrations lower than LOQ (1.6 g/L). This contradiction is possibly due to the fact that the LOD and LOQ are not robust parameters and can be affected by minor changes in the analytical system such as purity of reagents. According to Ripp (1996), the LOQ depends on the analytical technique, type of analyte as well as the general physical-chemical makeup of the sample. These parameters should therefore always be verified by laboratories adopting previously validated methods (Ripp, 2009).

5.2.2.3 *Specificity*

This study was done to determine if the surfactin concentration can be estimated in samples with impurities at a high accuracy and precision. This is necessary as surfactin samples are likely to contain some degree of impurities, especially in *B. subtilis* cultures. Specificity was studied using 2 g/L surfactin in simulated supernatant (prepared as discussed in section 4.2.1) and in water. The simulated supernatant and water with surfactin was spotted onto TLC plates, and the surfactin concentration was measured by TLC analysis as discussed in section 4.2.2 and compared to the actual surfactin concentration (Figure 5-5).

Figure 5-5 shows that there was no significant difference between the measured concentration and the actual concentration, in both water and simulated supernatant, since the actual concentrations lie within the error bars of the calculated concentrations. The measured concentration in water was also similar to that in the simulated supernatant, indicating that the presence of impurities in the simulated supernatant does not affect surfactin analysis. A single factor ANOVA was used to confirm this observation at a 95% confidence interval. TLC is therefore specific for surfactin analysis.

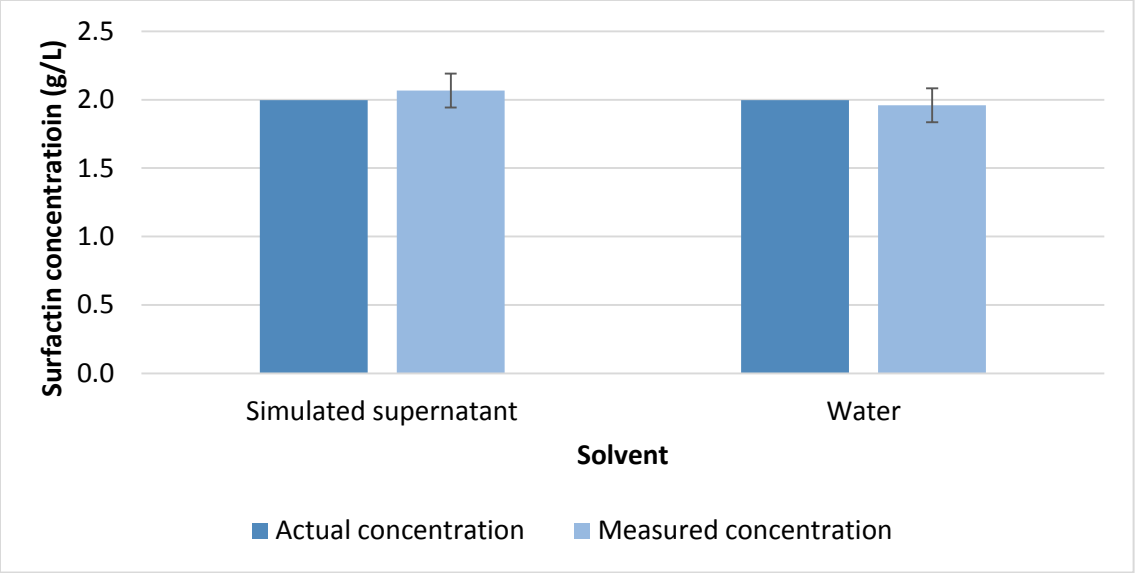

Figure 5-5: Specificity of TLC analysis for the measurement of surfactin concentrations, where error bars indicate the standard deviation of five replicates

To further confirm the specificity of the TLC analysis for measurement of surfactin concentration, the accuracy and precision of the measured concentrations was also determined according to Equation 5-3 and Equation 5-4 respectively. The accuracy in water and simulated supernatant was found to be 98% and 97% respectively, while the precision was found to be 95% and 94% respectively. The high accuracies and precisions (>90%) in the simulated supernatant as well as the similar accuracies and precisions between water and simulated supernatant confirmed that the TLC analytical technique is specific for surfactin analysis in the presence of impurities.

Geisslera et al. (2017), on the other hand, found that surfactin analysis by HPTLC can be affected by the presence of impurities as impurities tend to overlap with the substances of interest. This could cause the TLC technique to give measured surfactin concentrations which are lower than the actual concentrations. Although this was not the case in this study, it may be necessary to be pre-treat samples prior to quantification should overlapping occur.

5.2.2.4 *Robustness*

According to guidelines from the ICH Harmonized Tripartite (2005), robustness is the measure of the capacity of the TLC analytical procedure to remain unaffected by variations in method parameters. The variations which may be encountered in the TLC technique may arise due to analysis of surfactin in different solvents, having to analyse samples on different days as well as having to spot samples more than once on the TLC plate. An analytical technique is described as robust if results are not sensitive to

variations in the experimental conditions (ICH, 2005). Sensitivity to different solvents as well as sensitivity to multiple spotting were used to test the robustness of the TLC analytical technique in this study as discussed in section 5.2.2.4.1 and section 5.2.2.4.2.

5.2.2.4.1 Sensitivity to different solvents

This study was done to determine if the TLC analytical technique was suitable for surfactin analysis when surfactin was dissolved in different solvents. Sensitivity to different solvents was tested by comparing the actual and measured surfactin concentrations in different solvents (Table 5-3), and the results are displayed in Figure 5-6. Experiments for sensitivity to different solvents were repeated five times. The actual concentration was seen to lie within error of the measured concentration. A single factor ANOVA was used to confirm this observation at a 95% confidence interval. The TLC analytical technique was therefore found to be suitable for measurement of surfactin concentration when surfactin was dissolved in different solvents.

Table 5-3: Solvents used to study the suitability of the TLC analytical technique for surfactin analysis in various solvents and the concentration of spiked surfactin in the different solvents

Solvent	**Surfactin concentration (g/L)**
Methanol	1.70
Ethanol	2.10
Isopropanol	1.95
Water	2.00

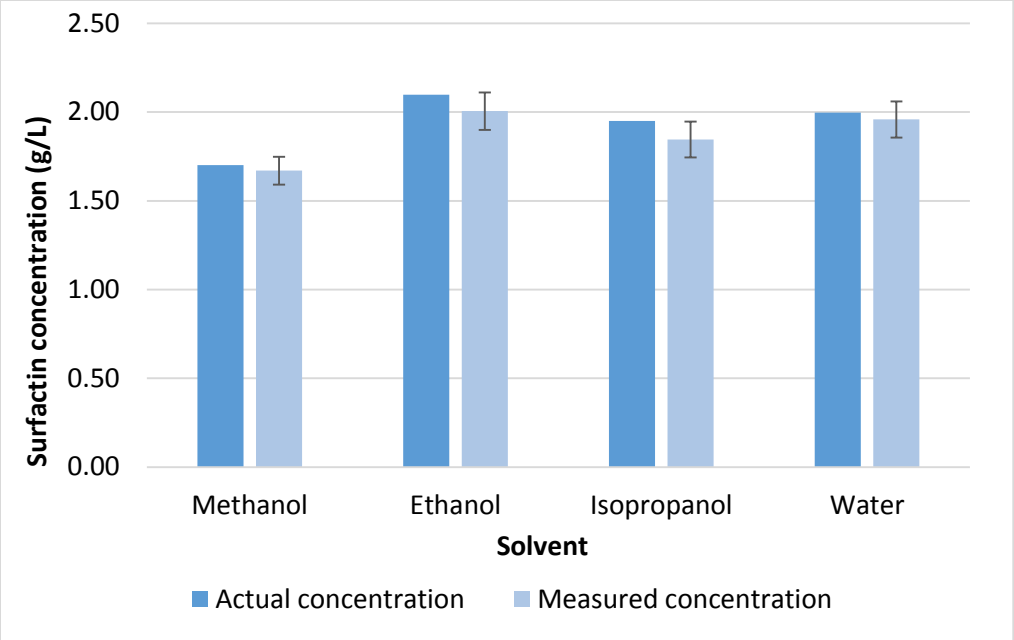

Figure 5-6: Comparison of actual and measured surfactin concentrations determined when surfactin was dissolved in different solvents, where the error bars indicate the standard deviation of five replicates.

To further confirm if TLC analysis was suitable for surfactin quantification in different solvents, the accuracy and precision of the measured concentrations were determined according to Equation 5-3 and Equation 5-4 respectively. High accuracies (≥94%) and precision (≥94%) were obtained (Table 5-4), thus this method is suitable for analysis of surfactin concentration when surfactin is dissolved in different solvents.

Table 5-4: Accuracy and precision of the TLC technique when used for surfactin analysis in various solvents

Solvent	Accuracy	Precision
Methanol	98%	94%
Ethanol	95%	95%
Isopropanol	94%	94%
Water	98%	95%

The TLC analytical technique was found to be suitable for analysis of surfactin concentration when surfactin is dissolved in different solvents. This is possibly due to the fact that when surfactin is spotted on the TLC plates, the solvents are allowed to evaporate before the plate is developed. The solvent in

which surfactin was dissolved therefore does not affect the polarity during analysis hence the migration of surfactin during the development of the plate. This is significant as this procedure can be used for analysis of surfactin during solvent extraction. In solvent extraction studies, surfactin concentration in various solvents used for extraction may be required, while analytical techniques such as HPLC are not capable of quantifying surfactin concentration when surfactin is dissolved in solvents.

5.2.2.4.2 Sensitivity to multiple spotting

Multiple spotting refers to spotting samples more than once on the TLC plate with intermediate drying. This is used to increase the amount of surfactin on a single spot on a TLC plate, which is necessary if the surfactin concentration in the samples being analysed is lower than the LOD (0.5 g/L). Multiple spotting was studied using simulated supernatant with 1 g/L surfactin (prepared by diluting the simulated supernatant prepared as discussed in 4.2.1 in water). The supernatants were then diluted 5 times and 10 times to form concentrations shown in Table 5-5. Experiments to determine the sensitivity of TLC analysis to multiple spotting were repeated 6 times.

Table 5-5: Surfactin concentration in media used for studying the multiple spotting error

Dilution	**Surfactin concentration (g/L)**
No dilution	1
5 times dilution	0.2
10 times dilution	0.1

The undiluted supernatant, 5 times diluted supernatant and 10 times diluted supernatant were spotted once, 5 times and 10 times respectively on the TLC plate with intermediate drying. Surfactin concentration of the three supernatants was then measured by TLC analysis and compared to the actual surfactin concentration (Figure 5-7). There was no significant difference between the measured and actual concentrations, since the actual concentrations lie within the error bars of the calculated concentrations. A single factor ANOVA was used to confirm this observation at a 95% confidence interval. The TLC analytical technique was therefore not sensitive to multiple spotting.

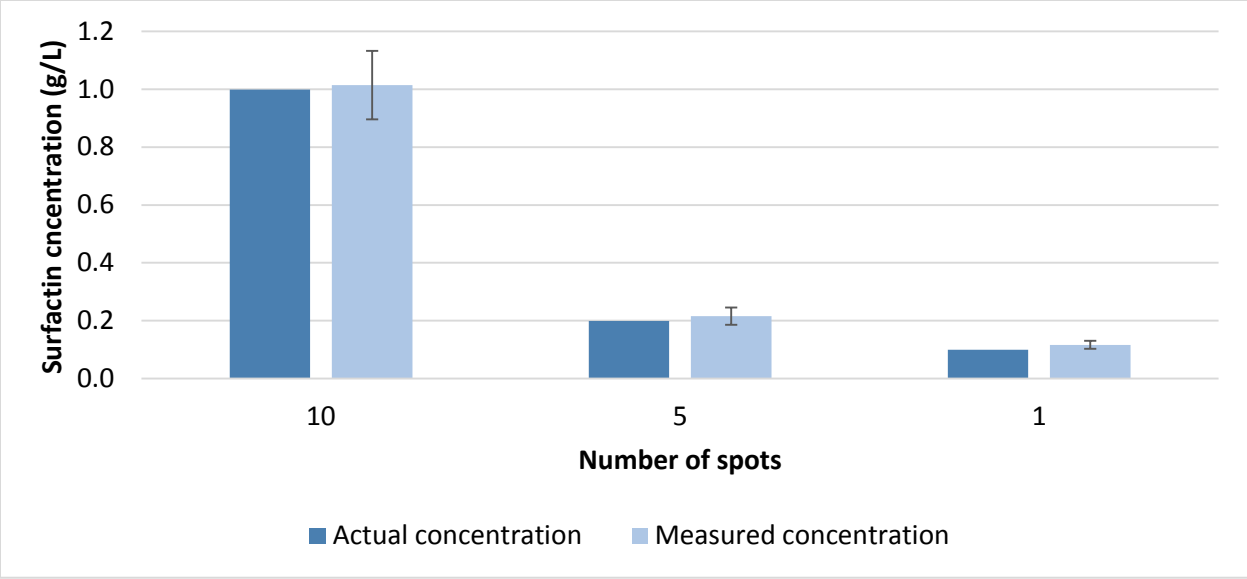

Figure 5-7: Comparison of actual and measured surfactin concentrations for 10 times, 5 times and non-diluted simulated supernatant which were spotted 10 times, 5 times and once on the same spot on the TLC plate respectively. The error bars indicate the standard deviation of six replicates.

To further confirm if TLC analysis was not sensitive to multiple spotting, the accuracy and precision of the measured concentrations was also determined according to Equation 5-3 and Equation 5-4 respectively. The accuracy ranged from 85% to 98%, while the precision ranged from 86% to 88% (Table 5-6). This confirmed that the TLC analytical technique is not sensitive to multiple spotting.

Table 5-6: Accuracy and precision obtained when surfactin was spotted by multiple spotting

Spots added	Accuracy	Precision
1	98%	88%
5	92%	86%
10	85%	88%

5.2.2.4.3 Sensitivity to analysis in different days

This study was done to determine if the TLC analytical technique is sensitive to surfactin analysis on different days, hence to determine the reproducibility of the TLC analytical technique. This study was carried out using 1.7 g/L surfactin in methanol (prepared as discussed in section 4.2.1. The concentration of surfactin in this preparation was then measured by TLC analysis in two different days (two weeks apart). Experiments for intraday error analysis were repeated 8 times for a particular day.

The measured surfactin concentration was compared to the actual surfactin concentration (Figure 5-8). The error bar of the measured surfactin concentration was higher at day 14, thus the precision got poor with increase in time. However, there was no significant difference between the actual concentration and the measured concentration on both days, as the actual concentrations lie with error range of the calculated concentrations. A single factor ANOVA was used to confirm this observation at a 95% confidence interval. The analysis of surfactin concentration by TLC is therefore reproducible.

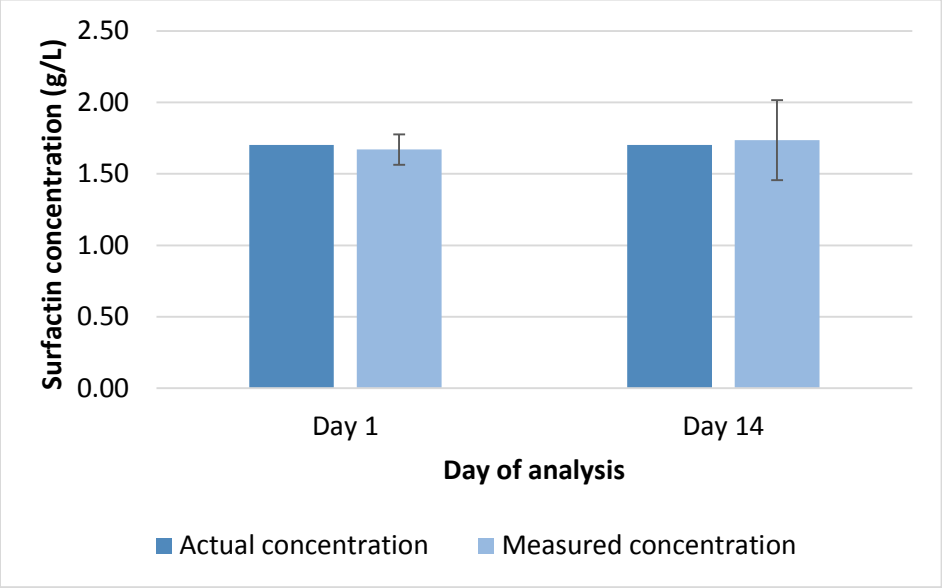

Figure 5-8: Comparison of the measured and calculated surfactin concentrations when surfactin was analysed in different days

The reproducibility of the TLC analytical technique at different days was confirmed by determining the accuracy and precision of surfactin analysis at different days according to Equation 5-3 and Equation 5-4 respectively. The TLC technique was seen to be reproducible as accuracies of 98% and precisions greater than 84% were achieved on both days when intraday assays were studied (Table 5-7). The precision dropped to 84% on the second day, but it was still above 80% thus acceptable.

Table 5-7: Accuracy and precision obtained when surfactin concentration was analysed on different days using the TLC analytical technique

Day	Accuracy	Precision
Day 1	98%	94%
Day 2	98%	84%

5.2.3 Impurity assays and extension of the TLC technique to lipopeptide analysis

Surfactin with different degrees of purities is obtained during initial recovery by acid precipitation or other purification procedures (Rangarajan and Clarke, 2016). Impurity assessments would suggest the course of purification procedures to be followed to get rid of the impurities in surfactin purification studies. The TLC analytical technique can be used to determine the level of impurities present in the surfactin sample using the primuline reagent (which would reveal the presence of lipid impurities) and ninhydrin reagent (which would reveal the presence of peptide/protein impurities).

To demonstrate the suitability of TLC analysis for detection and quantification of impurities, impurity assays were done on resolubilised acid precipitates of *B. subtilis* with a surfactin concentration of 2.48 g/L. The resolubilised precipitates were spotted 5 times on TLC plates and developed in the TLC chamber. Multiple spotting was used for better visualisation of the impurities.

Figure 5-9 shows the developed plates when primuline (Figure 5-9a) and ninhydrin (Figure 5-9b) were used as detection agents. Lipid impurities show as white spots and peptide/protein impurities show as dark spots on the TLC plates. Components with lipid parts were seen to move up the TLC plate while those with peptide parts did not move up the TLC plate. Impurity assays provide a qualitative analysis of impurities and proteins in *B. subtilis* cultures. A high concentration of protein impurities is noted by darker regions on the developed TLC plates, while higher lipid concentrations are noted by wider bands on the TLC plate.

Figure 5-9a shows that surfactin, fengycin and iturin can be detected using the primuline reagent. Surfactin and iturin, however cannot be detected using the ninhydrin detector. According to the certificate of analysis of surfactin (from Kaneka, Japan), surfactin does not respond to the ninhydrin detector. There was little or no iturin the *B. subtilis* cultures as it was not seen on the TLC plates.

Figure 5-9b shows that developing plates with a ninhydrin showed the presence of protein impurities in the same place where samples were originally spotted as dark spots on the TLC plates. The proteins in *B. subtilis* cultures therefore possibly have a poor solubility in the mobile phase (chloroform:methanol:water mixture in the volume ratio 65:25:4). According to Bele & Khale (2011), compounds which are insoluble in the mobile phase do not move up the TLC plate during development. The dark regions in the R_f range of fengycin in Figure 5-9b were assumed to be fengycin.

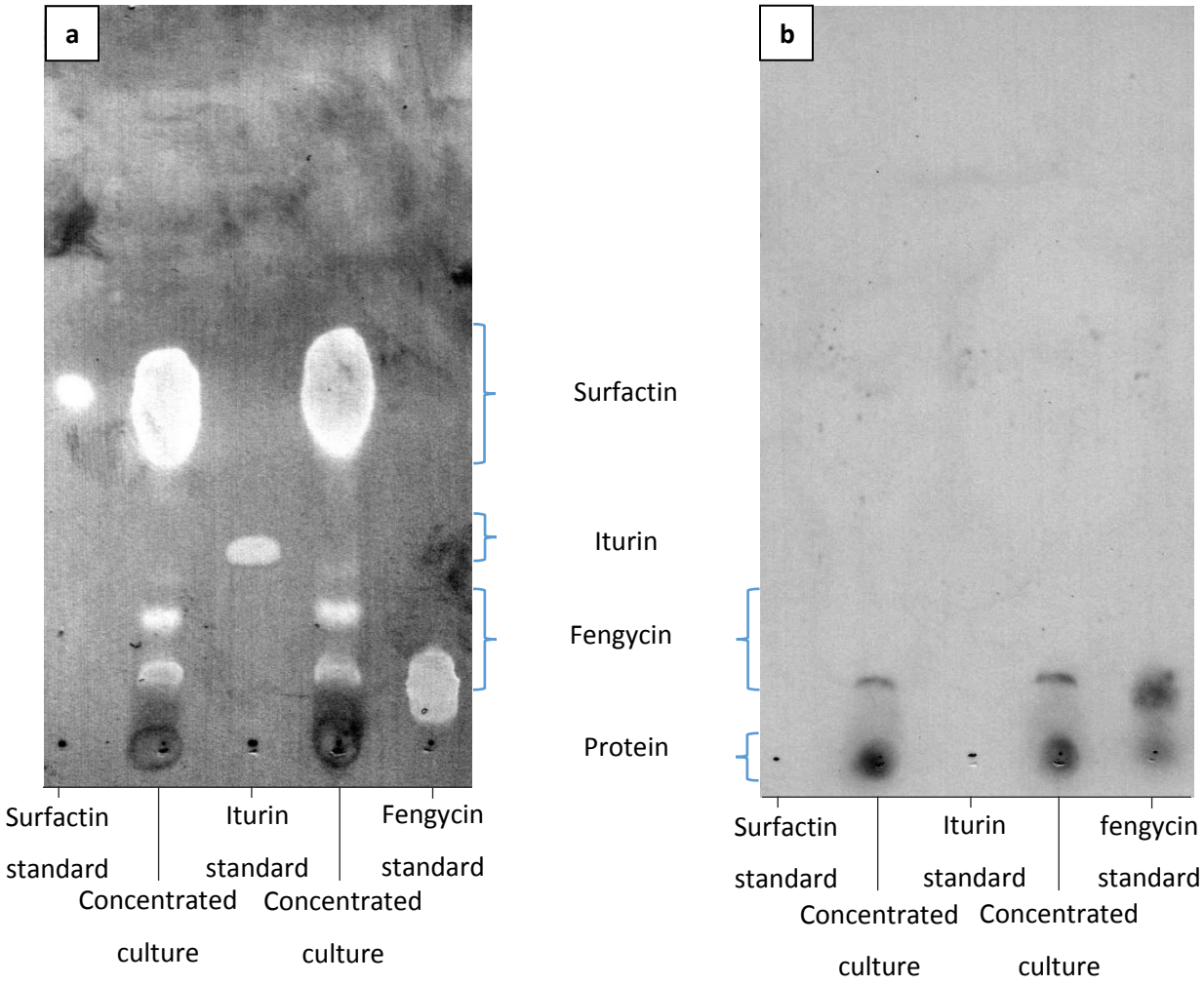

Figure 5-9: Developed plates for concentrated B. subtilis cultures (after purification by acid precipitation) with (a) primuline and (b) ninhydrin reagent

To determine if TLC analysis can be extended for analysis of other lipopeptides, selectivity studies were carried out based on R_f values of surfactin, iturin and fengycin. Selectivity studies were carried out using surfactin, iturin and fengycin (all from Sigma-Aldrich), prepared in methanol. The lipopeptide standards were spotted on different spots as well as on the same spot (with intermediate drying) on TLC plates, and the R_f values were determined as discussed in section 4.2.2 and compared (Figure 5-10).

Figure 5-10 shows that the migration of surfactin, iturin and fengycin on TLC plates is similar, whether these lipopeptides are spotted on the same spot or on different spots on the TLC plates as the error bars overlap. This observation was confirmed statistically using a single way ANOVA, which showed that there was no significant difference between the R_f values at a 95% confidence interval. The TLC method is therefore selective for surfactin, iturin and fengycin.

The R_f values of surfactin, iturin and fengycin were found to be 0.45, 0.23 and 0.05 respectively. The R_f values were comparable to those obtained by Geisslera et al. (2017), who found that surfactin, iturin and fengycin had R_f values of 0.44, 0.19 and 0.07 respectively when a chloroform:methanol:water in the ratio 65:25:4 (v/v) was used. Surfactin travels the longest distance along the TLC plate, followed by iturin then fengycin. Based on the R_f values, it can be concluded that surfactin is the least polar lipopeptide, followed by fengycin then iturin. According to Bele & Khale (2011), more polar compounds interact stronger with the stationary phase (silica). They are therefore more able to dispel the mobile phase from the binding places, hence moving shorter distances up TLC plates. More polar compounds thus have relatively low R_f values compared to less polar compounds.

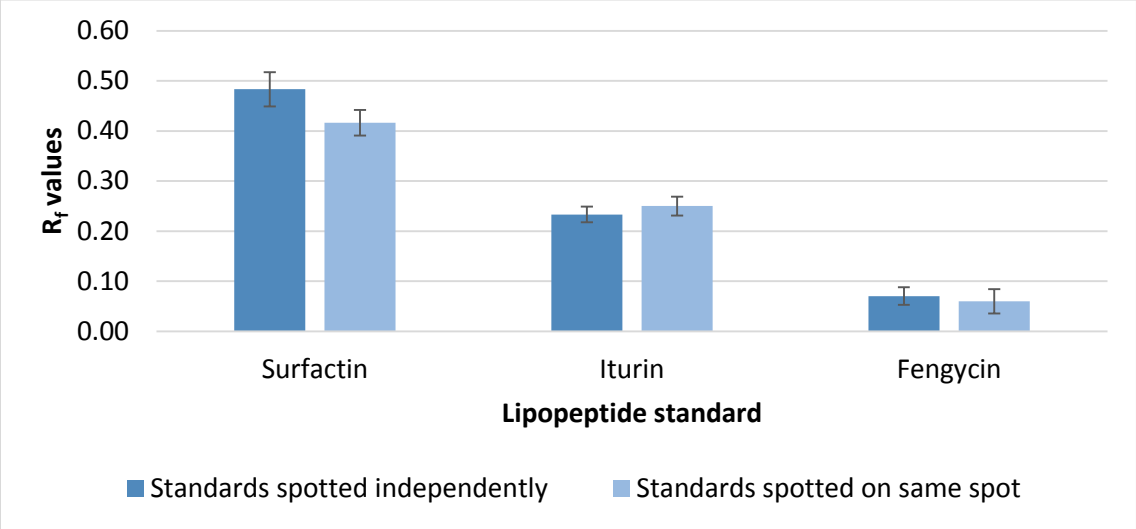

Figure 5-10: Comparison of the R_f values of surfactin, iturin and fengycin, when they were spotted on the same spot and when the lipopeptide standards were spotted on different spots. The error bars indicate the standard deviation of four replicates.

5.2.4 Problems encountered and recommendations for carrying out successful surfactin analysis by TLC

TLC analysis needs to be meticulously done as careless procedures (such as not ensuring proper saturation of TLC chamber prior to analysis) may affect the accuracy of the analysis. One problem encountered during TLC analysis is ensuring the mobile phase forms a straight front as it rises up the TLC plate. This problem may arise when the TLC plate is not parallel to the mobile phase when inserted in the TLC chamber. Surfactin will migrate a higher distance on the end where the solvent moves higher up the TLC plate, and the bands will be of poor quality as they will not be circular.

Another problem may arise when TLC is carried out in a chamber that is not properly saturated. This may arise due to loss of saturating vapour when spotted TLC plates are placed in the chamber as the chamber must be opened. This can be avoided by inserting spotted TLC plates in the chamber as quickly as possible, while making sure of a straight solvent front. Improper tank saturation may also arise when insufficient time was allowed for tank saturation prior to analysis. Analysis in an unsaturated tank causes surfactin to migrate higher up the plate (Geisslera, et al., 2017).

Another problem may arise when surfactin is analysed in solvents other than water. Solvents with dissolved surfactin should be stored at -18 °C before analysis, and spotting should be carried out while the temperature of the solvents is below 0 °C to minimise volatilisation during spotting.

Surfactin analysis may be affected by unclean TLC plates and the presence of impurities in samples. Impurities may overlap with the substances of interest. Samples of cell-free supernatants may need to be pre-treated prior to quantification should overlapping occur. Pre-treatment can be achieved by solvent extraction using chloroform:methanol (2:1, v/v) (Geisslera, et al., 2017). TLC plates must be clean and dust-free. Pre-washing of the TLC plates with methanol and storage in a dust-free environment after drying can be considered.

5.2.5 Comparison of TLC and HPLC analytical techniques for surfactin analysis

To compare surfactin analysis by TLC and HPLC, the surfactin purity in the precipitate (obtained as discussed in section 4.5.3) was determined based on concentrations determined by HPLC and TLC analysis (Figure 5-11). Figure 5-11 shows that the TLC purities are lower than the HPLC purities. The TLC results show greater error bars compared to the HPLC results, thus HPLC is more precise analytical technique. The error bars overlap thus there is no significant difference between the results determined by HPLC and TLC.

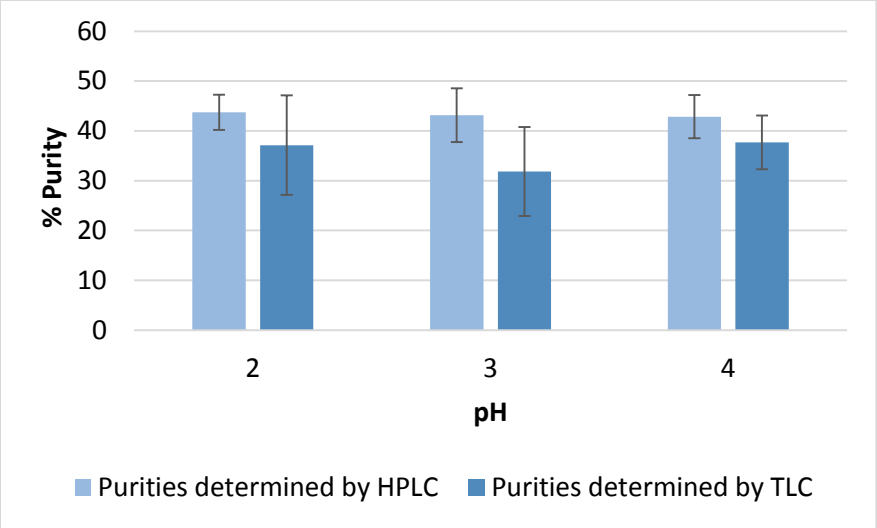

Figure 5-11: Comparison of purities from acid precipitation step determined by HPLC and TLC analysis, where the error bars indicate the standard deviation of four replicates

5.3 Surfactin purification by acid precipitation

Acid precipitation was used to isolate surfactin from supernatants of *B. subtilis* cultures, to obtain the partially purified product in solid form. Low pH causes the charges of amino acids in surfactin to neutralise, hence surfactin loses its solubility in water and precipitates (Kosaric & Sukan, 2014). Acid precipitation was chosen as it is capable of achieving high recoveries (>97%) and is a relatively cheap method compared to other techniques such as salting out (Chen & Juang, 2008b and Chen, et al., 2008c). Additionally, acid precipitation is capable of simultaneously achieving maximums of both recovery and purity, unlike foam fractionation where maximum surfactin recoveries cannot be obtained at maximum purities (Davis, et al. 2001).

Surfactin recovery by acid precipitation has been studied at pH 4 and pH 2 in literature (Table 2-2). No study was, however, carried out to optimise the operating pH between these two pH (pH 2 and pH 4) in literature, thus the influence of pH on acid precipitation was investigated in this study. Acid precipitation was optimised based on surfactin recovery and purity (section 5.3.1), improvement in selectivity (section 5.3.2) as well as impurity assays (sections 5.3.3). These recoveries, purities and improvement in selectivities were obtained based on surfactin and antifungal concentrations determined by HPLC analysis. HPLC analysis was used for analysis at it has a higher precision in comparison to TLC analysis.

5.3.1 Optimisation of acid precipitation based on surfactin recovery and purity

The surfactin and antifungal recoveries (determined according to Equation 4-3 and Equation 4-5) as well as total lipopeptide recoveries obtained from acid precipitation at different pHs are shown in Figure 5-12, and were found to be approximately 97% at the different pHs. A single factor ANOVA analysis was used to show that there was no significant difference between the surfactin, antifungal and total lipopeptide recoveries at the different pH at a 95% confidence interval. The high recoveries attained are in accordance with the study by Chen et al. (2007), who found that total lipopeptide recoveries greater than 97% are obtaned at pH 4. The optimum pH can therefore not be obtained based on recoveries.

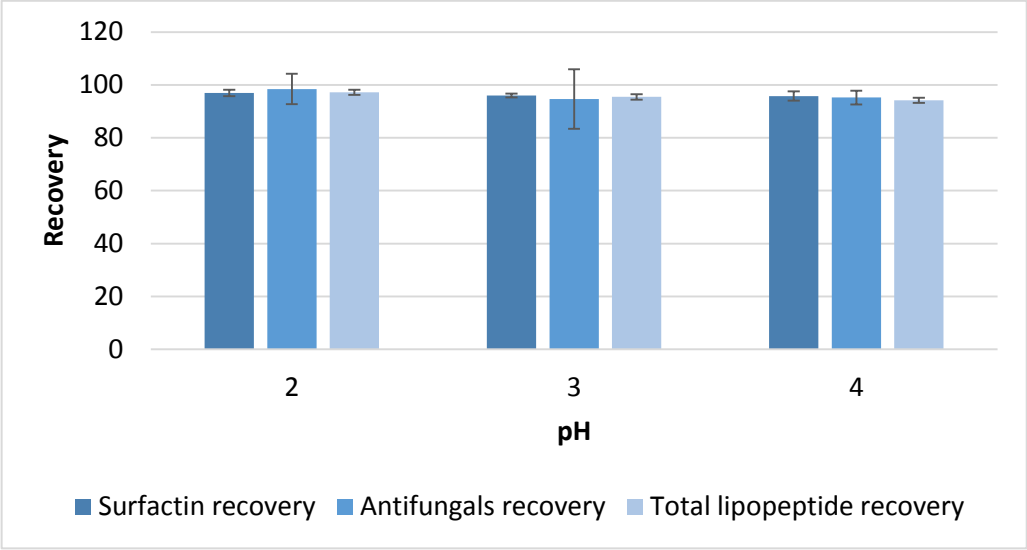

Figure 5-12: Surfactin, antifungal and total lipopeptide recoveries after surfactin purification by acid precipitation, where the error bars indicate the standard deviation of four replicates

The surfactin purities (determined according to Equation 4-2) and total lipopeptide purities (determined similarly to surfactin purities) at the different pHs are shown in Figure 5-13. The surfactin purities at the different pHs were similar, at approximately 43%, while the total lipopeptide purities were approximately 50%. The difference between the total lipopeptide purities and surfactin purities at the different pHs are a result of the antifungal purities. This difference is also similar at the different pHs. The surfactin purity after the acid precipitation step was not affected by pH at pH below pH 4. The optimum pH can therefore not be obtained based on surfactin or total lipopeptide purities.

The surfactin purities are greater than those obtained in the study by Wang et al. (2010) where the surfactin purity of 33.2% was obtained at pH 4. The total lipopeptide purities are however aproximately similar to those in the studies by Wang et al. (2010), Chen *et al*. (2007) and Chen & Juang (2008b) who found total lipopeptide purities of 56.6%, 55% and 53% respectively.

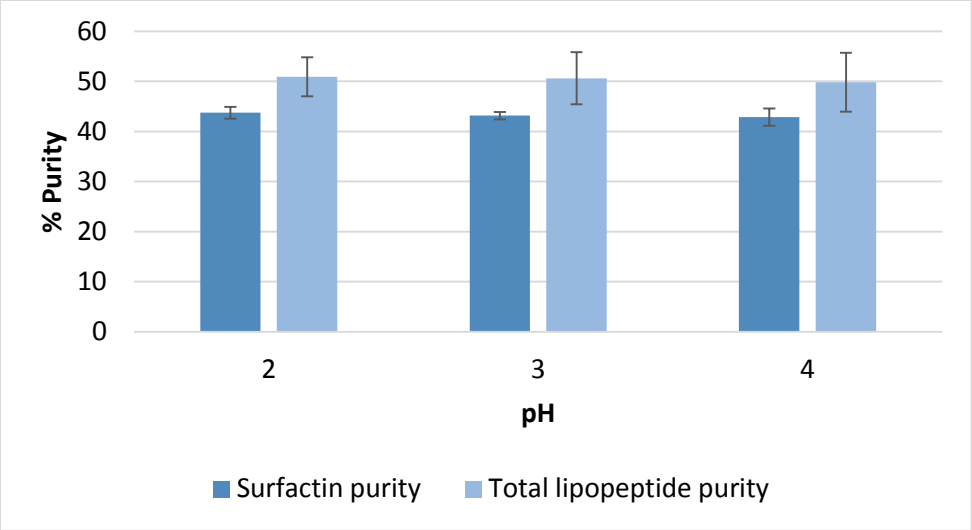

Figure 5-13: Surfactin and total lipopeptide purities after acid precipitation at different pH, where the error bars indicate the standard deviation of four two replicates

5.3.2 Optimisation of acid precipitation based on improvement in surfactin selectivity

This section compares the surfactin selectivity (determined according to Equation 4-1) as well as improvement in selectivity (IS, determined according to Equation 4-4) after acid precipitation at the different pHs (Figure 5-14). Like surfactin purity and recovery, the selectivity and IS were not affected by pHs in the studied range. The surfactin selectivity at the different pHs was constant at approximately 5.7. This is approximately similar to the selectivity in the supernatant before acid precipitation (5.5). Acid precipitation was not expected to improve surfactin selectivity as high recoveries (>97%) were expected for both surfactin and antifungals.

The improvement in selectivity at the different pHs, was therefore approximately 1 which means that acid precipitation did not improve the selectivity. This is due to the fact that approximately the same surfactin and antifungal recoveries were achieved during the acid precipitation step. No other studies have reported surfactin selectivity after acid precipitation so far, possibly due to the lack of fengycin and iturin standards for HPLC analysis. The optimum pH can therefore not be obtained based on surfactin selectivity and improvement in selectivity.

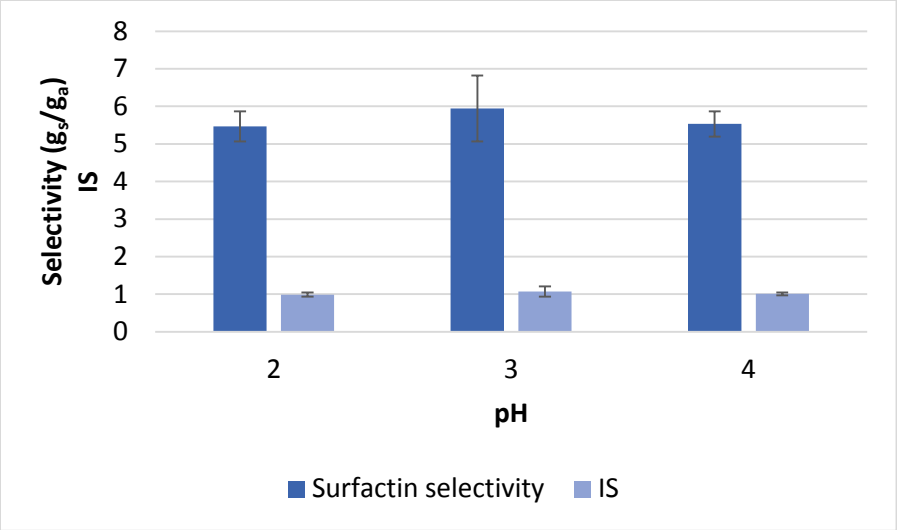

Figure 5-14: Surfactin selectivity and improvement in selectivity (IS) after surfactin purification by acid precipitation, where the error bars indicate the standard deviation of two replicates

5.3.3 Optimisation of acid precipitation based on impurity assays

Impurity assays were also used for optimisation of surfactin purification by acid precipitation. Samples of cell-free supernatant and resolubilised precipitates after acid precipitation at the different pHs (obtained as discussed in section 4.3.2) were spotted 10 times on TLC plates by multiple spotting. The plates were then developed as discussed in section 4.2.2, before primuline and ninhydrin were used to visualise the components with lipid and peptide moieties as discussed in section 4.2.3. The relative quantity of impurities with lipid and peptide parts in cell-free supernatants and resolubilised precipitates from acid precipitation at different pHs was then compared (Figure 5-15).

Figure 5-15a shows lipids or other components with lipid parts as white bands on the TLC plate. In Figure 5-15a, a component with lipid parts which is neither surfactin nor fengycin was seen near the top of the plate. It is unknown what type of lipid or biosurfactant this is, and so the area of its band cannot be related to its concentration. However, a bigger area would likely result from a higher concentration. The areas of these lipids were similar at the different pHs, thus it can be assumed that they are in similar concentrations in the precipitates obtained through acid precipitation at the different pHs. The lipid assays can therefore not be used to determine the optimum pH.

Figure 5-15b shows protein or other components with peptide parts as dark regions on the TLC plates. The proteins only showed on the lower parts of the TLC plate. Protein showed on the places where samples were originally spotted. Dark regions also showed in the R_f range of fengycin, and these were due to presence of fengycin in the cell-free supernatants and resolubilised acid precipitates. Darker

regions in (Figure 5-15b) are due to higher protein concetrations. Since the supernatant showed a darker and broader shading compared to the resolubilised precipitates obtained after acid precipitation at different pHs, the crude supernatant contains more protein in comparison to the resolubilised precipitates. The shading of the different resolubilised precipitates on the TLC plates (Figure 5-15b) are similar, hence the precipitates obtained by acid precipitation at different pHs can be assumed to have similar protein quantities. The analysis of protien impurities could therefore not be used to determine the optimum pH.

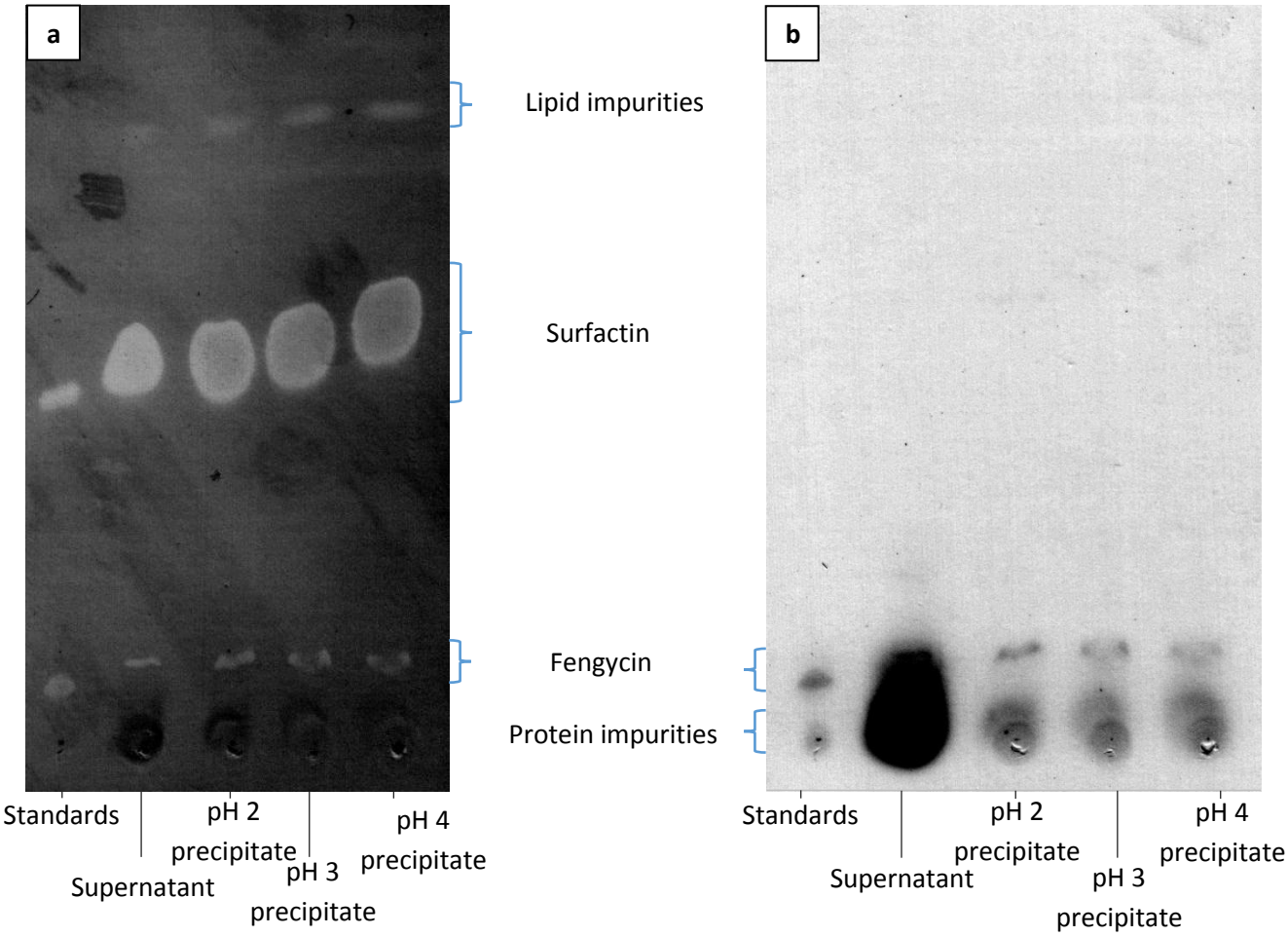

Figure 5-15: (a) lipid and (b) protein impurities before and after surfactin purification by acid precipitation

The optimum pH could not be determined in terms of recovery, purity, selectivity as well as impurity assays. The maximum surfactin recovery, purity and selectivity had already been reached at the highest pH used in this study. Further studies on pH optimisation are therefore recommended, and a suitable pH range for these studies would be between pH 4 and pH 6. pH 6 would be suitable as the highest pH as surfactin starts precipitating at pH below 6 (Liu, et al., 2007). Based on this study, pH 4 was

recommended for acid precipitation as it can be attained using less hydrochloric acid. This is advantageous as it is cost effective since less acid is used and the HCl is also impurity in our product. Additionally, less sodium hydroxide is required for solubilising dry precipitates at pH 4.

5.4 Surfactin purification by solvent extraction

Solvent extraction purifies surfactin from the acid precipitation by selective extraction of surfactin into organic solvents. Solvent extraction was chosen because of its simplicity compared to other purification techniques such as ultrafiltration and chromatographic techniques. Unlike ultrafiltration, it does not require high pressure operation equipment and there are fewer operational complications such as plugging of filter membranes. Unlike chromatographic techniques, solvent extraction is relatively inexpensive (Kosaric & Sukan, 2014).

This section presents results for optimisation of the solvent extraction process. Solvents of differing polarity were used in this study. These solvents, in order of decreasing polarity, were methanol, *i*-propanol, (C/M 1:1), acetonitrile, (C/M 2:1), acetone, chloroform, ethyl acetate, methyl *tert*-butyl ether (MTBE), petroleum ether and *n*-hexane. To determine the optimum solvent, the surfactin recovery and purity achieved after solvent extraction using the different solvents was compared (section 5.4.1). The surfactin recovery and purity were calculated based on surfactin based on surfactin concentrations determined by TLC analysis. TLC was used for surfactin analysis as it is capable of measuring surfactin concentrations when surfactin is dissolved in all of the organic solvents used in this study, unlike HPLC. Optimisation was also done base on impurity assays (section 5.4.2).

5.4.1 Optimisation of solvent extraction based on surfactin recovery and purity

The change in surfactin recovery (determined according to Equation 4-6) with solvent polarity is shown in Figure 5-16.. 100% recoveries were achievable when methanol, MTBE and acetone were used as solvents, while isopropanol, C/M (1:1), C/M (2:1) and ethyl acetate were also capable for achieving 100% recoveries. These solvents were therefore the best for extraction, based on surfactin recoveries.

According to a statistical single factor ANOVA and a post-hoc analysis by least significant difference (LSD), there is no significant difference between the surfactin recoveries by methanol, isopropanol, C/M (1:1), C/M (2:1), acetone, ethyl acetate and MTBE. Surfactin recoveries by acetonitrile and chloroform are significantly lower than those of methanol and MTBE. Surfactin is partially soluble in *n*-hexane and petroleum ether, and soluble in the rest of the solvents used (Arima, et al., 1972). The partial extraction in isopropanol, C/M (1:1), acetonitrile, C/M (2:1), acetone, chloroform and ethyl acetate is therefore

possibly due to some surfactin molecules not being exposed to the solvents due to interference of the impurities, hence they were not extracted. Surfactin recovery by ethyl acetate was found to be 84%, which is comparable to that in the study by Chen & Juang (2008b) (78%).

N-hexane and petroleum ether, which are the least polar solvents among the solvents studied, showed the lowest surfactin recoveries (15% and 7% respectively). This was in line with the hypothesis that less polar solvents give low surfactin recoveries. According Chen & Juang (2008b), up to 60% of the surfactin can be recovered by solvent extraction using the solid-liquid extraction technique when hexane is used as a solvent. In the study by Chen & Juang (2008b), repeated extraction was used to improve the surfactin recovery when hexane was used as the solvent. This is possible as surfactin is partially soluble in hexane (Arima, et al., 1972), thus repeated extraction using a fresh solvent is expected to improve total surfactin extraction.

Besides repeated extraction, another factor to consider for improving recovery is liquid-liquid extraction for extraction using polar solvents. Geisslera et al. (2017) found that solvent extraction on supernatants gave higher overall recoveries compared to acid precipitation followed by solvent extraction. Additionally, solvent extraction directly on supernatants is also less time consuming compared to acid precipitation followed by solvent extraction. Chen & Juang (2008b) found that extraction by ethyl acetate improved from 84% to 99% when liquid-liquid extraction was used rather than solid-liquid extraction, while the extraction by hexane decreased from 62% to 21%.

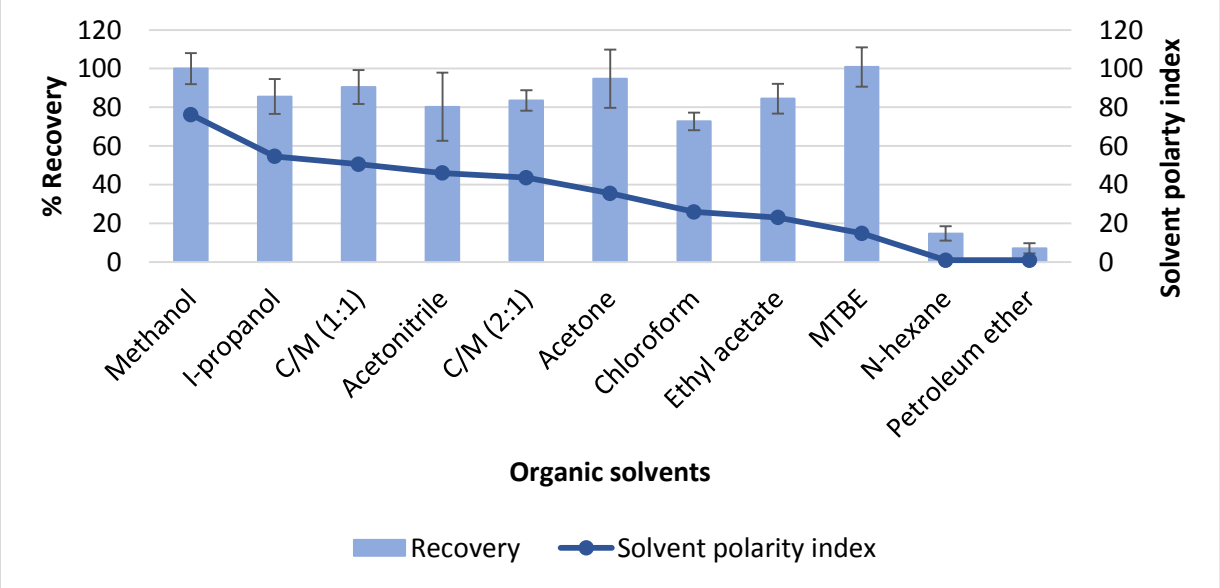

Figure 5-16: Surfactin recoveries after surfactin purification by solvent extraction, where the error bars indicate the standard deviation of two replicates. The solvent polarity index shows relative polarities of solvents on the scale of water=100. C/M (1:1)

and C/M (2:1) represent mixtures of chloroform and methanol in the volume ratio 1:1 and 2:1 respectively, while MTBE represent methyl tert-butyl ether.

The change in surfactin purity (determined according to Equation 4-7) with solvent polarity is shown in Figure 5-17. MTBE showed the highest surfactin purity (80%), hence it the best solvent for extraction based on surfactin purity. According to a statistical single factor ANOVA, there was no significant difference between the purities obtained when methanol, propanol, C/M (1:1), acetonitrile, C/M (2:1), acetone, chloroform and ethyl acetate at a 95% confidence interval. The purity after extraction by ethyl acetate (62%) was lower than that in the study by Chen & Juang (2008b) (84%). This could mean that the impurities in the precipitate used in this study are more soluble in ethyl acetate compared to those in the study by Chen & Juang (2008b).

Non-polar solvents (n-hexane and petroleum ether) showed the lowest purities (30%). This contradicted our hypotheses that purity increases with decrease in solvent polarity. Non-polar solvents were expected to extract surfactin and some lipid impurities from the acid precipitates. The low surfactin purities in non-polar solvents therefore possibly mean more polar lipid impurities were extracted into the solvents in comparison to surfactin. The surfactin purity after solvent extraction using hexane (30%) was lower than that obtained in the study by Chen & Juang (2008b) (62%), where surfactin was extracted directly from the precipitates.

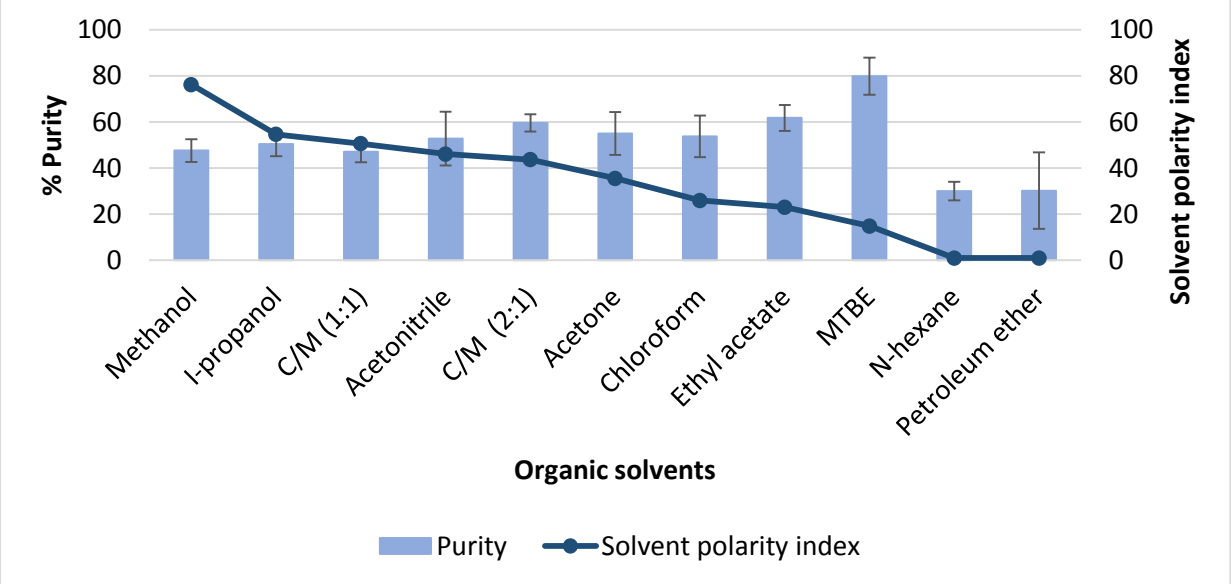

Figure 5-17: Surfactin purities after surfactin purification by solvent extraction, where the error bars indicate the standard deviation of two replicates. The solvent polarity index shows relative polarities of solvents on the scale of water=100. C/M (1:1) and C/M (2:1) represent mixtures of chloroform and methanol in the volume ratio 1:1 and 2:1 respectively, while MTBE represent methyl tert-butyl ether.

5.4.2 Optimisation of solvent extraction based on impurity assays

Impurity assays were also used for optimisation of surfactin purification by solvent extraction. Samples of solvents after extraction (as discussed in section 4.4.1) were spotted 10 times on TLC plates by multiple spotting. The plates were then developed as discussed in section 4.2.2, before primuline and ninhydrin were used to visualise the components with lipid and peptide moieties as discussed in section 4.2.3.

The relative quantity of impurities with lipid and peptide parts in the solvents after extraction was then compared (Figure 5-18 and Figure 5-19). It should be noted that alkaline water was used for resolubilisation, rather than purification of acid precipitates. This was done for comparison of impurity compositions as water was assumed completely resolubilise the acid precipitates (recovering 100% surfactin and impurities). Figure 5-18 shows the change of lipid impurities with solvent polarity, while Figure 5-19 shows the change in protein impurities with solvent polarity. The lipid impurity layer is shown by the white bands near the top of the TLC plate in Figure 5-18. The chloroform used in this study seemed to had vaporized during storage, thus could be showing a higher lipid concentration than the actual concentration.

The lipid layer was seen to be more prominent with decrease in polarity, except for chloroform (Figure 5-18). According to Breil et al. (2017) non-polar solvents dissolve lipids better than polar solvents. Methanol, *i*-propanol, and acetonitrile, which are among the most polar solvents, hardly showed lipid bands. Polar solvents were therefore more effective for surfactin recovery while eliminating lipid impurities. Although the lipid band was not seen in Figure 5-18, alkaline water is capable of solubilising lipid impurities. This was observed in acid precipitation studies (Figure 5-15b).

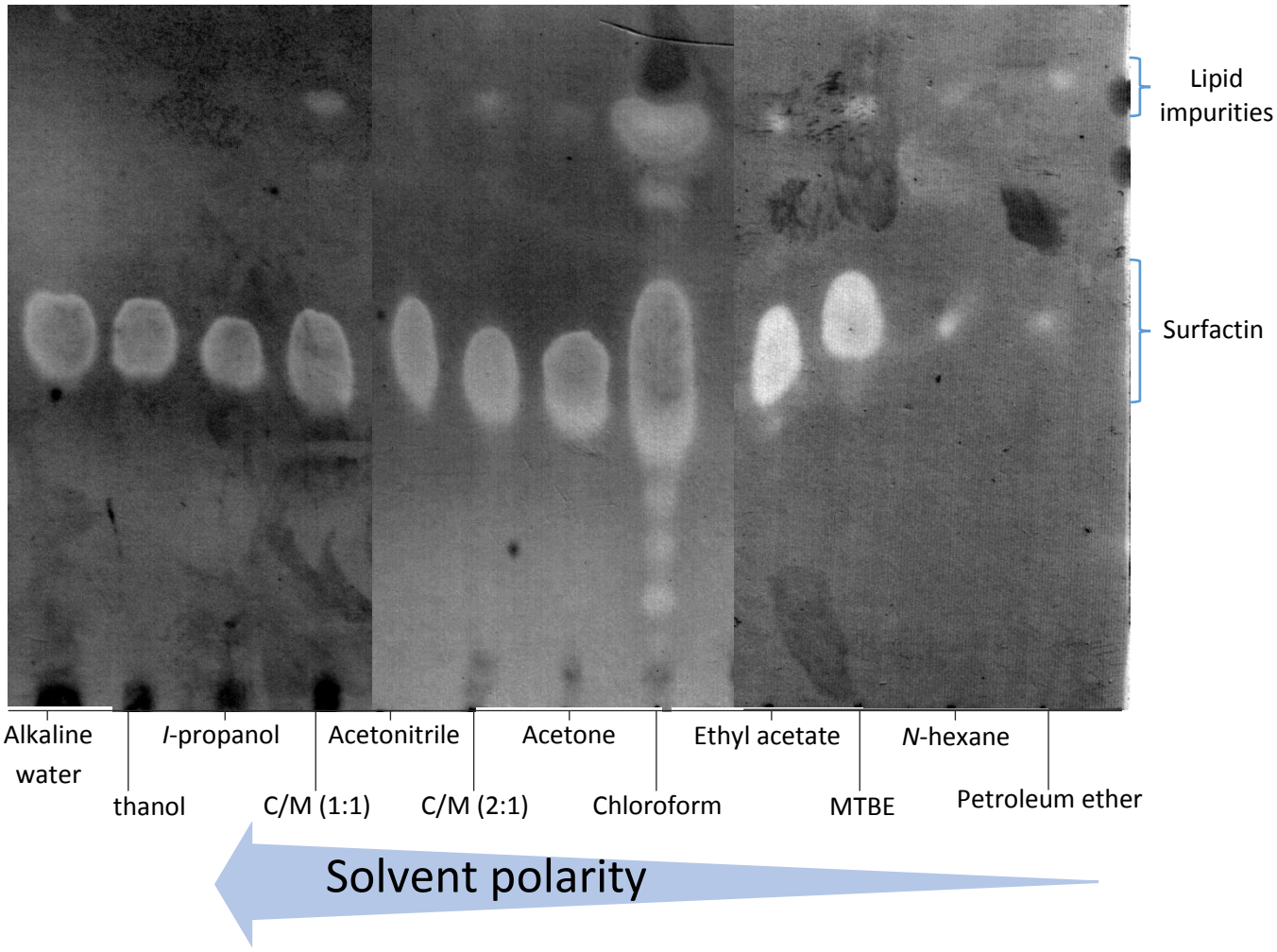

Figure 5-18: Lipid impurities at different solvent polarities after surfactin purification by solvent extraction. C/M (1:1) and C/M (2:1) represent mixtures of chloroform and methanol in the volume ratio 1:1 and 2:1 respectively, while MTBE represent methyl tert-butyl ether.

The change in protein impurities with solvent polarity after solvent extraction is shown in Figure 5-19. Protein impurities in solvents after extraction decreased with solvent polarity. This is accordance with findings of Breil et al. (2017), which state that polar solvents are better at dissolving proteins than non-polar solvents. Non-polar solvents are therefore more suitable for removal of protein impurities. MTBE, ethyl acetate and acetonitrile were seen to recover surfactin, while recovering low protein impurities.

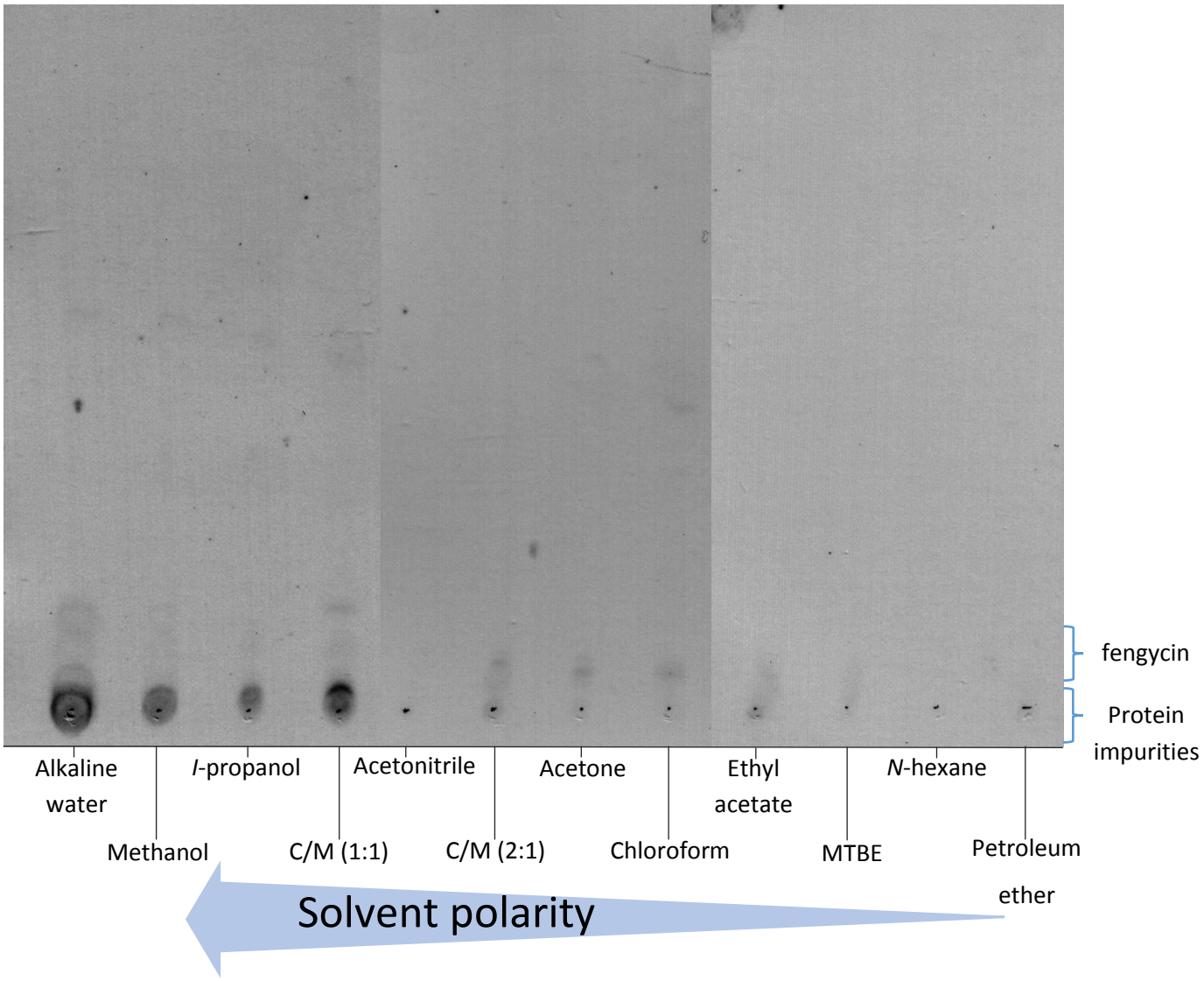

Figure 5-19: Protein impurities after surfactin purification by solvent extraction. C/M (1:1) and C/M (2:1) represent mixtures of chloroform and methanol in the volume ratio 1:1 and 2:1 respectively, while MTBE represent methyl tert-butyl ether

Since non-polar solvents (hexane and petroleum ether) had poor recoveries of surfactin, but were better at recovering lipids, a two-stage extraction can therefore be recommended. In the first stage, petroleum ether can be used for removal of lipid impurities, while surfactin is left in the solid precipitate. This can then be followed by extraction using MTBE, which has a high surfactin recovery, but recovers less protein as shown in Figure 5-20.

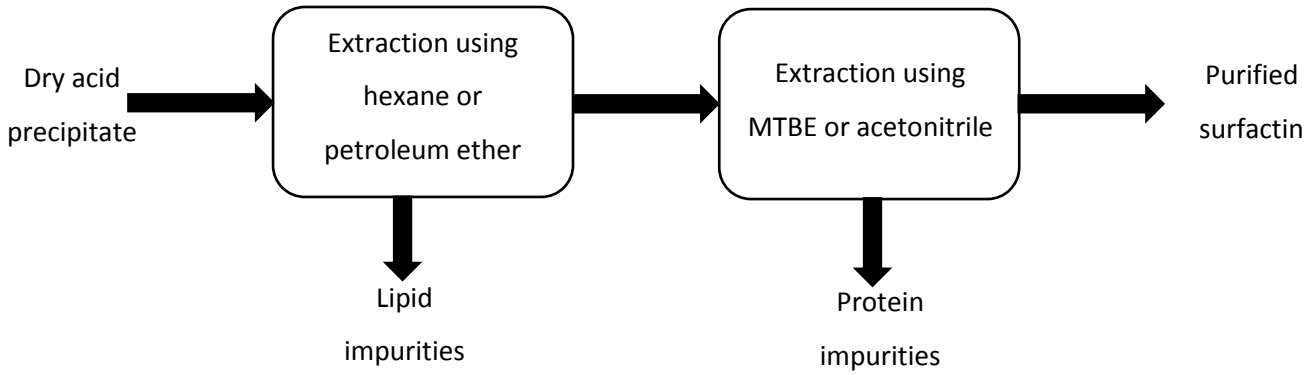

Figure 5-20: Flow diagram showing how surfactin precipitates can be purified by a two-stage solvent extraction process

5.5 Adsorption

Adsorption was also considered for surfactin purification in this study. Surfactin adsorption was chosen as it does not require high operating pressures and is not affected by concentration polarization like membrane filtration. Additionally, this method is capable of achieving the highest recoveries compared to solvent extraction and ultrafiltration, and it is capable of separating the different lipopeptide families. Lastly, surfactin purification by adsorption is relatively cheaper than other methods capable of recovering surfactin at high purities (i.e. chromatographic techniques). Surfactin purification by adsorption, however, has limitations as it is affected mass transfer limitations, requires an additional elution step, requires the use of organic solvents which may be toxic and has additional costs due to regeneration of the resins (Kosaric & Sukan, 2014).

From the literature survey, it was seen that surfactin adsorption is affected by the operating temperature, initial pH and resin concentration to surfactin concentration ratio (RC/SC ratio). Based on an understanding of the surfactin adsorption mechanism, it was also proposed that the presence of methanol in the adsorption liquid could improve adsorption. No studies have however been done to determine how methanol addition to adsorption liquids affect surfactin concentration. In this study, the effect of the initial pH was determined in the range 6.5 to 11.5, while the effect of the operating temperature was determined between 25 °C and 45 °C. The minimum initial pH and operating temperature were chosen as surfactin precipitates at pH below 6 (Liu, et al., 2007), and 25 °C is approximately room temperature.

The maximum initial pH and operating temperature ranges (11.5 and 45 °C respectively) were chosen in consideration of the antimicrobial properties of surfactin. It was believed that high temperatures and pH reduce the antimicrobial activity of surfactin. No studies have been done to determine if the exposure of surfactin to high pH and temperatures affect its efficacy against *M. tuberculosis*. Geetha & Manonmani (2010), however, studied the larvicidal efficacy of surfactin after exposure to high pH and temperatres. It was seen that surfactin produced by *B. subtilis* had maximum larvicidal efficacy between pH 3 and pH 10, while the surfactin was stable at temperatures between 25 °C and 42 °C.

The effect of the RC/SC ratio was determined between 4 g_r/g_s and 40 g_r/g_s. This range was chosen as it was assumed that surfactin reaches saturation somewhere in this range based on studies by Dhanarajan et al. (2015). The maximum methanol concentration was chosen as 30% (v/v) as this concentration is sufficient to disperse approximately all surfactin micelles in solution (Chen, et al., 2008b). The effect of

methanol concentration on surfactin adsorption is a new technique, investigated here for the first time, but the effect of operating temperature, initial pH and RC/SC ratio have been studied in literature (Chen, et al., 2008c; Dhanarajan, et al., 2015, Liu, et al., 2007, Montastruc, et al., 2008 and Wang, et al., 2010) using one factor at a time (OFAT) experiments. In this study, however, the effect of these parameters on surfactin adsorption was instead studied using a surface design.

A response surface design was chosen over OFAT design since OFAT designs are not effective if there are interactions within the system (Czitrom, 1999). The face-centred central factorial design (FCC design) was chosen over the circumscribed central factorial design (CCC design) since the CCC design requires points outside the design space which are not possible to achieve (e.g. temperatures below 25 ^{o}C are not achievable in an orbital shaker without a cooling system). The FCC was chosen over the inscribed central composite design (ICC design) since the FCC provides high quality predictions over the entire design space (Verseput, 2001). Lastly, a FCC design allows fewer runs for a comparison study between adsorption in the presence of methanol or in the absence of methanol compared to the CCC and ICC designs.

A FCC design has a drawback as it gives poor precision for estimating pure quadratic coefficients for the quadratic models used to model surface designs (Verseput, 2001). Verseput (2001), however, found that there is insufficient reduction in prediction error or difference in effects estimation resulting from using FCC designs rather than CCC or ICC designs. Additionally, for a reasonable overall experimental error (e.g. 5% relative standard deviation), a FCC design is preferable as it does not have the added complexity of running each variable at five levels. In FCC designs, each variable is only run at three levels, thus FCC designs are the simplest variety of CCD to carry and they are least vulnerable to experimental error associated with setup and operation (Verseput, 2001).

This study begins by providing the results and discussion of optimisation of surfactin adsorption based on the percentage of surfactin adsorbed onto resins when independent variables were altered. This is followed by the results and discussion of optimisation of surfactin adsorption based on improvement in surfactin selectivity when independent variables were altered. This section then provides the purity and recovery of the surfactin product after purification adsorption Lastly, batch kinetics and isotherms are then presented to determine the adsorption characteristics.

5.5.1 Optimisation of surfactin adsorption based on % SA

Surfactin adsorption was optimised based on the percentage of surfactin in adsorption liquids which is adsorbed onto resins during adsorption (% SA). The % SA was calculated based on Equation 4-11, using surfactin concentrations determined by HPLC analysis. HPLC analysis was used as it is has a higher precision than TLC analysis and it is capable of quantifying surfactin at low concentrations. The effects and interactions of the initial pH, operating temperature and RC/SC ratio on % SA are summarised in a Pareto chart (Figure 5-21) and their ANOVA is summarised in Table 5-8. It should be noted that the effect of methanol concentration was studied separately in section 5.5.1.4.

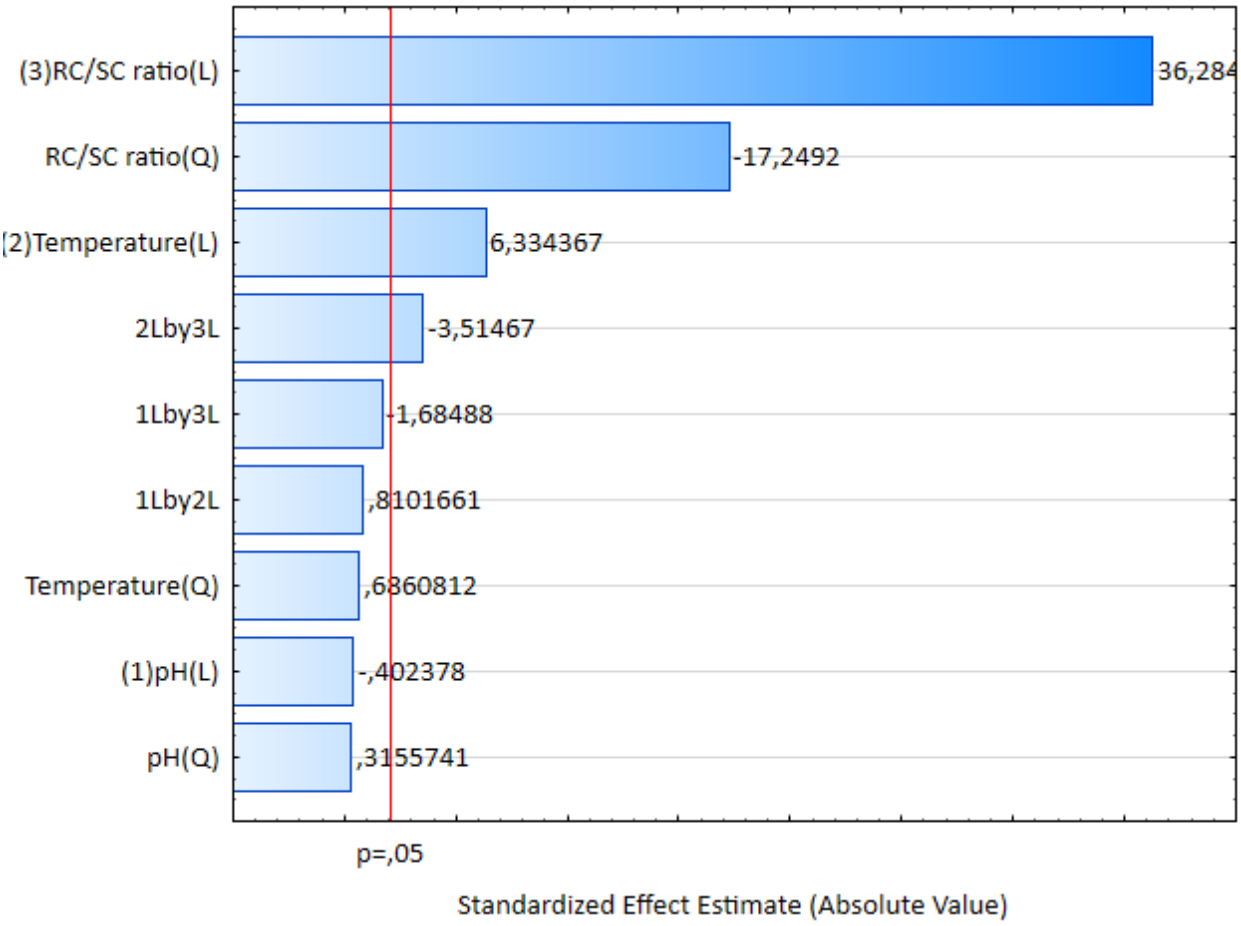

Figure 5-21: Linear (L) and quadratic (Q) effects of initial pH (1), operating temperature (2), resin concentration to surfactin concentration (RC/SC) (3) ratio and interactions of these independent variables on % Surfactin adsorption (% SA) at 95% confidence

Table 5-8: ANOVA table showing the linear (L) and quadratic (Q) effects of initial pH (1), operating temperature (2), resin concentration to surfactin concentration (RC/SC) ratio (3) and interactions of these independent variables on % SA at 95% confidence. The values written in red show parameters that had an effect on % surfactin adsorption (% SA) at a 95% confidence interval

Source of variation	Sum of squares	Degrees of freedom	Mean square	p
(1) pH (L)	2.33	1	2.33	0.690967
pH (Q)	1.43	1	1.43	0.755054
(2) Temperature (L)	578.03	1	578.03	0.000002
Temperature (Q)	6.78	1	6.78	0.499237
(3) RC/SC ratio (L)	18966.02	1	18966.02	0.000000
RC/SC ratio (Q)	4286.28	1	4286.28	0.000000
1L by 2L	9.46	1	9.46	0.425805
1L by 3L	40.90	1	40.90	0.104973
2L by 3L	177.96	1	177.96	0.001777
Error	345.74	24	14.41	
Total SS	26331.97	33		

5.5.1.1 *Effect of initial pH on surfactin adsorption*

The initial pH had no significant effect on adsorption. Additionally, the pH did not have any interactions with either temperature or RC/SC ratio (Figure 5-21 and Table 5-8). The surface plots of % SA against RC/SC ratio and temperature at different pH levels (Figure 5-22 to Figure 5-24) are identical which indicates pH has no effect or interaction with temperature or RC/SC ratio. The absence of initial pH-RC/SC ratio interactions can also be seen on surface plots of % SA against RC/SC ratio and pH at different operating temperature levels (Figure 8-14 to Figure 8-16) in the appendix (section 8.3.3.2). Additionally, the absence of initial pH- operating temperature interactions can be seen on surface plots of % SA against initial pH and operating temperature at different levels of the RC/SC ratio (Figure 8-17 to Figure

8-19) in the appendix (section 8.3.3.2). In Figure 8-14 to Figure 8-19, the change in % SA with operating temperature or RC/SC ratio is independent of the initial pH.

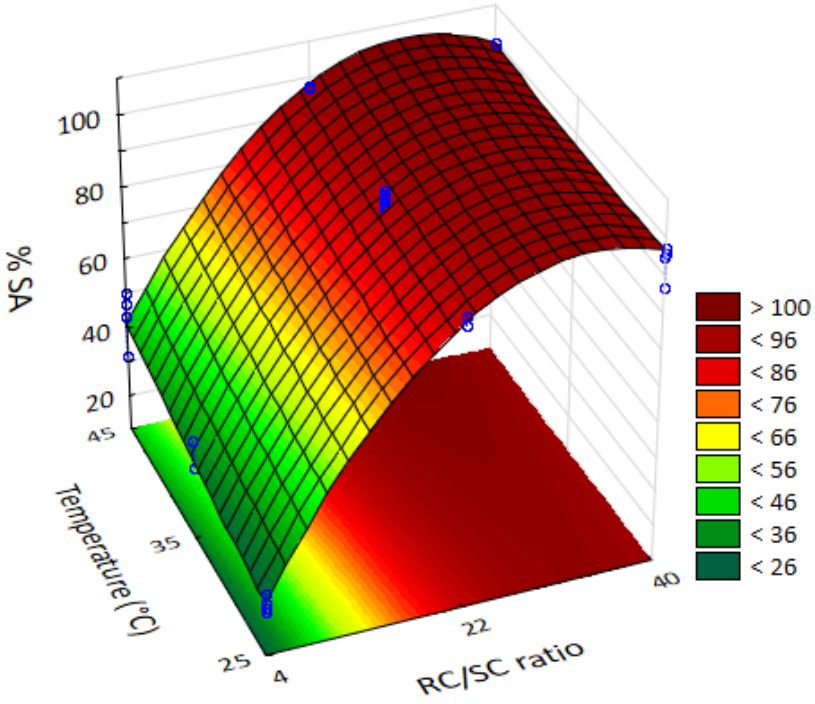

Figure 5-22: % Surfactin adsorption (% SA) obtained after surfactin adsorption in the absence of methanol at an initial pH of pH 6.5

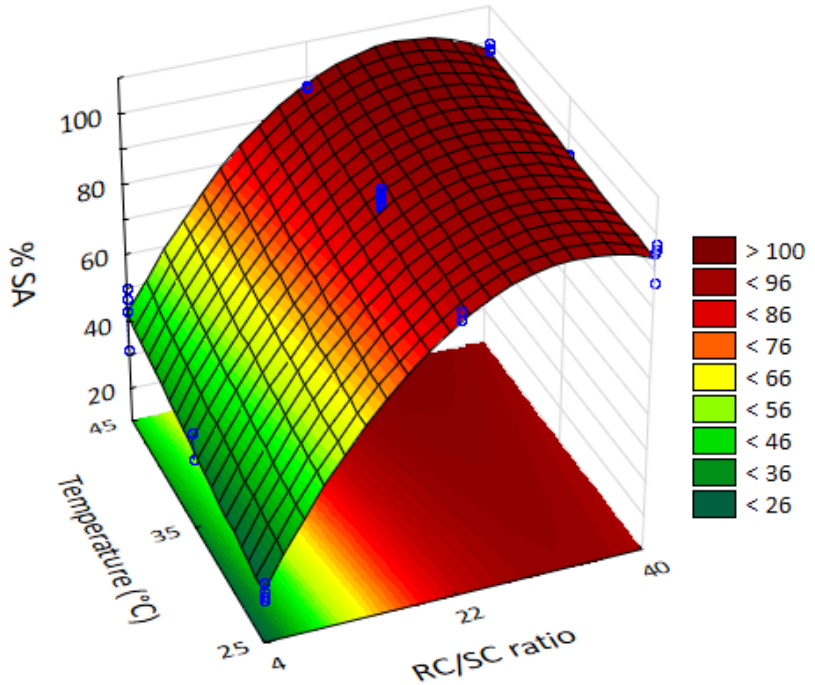

Figure 5-23: % Surfactin adsorption (% SA) obtained after surfactin adsorption in the absence of methanol at an initial pH of pH 9

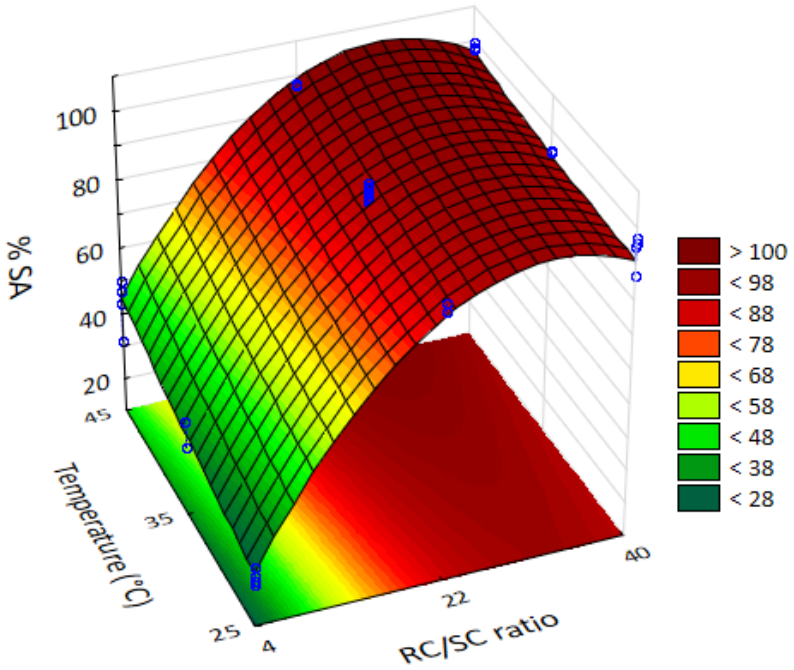

Figure 5-24: % surfactin adsorption (% SA) obtained after surfactin adsorption in the absence of methanol at an initial pH of pH 11.5

The % SA was expected to be relatively higher at pH 11.5. This is because surfactin forms smaller micelles, and the surfactin micelles have a more negative charge at this pH (Chen, et al., 2008c). In this study, however, the surfactin adsorption did not change with change in pH possibly due to the fact that charged resins were used in the study by Chen et al. (2008c), while the charge of the HP-20 resins used in this study is neutral. The absence of significant effect of pH on surfactin adsorption is, however, in agreement with the study by Liu et al. (2007), who studied adsorption on charged ion exchange resins (activated carbon).

The equilibrium pH was tested to determine if the insignificant effect of pH on surfactin adsorption was due to pH changes during adsorption. When the initial pHs were pH 6.5, the pH increased to an equilibrium pH of approximately pH 7 after adsorption. When the initial pH was pH 9 and pH 11.5, the equilibrium pH decreased to approximately 8 and 9 respectively. The equilibrium pH therefore increased from pH 7 to pH 9. % SA therefore did not change with either increase in initial pH or increasing equilibrium pH. Additionally, the operating temperature and RC/SC ratio did not have significant effects on the change in pH during adsorption (Figure 8-8 to Figure 8-10 in the appendix, section 8.3.3). In these

figures, the equilibrium pH does not change with either operating temperature or RC/SC ratio. The change in pH during adsorption was in agreement with the study by Chen et al. (2008c).

Due to the pH change during adsorption, the control of pH by the use of buffers seems to be a better option for studying the effect of pH on adsorption. Liu et al. (2007) studied the effect of pH on surfactin adsorption with pH between 6.5 and 8.5 while controlling the pH using buffer solutions. The pH was still seen not to affect surfactin adsorption even in the presence of buffers. The addition of buffers may also increase the ionic strength in the adsorption liquid (Breuer & Jeffrey, 2004). Liu et al. (2007) found that an increase in ionic strength in adsorption liquid reduces surfactin adsorption capacity as well as the surfactin adsorption rate.

Since the pH did not affect adsorption in the absence of methanol, an initial pH of pH 11.5 was selected. This was selected since pH 11.5 is not so extreme as to affect surfactin antibacterial activity, while it is too alkaline to prevent the growth of cells in the adsorption liquid, hence no sterilisation of the liquid before adsorption is required.

5.5.1.2 *Effect of RC/SC ratio on surfactin adsorption*

The RC/SC ratio was seen to have the most significant effect on surfactin adsorption (Figure 5-21 and Table 5-8). The effect of RC/SC ratio can also be seen from Figure 5-22 to Figure 5-24, which show that % SA increased from a minimum of approximately 25% at an RC/SC ratio of 4 g_r/g_s to 100% at an RC/SC ratio of 22 g_r/g_s. The % SA increased with RC/SC ratio until it reached a peak of approximately 105% at an RC/SC ratio of approximately 31 g_s/g_r, before lowering again (Figure 5-22 to Figure 5-24).

The increase in % SA with RC/SC ratio was due to the fact that an increase in RC/SC ratio results in an increase in amount of resin available to adsorb surfactin in the medium. An increase in RC/SC ratio is therefore expected to improve surfactin adsorption until approximately 100% of the surfactin has been adsorbed. The % SA is then expected to be constant with increase in RC/SC ratio (Dhanarajan, et al., 2015). This decrease in % SA adsorption after a % SA of 100% was achieved was due to the fact that a quadratic function was used to model the experimental data, since a quadratic function must have turning point thus is bound to show a decrease at some point. The quadratic model is therefore not suitable for modelling the change in % SA with RC/SC ratio

In this study, the RC/SC ratio was used as a single factor affecting adsorption since it was expected that an optimum RC/SC ratio exists, rather than an optimum resin concentration or optimum initial surfactin concentration independently. This was expected as a continuous increase in resin concentration, at a

constant initial surfactin concentration, increases % SA until an optimum resin concentration is reached where approximately all of the surfactin in the adsorption liquid has been adsorbed. When the initial surfactin concentration is increased, the optimum resin concentration is expected to increase as more resin will be required to adsorb the surfactin. Since the optimum resin concentration increases with increase in initial surfactin concentration, the RC/SC ratio was optimised as a single factor rather than the resin concentration and resin concentration independently.

In most studies (Dhanarajan, et al., 2015; Chen, et al., 2008 and Wang, et al., 2010), however, the resin concentration and the surfactin concentration were optimised independently. A study was therefore carried out to determine if using RC/SC ratio as a single factor affecting adsorption was similar or different to optimising the resin concentration and surfactin concentration independently. This study was carried out at resin concentrations of 5.5 g/L and 11 g/L, while maintaining the RC/SC ratio at 40 g_r/g_s as shown in the appendix section 8.3.2. This study showed that % SA was dependent on the RC/SC ratio rather than resin concentration and surfactin concentration independently (Figure 8-7). A single factor ANOVA was used to show that the % SA obtained at the different resin concentrations (5.5 g/L and 11 g/L) were similar at a 95% confidence interval. This was in agreement with the study by Liu et al. (2007), who also found that % SA depends on the RC/SC ratio rather than the resin concentration or the initial surfactin concentration, where an RC/SC ratio of 7.6 g_r/g_s was used. Additionally, the study by Liu et al. (2007) found that it also takes approximately the same amount of time to reach equilibrium if the surfactin concentration or resin concentration is varied as long as the RC/SC ratio is maintained.

The optimum RC/SC ratio predicted by the CCF was 31 g_r/g_s, corresponding to a resin concentration to total lipopeptide concentration (RC/TC) ratio of 35 g_r/g_{tl}. 31 g_r/g_s was chosen as the optimum as it is the RC/SC ratio where maximum % SA was obtained in this study (Figure 5-22 to Figure 5-24). The RC/TC ratio was expected to be similar to that in the study by Dhanarajan et al. (2015), who used the same resins as in this study. In the study by Dhanarajan et al. (2015), the optimum RC/TC ratio obtained when resin concentration was varied while keeping the initial total lipopeptide concentration constant (10 g_r/g_s) was different to the RC/TC ratio obtained when the initial total lipopeptide concentration was varied and the resin concentration held constant (3.33 g_s/g_r). This was because the dependent variable used when optimising the resin concentration at a constant initial total lipopeptide concentration (% SA) was different to the dependent variable used when optimising the initial total lipopeptide concentration at a constant resin concentration (q_e).

5.5.1.3 *Effect of operating temperature on surfactin adsorption*

Temperature had the second most influential effect on % SA, after RC/SC ratio linear and quadratic effects (Figure 5-21 and Table 5-8). The % SA was seen to continuously increase with temperature (Figure 5-22 to Figure 5-24). At the low RC/SC ratio (4 g_r/g_s), %SA increased from approximately 25% to approximately 43% (which is approximately a 1.72 fold increase) when the operating temperature was increased from 25 °C to 45 °C. However, at high RC/SC ratio (40 g_r/g_s), surfactin adsorption only increased from 94% to 98% (a 1.04 fold increase). The temperature therefore had a significant increase in surfactin adsorption at lower RC/SC ratio in comparison to higher RC/SC ratio, thus indicating RC/SC ratio-temperature interactions. This is possibly due to the increased space for adsorption at high RC/SC ratio, thus a high surfactin adsorption can be achieved regardless of the temperature. The recommended operating temperature for surfactin adsorption was therefore 45 °C.

The increase in % SA with increase in temperature was in agreement with the study by Dhanarajan et al. (2015). In the study by Dhanarajan et al. (2015), the temperature increased the total lipopeptide adsorption from 88% to 95% at a resin concentration to total lipopeptide concentration of 10 g_r/g_s. This is slightly lower than the increase from 94% to 98% obtained at a resin concentration to total lipopeptide concentration of 40 g_r/g_s in this study. The increase in % SA with temperature was however contrary to the results of Liu et al. 2007, who found that surfactin adsorption is improved by lower temperatures.

One limitation of the data from this study is that the starting temperature of the experiments did not necessarily correspond to the operating temperature: room temperature solution and adsorption resin were mixed and then placed in the heated incubator at the operating temperature. Thus the temperature of the adsorption liquid was not constant at the operating temperature during adsorption, but increasing from room temperature to the operating temperature. A recommendation for future studies is therefore to preheat the absorption fluid and media, separately to the operating temperature, before mixing.

We can surmise from these results that surfactin adsorption is likely an endothermic process, since it improved with increase in operating temperature. While it fell outside of the scope of this study, we can speculate that this result would be further bolstered by thermodynamic studies, where endothermic processes would be indicated by positive enthalpies. These thermodynamic studies are recommended for future work as they also give useful information such as whether adoption is physical or chemical, if

adsorption is spontaneous or not, and whether the adsorption system becomes more ordered or not during adsorption (Du, et al., 2008 and Ming, et al., 2006).

5.5.1.4 *Effect of methanol concentration on surfactin adsorption*

The CCF design used for studying the effect of pH, RC/SC ratio and temperature on % SA was extended to study the effect of methanol on % SA. This allowed the effects of methanol as well as the effects of interactions of methanol with pH, RC/SC ratio and temperature on % SA to be quantified. The effect of methanol as well as the interactions of methanol with and methanol interactions with pH, RC/SC ratio and temperature on % SA are shown in Figure 5-25 and Table 5-9. The effect of interactions between methanol concentration with either operating temperature or RC/SC ratio did not have significant effects on % SA. The effect of the interaction between initial pH and methanol concentration, however, did have a significant effect on % SA.

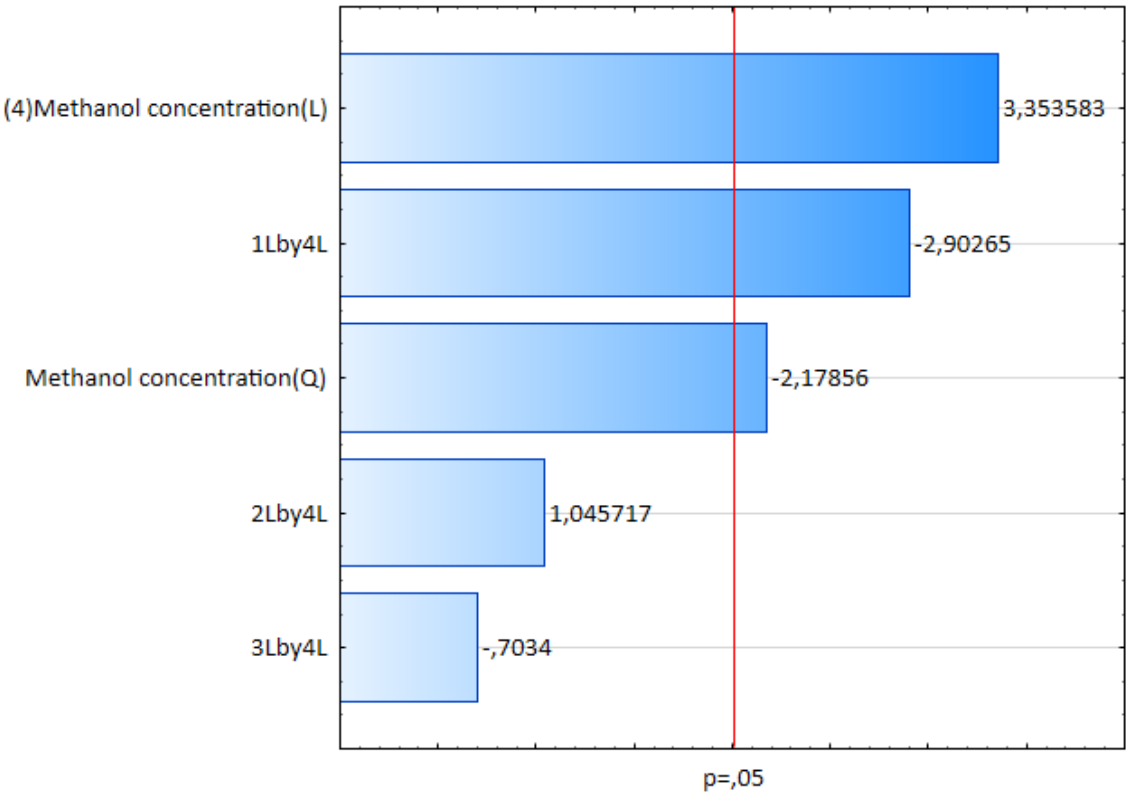

Figure 5-25: Effects of methanol concentration (4)as well as effects of interactions between methanol concentration and initial pH (1), operating temperature (2) and resin concentration to surfactin concentration (RC/SC) ratio (3) on % surfactin adsorption (% SA) at 95% confidence

Table 5-9: ANOVA table showing the effects of methanol concentration (4) as well as effects of interactions between methanol concentration and initial pH (1), operating temperature (2) and resin concentration to surfactin concentration (RC/SC) (3) ratio on % surfactin adsorption (% SA) at a 95% confidence. The values in red indicate parameters which had a significant effect on % SA at 95% confidence interval.

	Sum of Squares	Degrees of freedom	Mean Square	p
Methanol concentration (Q)	4764.77	1	4764.773	0.001604
Methanol concentration (L)	2010.77	1	2010.772	0.034526
1L by 2L	209.62	1	209.618	0.485352
1L by 3L	463.29	1	463.289	0.301156
1L by 4L	3569.55	1	3569.555	0.005664
Error	19488.65	46	423.666	
Total SS	30506.66	51		

The relationship between methanol concentration and % SA was shown through surface plots. These surface plots (Figure 5-26 to Figure 5-34) show % SA against methanol concentration and temperature at each of the pHs 6.5, 9 and 11.5 with RC/SC ratios of 4 g_r/g_s (Figure 5-26 to Figure 5-28), 22 g_r/g_s (Figure 5-29 to Figure 5-31) and 40 g_r/g_s (Figure 5-32 to Figure 5-34).

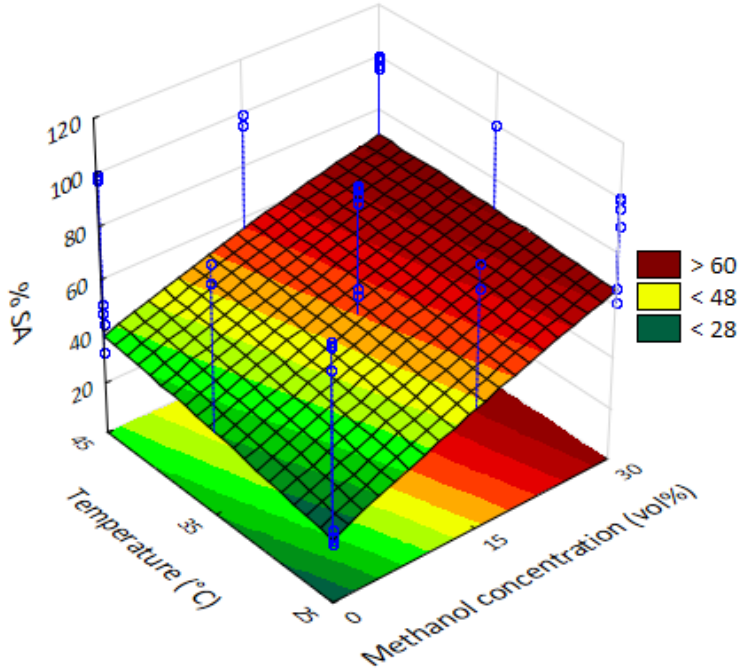

Figure 5-26: % Surfactin adsorption (% SA) adsorption obtained after surfactin adsorption in the presence of methanol at an initial pH and RC/SC ratio of 6.5 and 4 g_r/g_s respectively

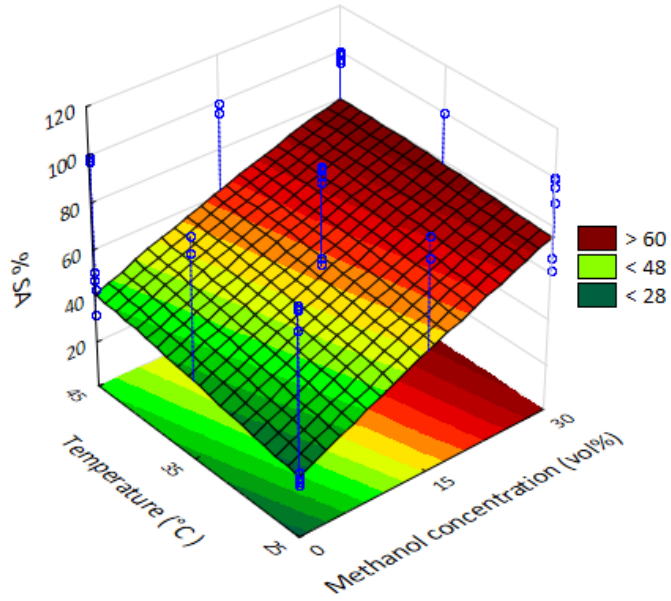

Figure 5-27: % Surfactin adsorption (% SA) obtained after surfactin adsorption in the presence of methanol at an initial pH and RC/SC ratio of 9 and 4 g_r/g_s respectively

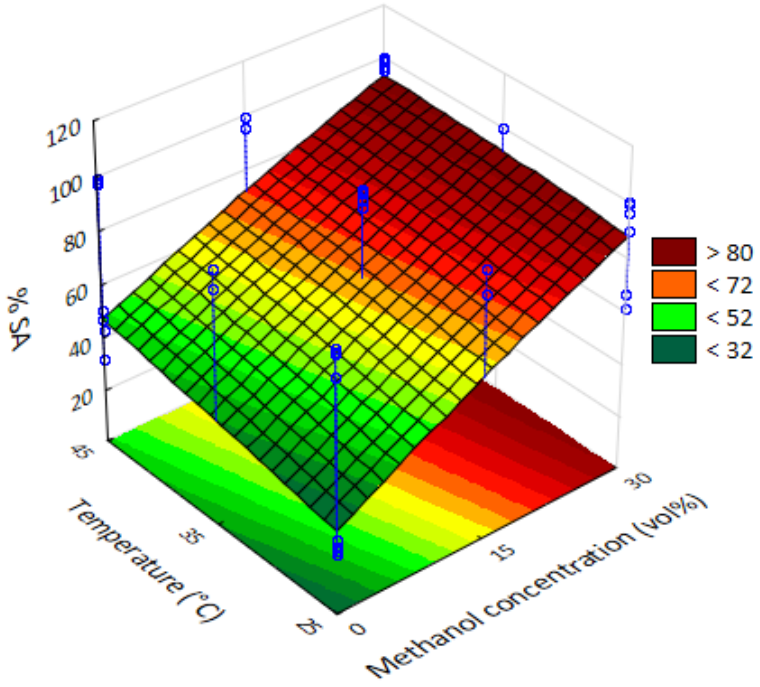

Figure 5-28: % Surfactin adsorption (% SA) obtained after surfactin adsorption in the presence of methanol at an initial pH and RC/SC ratio of 11.5 and 4 g_r/g_s respectively

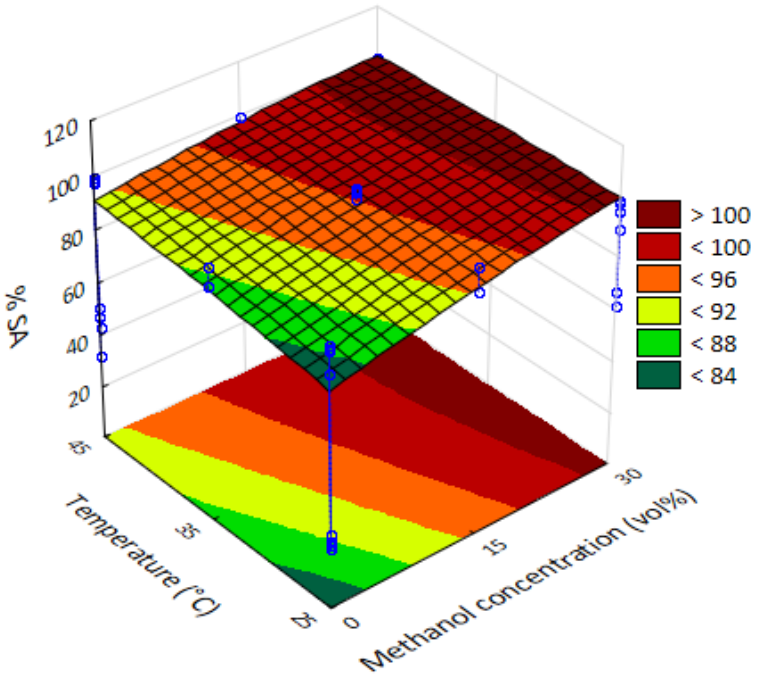

Figure 5-29: % Surfactin adsorption (% SA) obtained after surfactin adsorption in the presence of methanol at an initial pH and RC/SC ratio of 6.5 and 22 g_r/g_s respectively

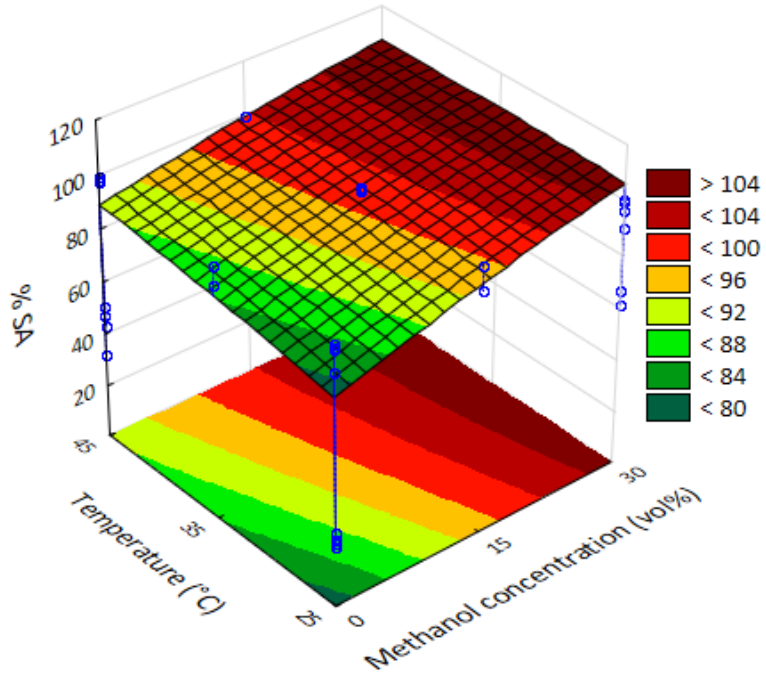

Figure 5-30: % surfactin adsorption (% SA) obtained after surfactin adsorption in the presence of methanol at an initial pH and RC/SC ratio of 9 and 22 g_r/g_s respectively

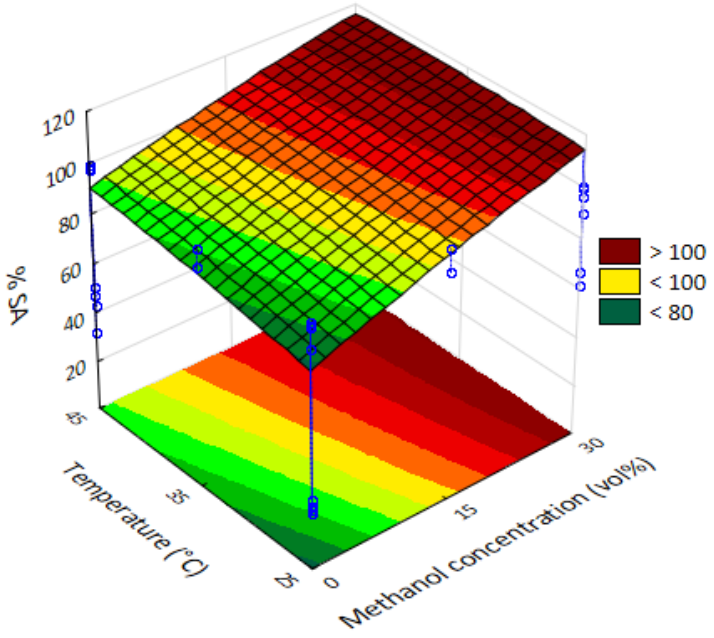

Figure 5-31: % SA obtained after surfactin adsorption in the presence of methanol at an initial pH and RC/SC ratio of 11.5 and 22 g_r/g_s respectively

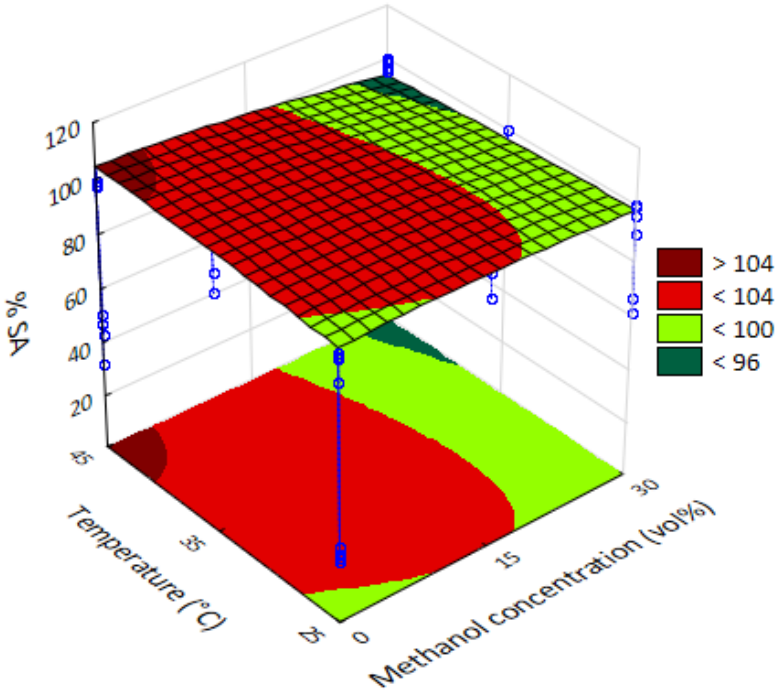

Figure 5-32: % Surfactin adsorption (% SA) obtained after surfactin adsorption in the presence of methanol at an initial pH and RC/SC ratio of 6.5 and 40 g_r/g_s respectively

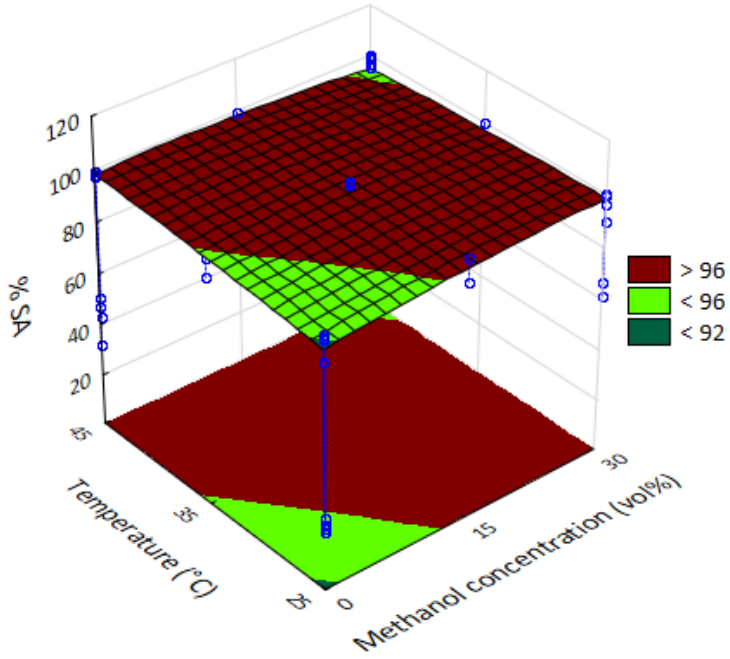

Figure 5-33: % Surfactin adsorption (% SA) obtained after surfactin adsorption in the presence of methanol at an initial pH and RC/SC ratio of 9 and 40 g_r/g_s respectively

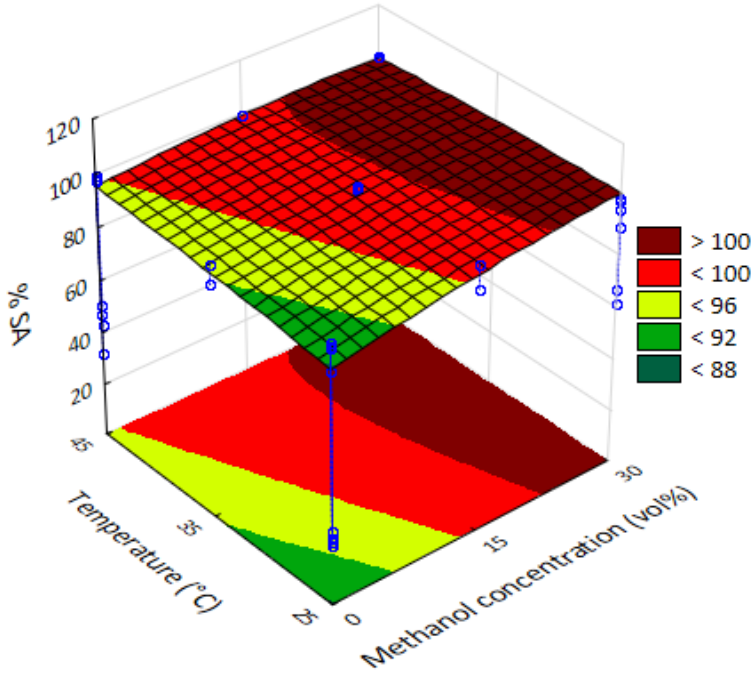

Figure 5-34: % Surfactin adsorption (% SA) obtained after surfactin adsorption in the presence of methanol at an initial pH and RC/SC ratio of 11.5 and 40 g_r/g_s respectively

An increase in methanol concentration was seen to improve the % SA. This effect is seen predominantly at the lowest RC/SC ratio of 4 g_r/g_s (Figure 5-26 to Figure 5-28). Here, an increase in methanol concentration from 0% (v/v) to 30% (v/v) was seen to increase % SA from approximately 25% to 95%. At RC/SC ratios of 22 g_r/g_s (Figure 5-29 to Figure 5-31) and 40 g_r/g_s, (Figure 5-32 to Figure 5-34), however, the % SA was close to 100% at all methanol concentrations.

An increase in pH resulted in an increase in % SA in the presence of methanol. This is particularly evident when comparing Figure 5-26, Figure 5-27 and Figure 5-28, where pH was increased stepwise while the RC/SC ratio was kept constant at 4 g_r/g_s and Figure 5-29, Figure 5-30 and Figure 5-31 where the pH increased stepwise while the RC/SC ratio is kept constant at 22 g_r/g_s. An increase in pH was seen to improve surfactin in the presence of methanol in the adsorption liquid, while it had not effect on surfactin adsorption in when there was no methanol in the adsorption liquid. Additionally, the pH did not change during adsorption in the presence of methanol in the adsorption liquid. Figure 8-11 to Figure 8-13 in the appendix (section 8.3.3) show that the equilibrium pH was similar to the initial pH during adsorption at 30% (v/v) methanol. Since surfactin adsorption continuously increased with increase in initial pH, the recommended initial pH for surfactin adsorption in the presence of methanol is 11.5.

The pH is known to affect % SA by changing the charge of the surfactin molecule. From the behaviour of the % SA with pH in the presence of methanol, it can be assumed that the surfactin charge decreases with an increase in methanol concentration, and surfactin has a more negative charge in adsorption solutions with methanol. Surfactin has an overall negative charge in solution, but the surfactin charge changes differently with pH in the presence of methanol and in the absence of methanol (Chen, et al., 2010c and Isa, et al., 2007). At 0% (v/v) methanol, Chen et al. (2010c) found that the zeta potential of surfactin (at 0.5 g_s/L) increased from -26 mV to -18 mV when the pH was increased from 6.5 to 7, before decreasing again from -18 mV to -26 mV when the pH was increased from 7 to 10. At 50% (v/v) methanol, Isa et al. (2007) found that the zeta potential of surfactin at 0.583 g_s/L remained constant at approximately -28 mV in the pH range between 4 and 10. The surfactin therefore has a more negative charge in the presence of methanol. To understand the relationship between % SA and pH in adsorption liquids with methanol, the zeta potential of surfactin at different pHs (between 6.5 and 11.5) needs to be studied at a methanol and surfactin concentration of 30% (v/v) and 0.5 g_s/L respectively.

Based on this study, the recommended methanol concentration for surfactin adsorption was 30% (v/v), which corresponds to an optimum RC/SC ratio of 5 g_r/g_s. This RC/SC ratio was selected as the % SA was approximately 98% at an initial pH, operating temperature, RC/SC ratio and methanol concentration of 11.5, 45 °C, 4 g_r/g_s and 30% (v/v) methanol. The % SA then increased, reaching a maximum at 120% (at an RC/SC ratio of 22 g_r/g_s, before decreasing to 100% at an RC/SC ratio of 40 g_r/g_s (Figure 5-35). This quadratic behaviour of the change in % SA with increase in RC/SC ratio was due to the fact that a quadratic model was used to model the effects of RC/SC ratio in this study. The % SA is actually expected to increase to 100%, where it would remain constant when the RC/SC ratio is increased. The optimum RC/SC ratio for surfactin adsorption is thus expected to be lower than 22 g_r/g_s, which is predicted by the quadratic model (Figure 5-35), but slightly higher than 4 g_r/g_s since surfactin is not fully adsorbed at this RC/SC ratio. An optimum RC/SC ratio of 5 g_r/g_s was therefore selected in this for surfactin adsorption in the presence of methanol.

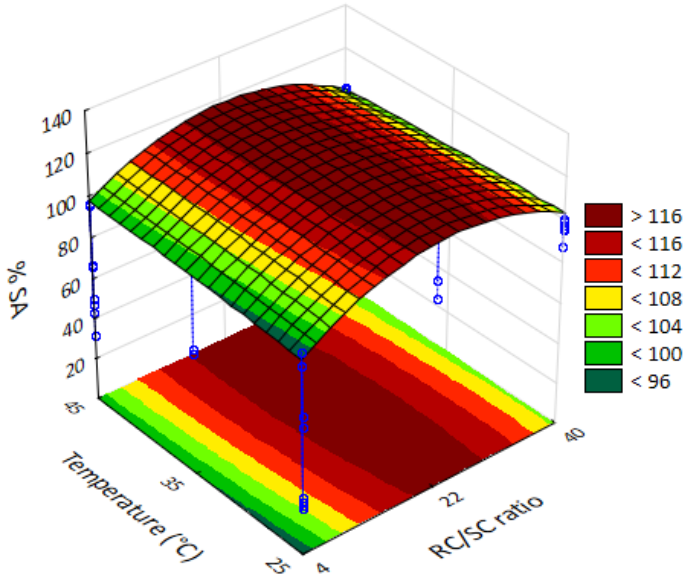

Figure 5-35: % Surfactin adsorption (% SA) obtained after surfactin adsorption at an initial pH and methanol concentration of 11.5 and 30 % (v/v) respectively

The addition of methanol in the adsorption liquid was expected to improve the surfactin adsorption. This is because methanol disperses micelles to form monomers (Isa, et al., 2011). In the absence of methanol, surfactin exists as micelles in dynamic equilibrium with monomers. During adsorption, only surfactin monomers are adsorbed and the micelles break to maintain dynamic equilibrium between micelles and monomers. When methanol is added, the micelles disperse such that surfactin only exists as monomers in the adsorption liquid, hence surfactin is expected to be adsorbed much quicker (Liu, et al., 2007). This is then expected to improve the adsorption capacity of surfactin since there is competitive adsorption between the adsorption of surfactin and impurities. Methanol has an additional advantage as it inhibits bacterial growth during adsorption.

5.5.2 Optimisation of surfactin adsorption based on improvement in selectivity (IS)

5.5.2.1 *Effect of initial pH, operating temperature and RC/SC ratio on IS*

Surfactin adsorption was also optimised based on the factor by which the surfactin selectivity improved during surfactin purification by adsorption (IS). IS was calculated based on Equation 4-12, using surfactin concentrations determined by HPLC analysis. HPLC analysis was used as it is has a higher precision compared to TLC and it is capable of quantifying surfactin and antifungals at low concentrations. To determine the effects of initial pH, operating temperature and RC/SC ratio on IS, a Pareto chart (Figure 5-36) and an ANOVA table (Table 5-10) were used to display the relative effects of independent variables as well as their interactions on improvement in surfactin selectivity respectively.

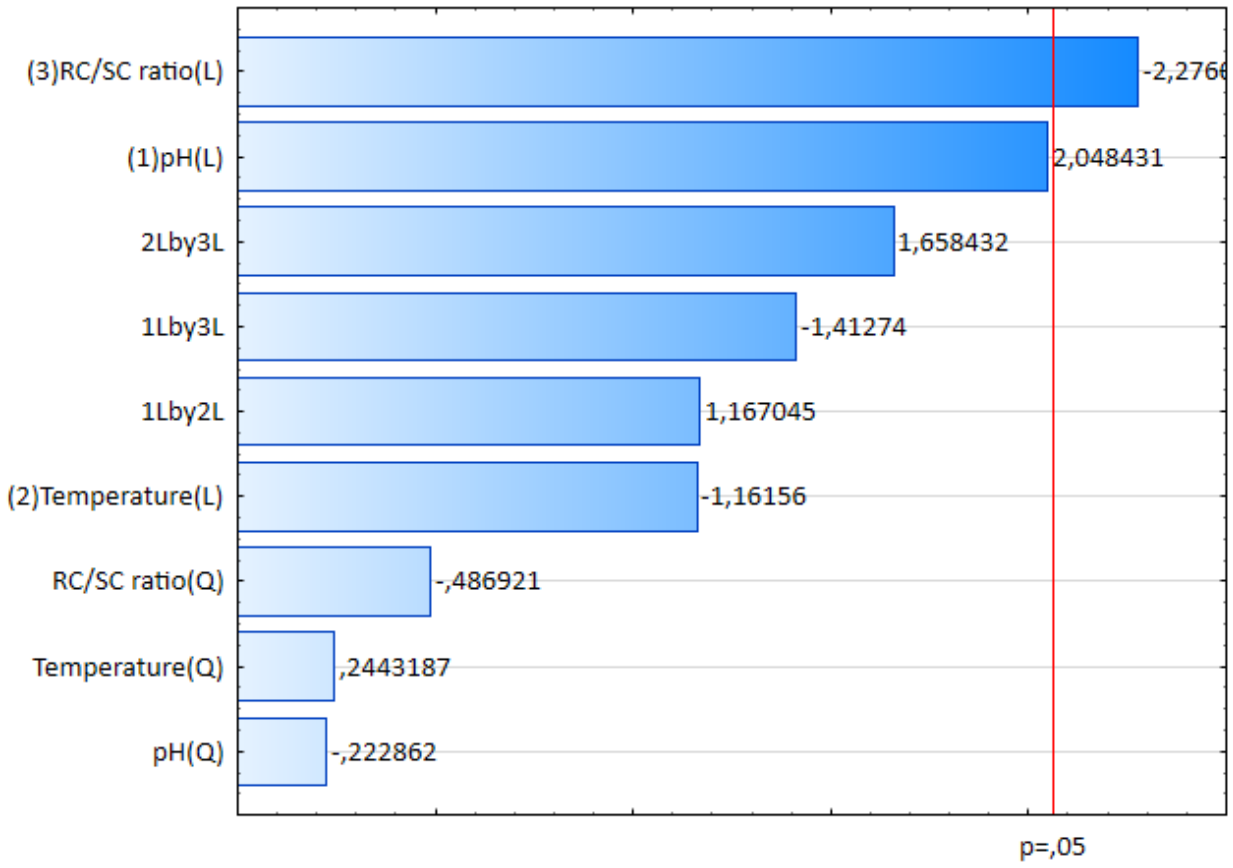

Figure 5-36: Linear (L) and quadratic (Q) effects of initial pH (1), operating temperature (2), resin concentration to surfactin concentration (RC/SC) (3) ratio and interactions of these independent variables on improvement in selectivity (IS) at 95% confidence

Table 5-10: ANOVA table showing the linear (L) and quadratic (Q) effects of initial pH (1), operating temperature (2), resin concentration to surfactin concentration (RC/SC) (3) ratio and interactions of these independent variables on improvement in selectivity (IS) at 95% confidence. The values in red indicate parameters which had a significant effect on IS at a 95% confidence interval

Source of variation	Sum of squares	Degrees of freedom	Mean square	p
(1) pH (L)	0.340605	1	0.340605	0.051607
pH (Q)	0.004032	1	0.004032	0.825529
(2) Temperature (L)	0.109520	1	0.109520	0.256836
Temperature (Q)	0.004845	1	0.004845	0.809061
(3) RC/SC ratio (L)	0.420500	1	0.420500	0.032056
RC/SC ratio(Q)	0.019245	1	0.019245	0.630732
1L by 2L	0.110556	1	0.110556	0.254657
1L by 3L	0.162006	1	0.162006	0.170570
2L by 3L	0.223256	1	0.223256	0.110245
Error	1.948137	24	0.081172	
Total SS	3.349474	33		

The change in IS during adsorption was seen to be independent of the operating temperature (Figure 5-36 and Table 5-10). The change in IS during adsorption was also independent of operating temperature- RC/SC ratio and operating temperature- initial pH interactions. This can be supplemented by surface plots of IS against operating temperature and pH at RC/SC ratio levels (Figure 8-20 to Figure 8-22) as well as surface plots of IS against operating temperature and pH at different RC/SC ratio levels (Figure 8-23 to Figure 8-25) shown in the appendix (section 8.3.3.3).

The IS was seen to be dependent only on the RC/SC ratio (Figure 5-36 and Table 5-10) at a 95% confidence interval. The IS was seen to improve with a decrease in RC/SC ratio (Figure 5-37 and Figure 5-38). At an operating temperature of 25 °C and initial pH 11.5, the IS was seen to increase from a minimum of approximately 1 to a maximum of approximately 1.8 (Figure 5-37). This behaviour was

possibly due to the ease of adsorption of surfactin compared to antifungals. Surfactin is the least polar lipopeptide in comparison to other antifungals. It is therefore expected that it is more easily adsorbed onto non-polar resins compared to antifungals. Therefore, at low RC/SC ratio, the surfactin is adsorbed to fill the adsorption sites before antifungals are adsorbed, hence the higher IS. At high RC/SC ratio, however, there are enough adsorption sites for adsorption of surfactin and antifungals hence both surfactin and antifungals are adsorbed.

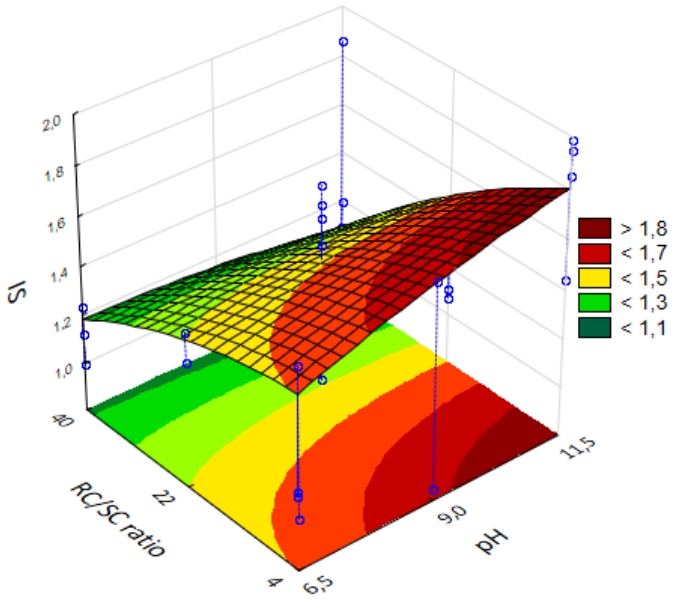

Figure 5-37: Improvement in selectivity (IS) obtained after surfactin adsorption in the absence of methanol at an operating temperature of 25 °C

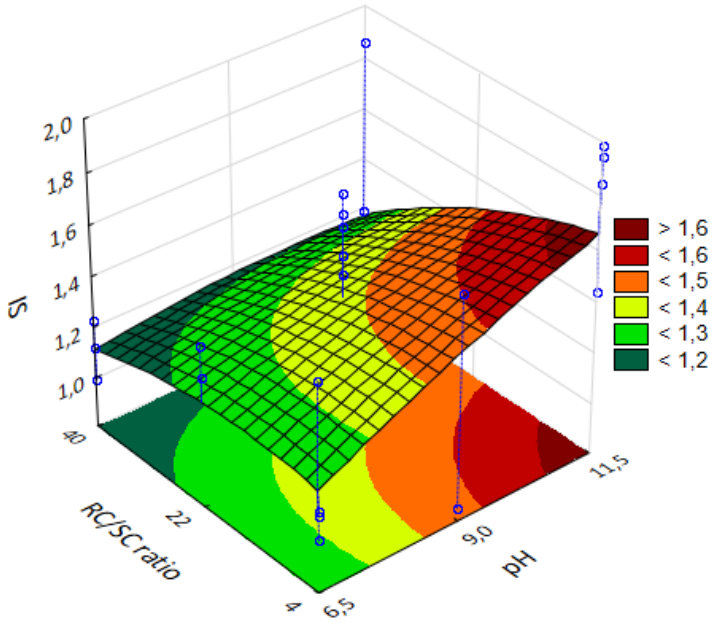

Figure 5-38: Improvement in selectivity (IS) obtained after surfactin adsorption in the absence of methanol at an operating temperature of 35 °C

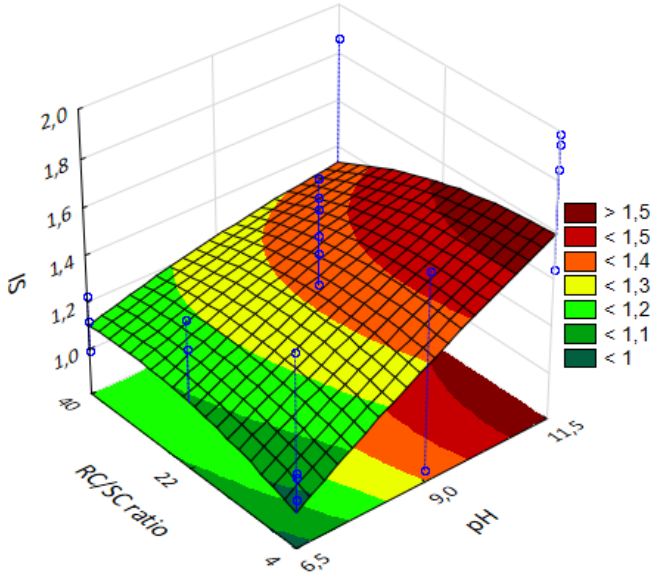

Figure 5-39: Improvement in selectivity (IS) obtained after surfactin adsorption in the absence of methanol at an operating temperature of 45 °C

The IS was seen to be slightly affected by the change in pH. An increase in pH was seen to slightly improve the surfactin selectivity. This behaviour is possibly due to ionization of surfactin and antifungals with increase in pH. According to Wiczling & Kaliszan (2010), an acidic group is generally ionized (deprotonated) at a pH above its pKa, while a basic group is ionized (protonated) at a pH below its pKa. A change in pH can thus result in ionization of the side chains of amino acids present in the peptide moiety of lipopeptides thus varying their adsorption properties. Since surfactin has different amino acid composition to antifungals, variation in pH may affect the IS. The operating temperature was not seen to have any effect on IS at a 95% confidence interval.

5.5.2.2 *Effect of methanol concentration on IS*

To determine the effects of methanol concentration on IS during adsorption, a Pareto chart (Figure 5-40) and an ANOVA table (Table 5-11) were used to display the relative effects of independent variables as well as their interactions on improvement in surfactin selectivity respectively.

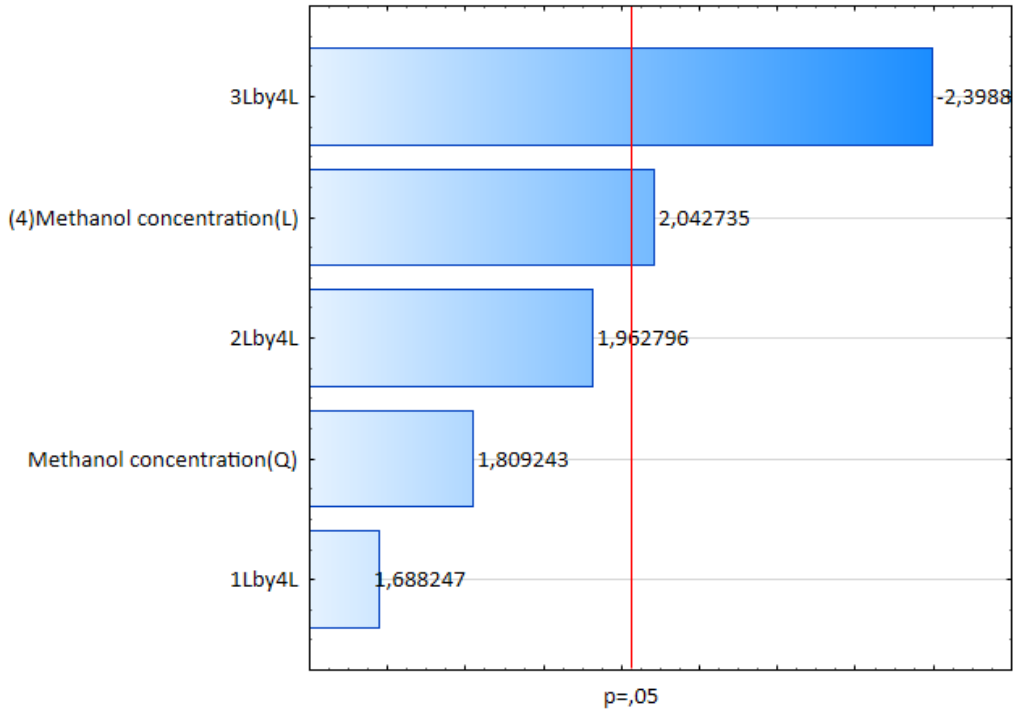

Figure 5-40: Linear (L) and quadratic (Q) effects of methanol concentration (4) as well as effects of interactions of methanol concentration with initial pH (1), operating temperature (2) and resin concentration to surfactin concentration (RC/SC) ratio (3) on improvement in selectivity (IS) at 95% confidence

Table 5-11: ANOVA table showing the linear (L) and quadratic (Q) effects of methanol concentration (4) as well as effects of interactions of methanol concentration with initial pH (1), operating temperature (2) and resin concentration to surfactin concentration (RC/SC) ratio (3) on improvement in selectivity (IS) at 95% confidence. The values in red indicate parameters which had a significant effect on IS at a 95% confidence interval

	Sum of squares	Degrees of freedom	Mean square	p
Methanol concentration (L)	2.49114	1	2.491136	0.046710
Methanol concentration (Q)	1.95419	1	1.954190	0.076812
1L by 4L	1.70155	1	1.701551	0.097990
2L by 4L	2.29998	1	2.299976	0.055604
3L by 4L	3.43548	1	3.435476	0.020465
Error	28.05893	47	0.596998	
Total SS	39.94126	52		

Surface plots of a change in IS with change RC/SC ratio and methanol concentration at different operation temperatures are shown in Figure 5-41 to Figure 5-49.

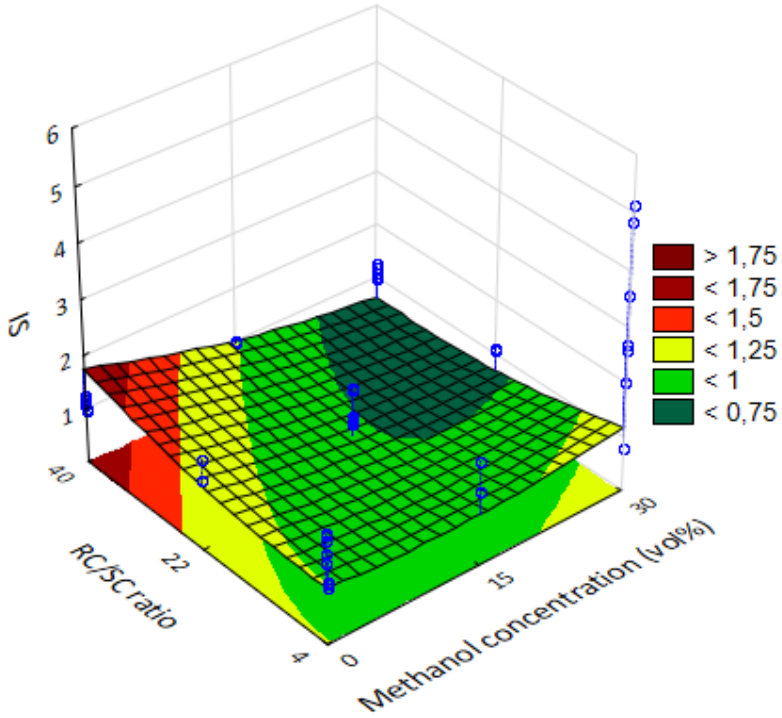

Figure 5-41: IS obtained after surfactin adsorption in the presence of methanol at an operating temperature and initial pH of 25 °C and 6.5 respectively

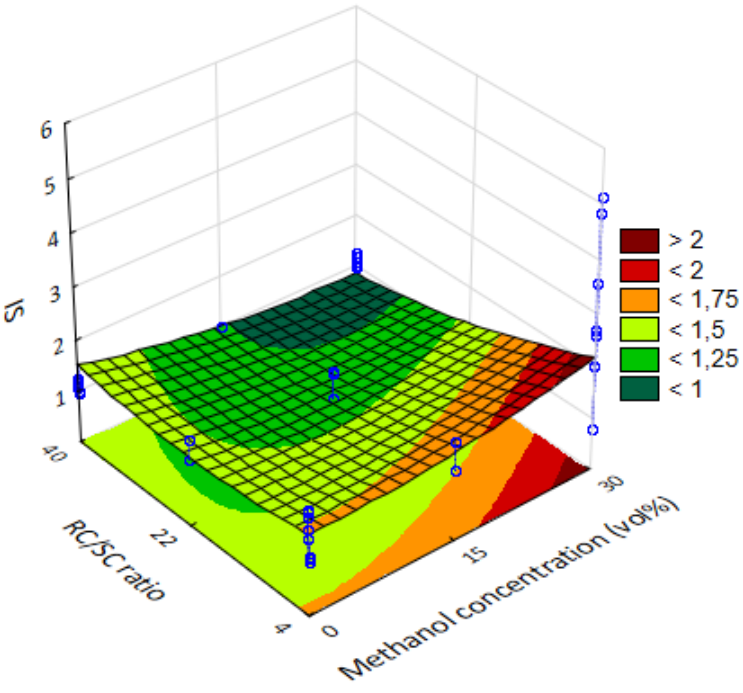

Figure 5-42: Improvement in selectivity (IS) obtained after surfactin adsorption in the presence of methanol at an operating temperature and initial pH of 25 °C and 9 respectively

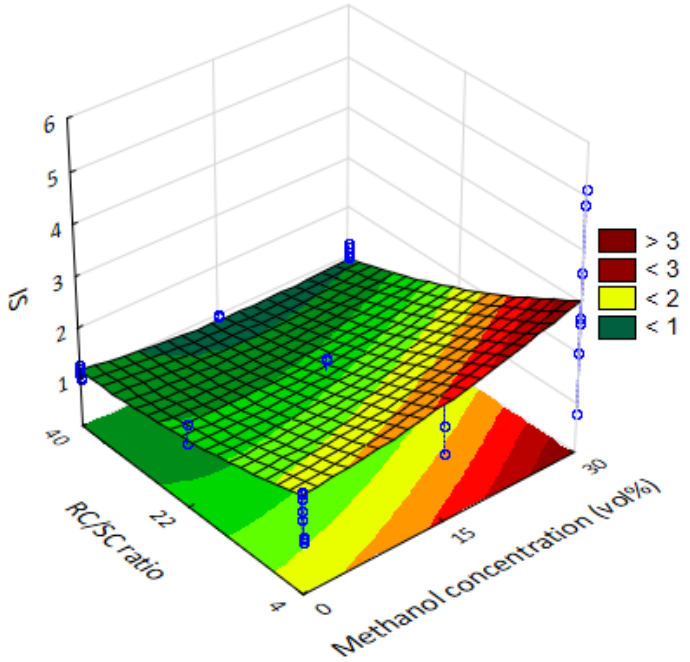

Figure 5-43: Improvement in selectivity (IS) obtained after surfactin adsorption in the presence of methanol at an operating temperature and initial pH of 25 °C and 11.5 respectively

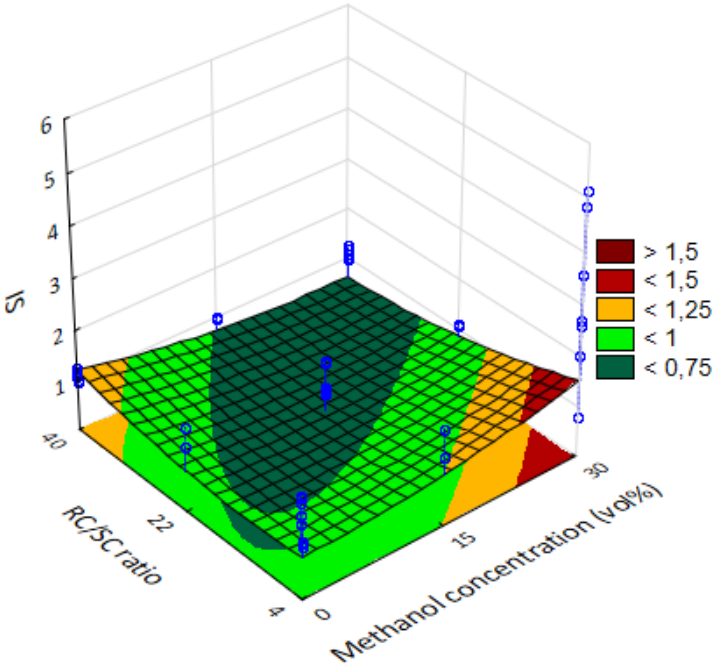

Figure 5-44: Improvement in selectivity (IS) obtained after surfactin adsorption in the presence of methanol at an operating temperature and initial pH of 35 °C and 6.5 respectively

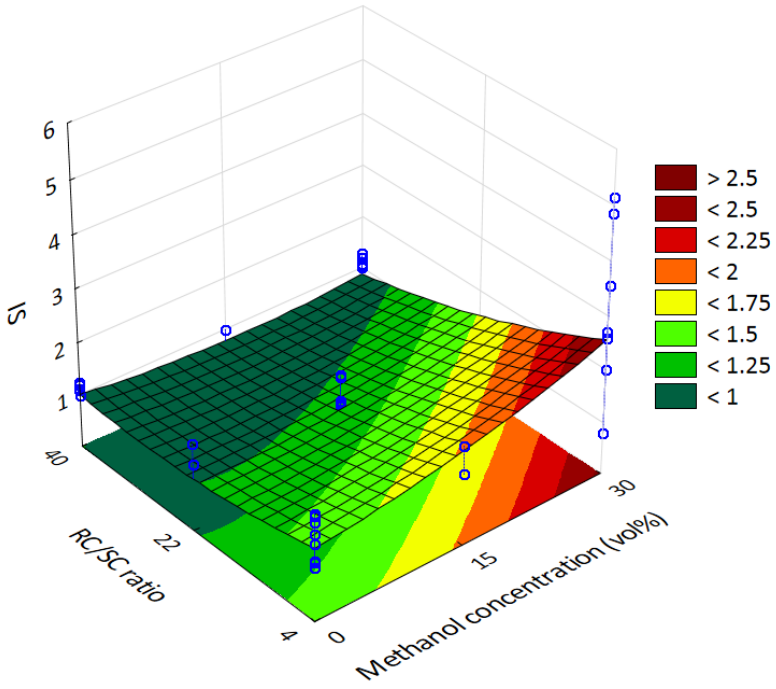

Figure 5-45: Improvement in selectivity (IS) obtained after surfactin adsorption in the presence of methanol at an operating temperature and initial pH of 35 °C and 9 respectively

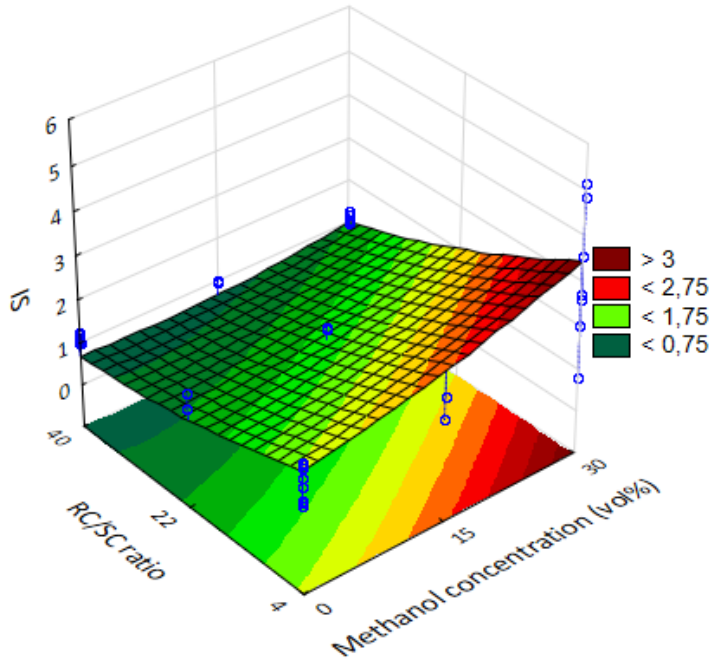

Figure 5-46: Improvement in selectivity (IS) obtained after surfactin adsorption in the presence of methanol at an operating temperature and initial pH of 35 °C and 11.5 respectively

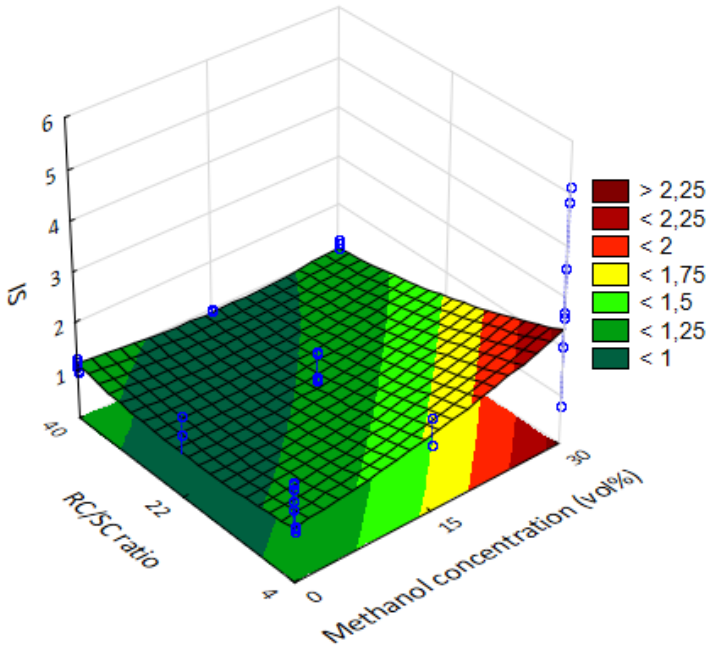

Figure 5-47: Improvement in selectivity (IS) obtained after surfactin adsorption in the presence of methanol at an operating temperature and initial pH of 45 °C and 6.5 respectively

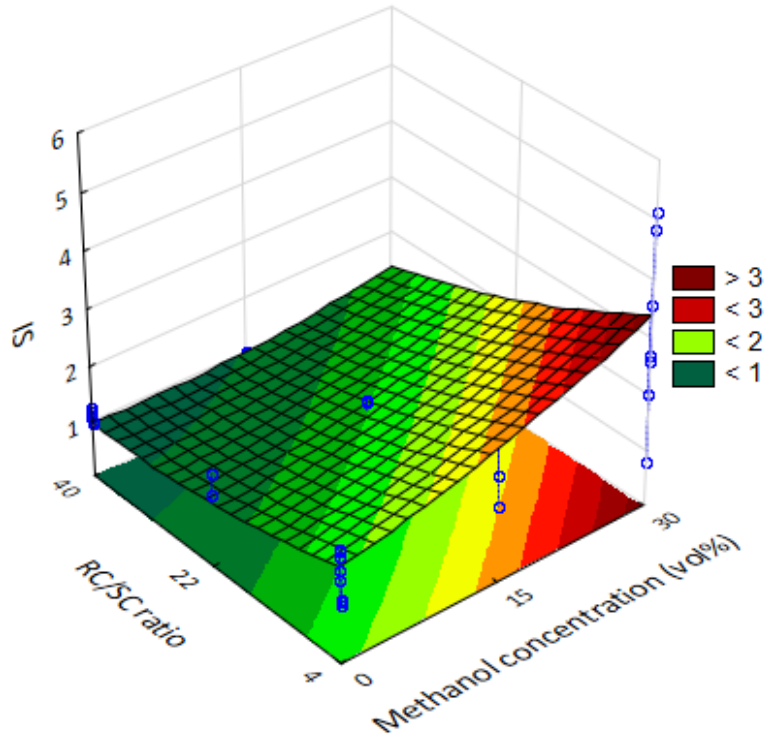

Figure 5-48: Improvement in selectivity (IS) obtained after surfactin adsorption in the presence of methanol at an operating temperature and initial pH of 45 °C and 9 respectively

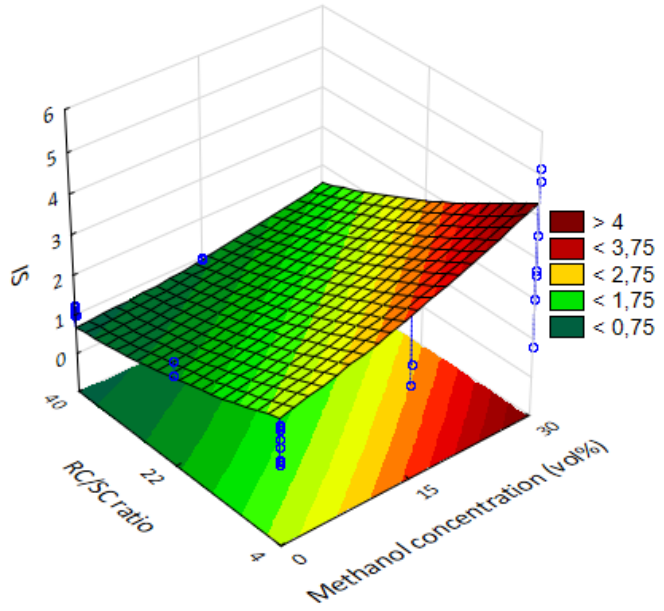

Figure 5-49: Improvement in selectivity (IS) obtained after surfactin adsorption in the presence of methanol at an operating temperature and initial pH of 45 °C and 11.5 respectively

The improvement in selectivity (IS) was seen to improve with increase in methanol concentration (Figure 5-41 to Figure 5-49). The lowest IS was approximately 1 at a methanol concentration of 0% (v/v), while the highest IS was approximately 4 at a methanol concentration of 30% (v/v) (Figure 5-49). The effect of methanol concentration was dependent on the RC/SC ratio. The methanol concentration resulted in a greater IS at low RC/SC ratio compared to high RC/SC ratio. This is because at high RC/SC ratio there are enough adsorption sites for both surfactin and antifungals, while there is selective adsorption at low RC/SC ratio.

The IS was improved possibly due to the ease of adsorption of surfactin compared to antifungals at increased methanol concentrations. The presence of methanol in adsorption liquid affects the polarity of the solution. Based on elution studies by Dhanarajan et al. (2015), it is expected that when organic solvents (methanol or acetone) are added in adsorption liquids (to reduce its polarity), more surfactin will be adsorbed compared to other lipopeptide. However, a high concetration of the organic solvents in water will limit surfactin adsorption, and this favours surfactin desorption.

The pH was seen to have a greater effect on IS when there was methanol in the solution. This is evident in Figure 5-47 to Figure 5-49, where the IS increased from approximately 2 to approximately 4 when the methanol concentration was increased from 0% (v/v) to 30% (v/v) at an RC/SC ratio of 4 g_r/g_s. The

improved effects of pH in the presence of methanol are possibly to similar ionizaion effects disussed in section 5.5.2.1. The ionization however would have a greater effect in IS in the presence of methanol.

5.5.3 Surfactin purity and recovery after purification by adsorption process

To study surfactin purity and recovery after adsorption, surfactin was first adsorbed onto resins as discussed in section 4.5.1. Surfactin adsorption was carried out at the recommended adsorption operating conditions, which were an initial pH, operating temperature, RC/SC ratio and methanol concentration of 11.5, 45 °C, 5 g_r/g_s and a methanol concentration of 30% (v/v) in the adsorption liquid. After adsorption, surfactin was then recovered from the resins by desorption using absolute methanol (section 4.5.2). Like adsorption, desorption was carried out for 24 hours. Desorption was seen to occur as soon as the resins were contacted with the desorption liquid (methanol). In a study by Wang et al. (2010), desorption was carried out for 6 minutes, and up to 100% desorption from resins was achieved. In this study, desorption was carried out for 24 hours to assure surfactin desorption was complete as desorption was carried in columns in the study by Wang et al. (2010) while it was carried out in shake flasks in this study.

The surfactin recovery was calculated using Equation 4-9, while the surfactin purity was calculated using Equation 4-10. The surfactin purity and recovery were calculated using surfactin concentrations determined by TLC analysis. TLC analysis was used due to its simplicity and quickness in comparison to HPLC analysis. The surfactin recovery and purity after surfactin purification by adsorption were 91±8% and 58±6% respectively. The surfactin recovery was greater than that in the study by Dhanarajan et al. (2015) (89.4%), while the surfactin purity was lower than that in the study by Dhanarajan et al. (2015) (91.6%). The purity after adsorption was lower than the maximum purity achieved by solvent extraction (80%).

The purity could be improved by resin washing, using dynamic rather than static adsorption, gradient elution and carrying out the solvent extraction and adsorption purification steps sequentially. Resins can be washed using deionised water after adsorption. Some of the adsorption liquid, which contains impurities, may be trapped in the resins when the resins are recovered before surfactin desorption. Since surfactin is harder to elute than other impurities, methanol can also be added to the deionised water used for resin washing without affecting surfactin recovery. The methanol concentration would, however, have to be optimised to see the best concentration for resin washing. A suitable range for optimisation of methanol in the adsorption liquid would be between 0% and 80% (v/v).

Adsorption can also be improved by carrying out the adsorption and desorption steps in a column (dynamic adsorption) rather than in shake flasks (static adsorption). Wang et al. (2010) has shown that dynamic adsorption improves % SA compared to static adsorption. Gradient elution, which is used to separate lipopeptide families, can also be done to elute the adsorbed surfactin in column operation. Gradient elution can be carried out using organic solvents (such as methanol and acetone) in water. Organic solvents can be tested to determine which solvent is best for elution, and their concentration in water would have to be optimised. A recommended organic solvent concentration range in water would be between 0% (v/v) and 90% (v/v). Another factor which would need to be optimised for elution studies would be the pH of the liquid used for elution.

Lastly, the purity can be improved by combining the adsorption with the solvent extraction step. In this case, the precipitate from the acid precipitation step would be purified by methanol extraction. Instead of evaporating the methanol to recover the surfactin product, the methanol-surfactin mixture would be diluted with water to a methanol concentration of 30% (v/v), with a pH of 11.5. This mixture would then be purified by surfactin adsorption at optimum operating temperature and RC/SC ratio.

5.5.4 Batch adsorption kinetics

Batch kinetics were studied to determine if the addition of methanol in the adsorption liquid improves the surfactin adsorption rate. This was done by comparing the time it takes to reach equilibrium when adsorption was carried out with 0% (v/v) methanol and 30% (v/v) methanol in the adsorption liquid. Adsorption rates were also determined using the pseudo first-order and pseudo second-order models (shown in Equation 4-15 and Equation 4-16 respectively). Lastly, batch kinetics were studied to determine if the intraparticle diffusion was rate limiting.

To determine the time it takes to reach equilibrium, batch kinetics were studied by observing the change in surfactin adsorption capacity at different time intervals during adsorption (q_t) (determined according to Equation 4-14) with time during adsorption (Figure 5-50). Surfactin adsorption capacity was initially increased rapidly, before increasing slower and slower until equilibrium was reached, where the adsorption capacity did not increase with time (Figure 5-50). This was possibly due to the presence of more vacant surface sites available for adsorption during the initial stage, and the vacant sites decrease as more surfactin is adsorbed making further adsorption more difficult. Additionally, surface sites could be more difficult to occupy in later stages should there be repulsive forces between the adsorbed surfactin molecules with the molecules on the bulk phase.

The equilibrium was achieved much quicker (after 5.5 h) when methanol was added onto the adsorption liquid compared to when there was no methanol in the adsorption liquid (where equilibrium was reached after 25 h). Equilibrium was therefore reached approximately 5 times quicker when there was 30% (v/v) methanol in the adsorption liquid compared to when there was 0% (v/v) methanol. It took longer to reach equilibrium for surfactin adsorption at 0% (v/v) methanol in this study; compared to the study by Montastruc et al. (2007), where equilibrium was reached after 15 h. In the study by Montastruc et al. (2007), adsorption was carried out at using activated carbon at a concentration of 25 g/L, initial surfactin concentration of approximately 0.55 g_s/L, and temperature of 37 °C. Although equilibrium was not reached as later than in the study by Montastruc et al. (2007) at 0% (v/v) methanol, it was reached approximately three times faster in the presence of methanol. Methanol therefore improves the rate at which equilibrium is reached.

It should be noted that while the kinetics studies are sufficient to provide an indication of the effect of influence of the presence of methanol on surfactin adsorption, they do not accurately determine the time needed to reach equilibrium. Adsorption in optimisation studies is expected to have occurred more rapidly compared to kinetics studies, since more adsorption liquid (30 mL) was used per run to compensate for volume loss as samples are taken for surfactin analysis during adsorption. Higher volumes are likely to be more affected by mass transfer limitations during adsorption.

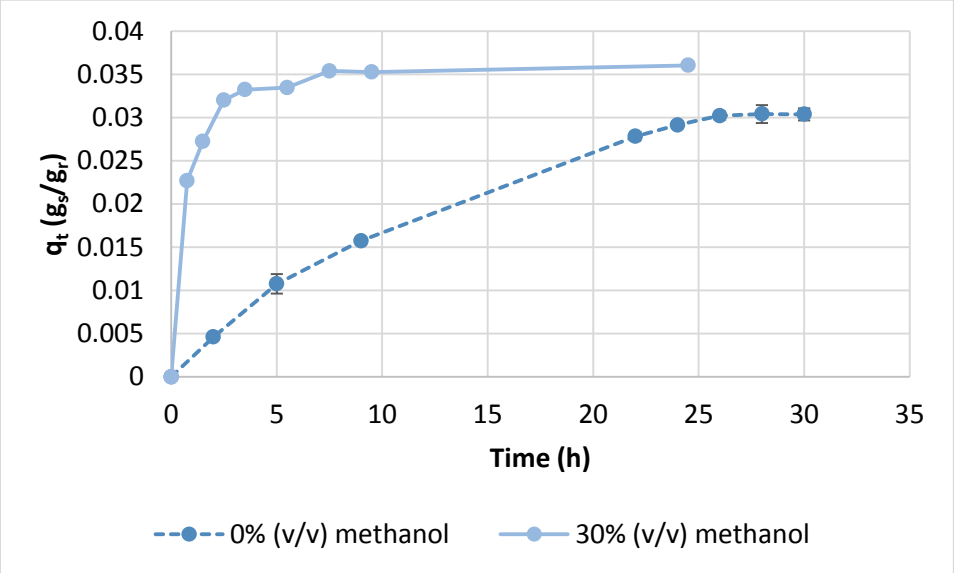

Figure 5-50: Surfactin adsorption kinetics at an initial pH, operating temperature and RC/SC ratio of 11.5, 45 °C and 27 g_r/g_s respectively. The error bars indicate the standard deviation of two replicates, and some error bars do not show on the graph as they are too small. q_t represents the adsorption capacity at a particular time interval.

To determine the adsorption rates, surfactin adsorption was modelled using the pseudo first-order and second-order models (Figure 5-51 and Table 5-12 respectively), which are generally used in literature to determine for modelling adsorption kinetics (Simonin, 2016). The pseudo first-order and second-order models were compared to determine which model best fits the experimental data, which would then be used to determine the surfactin adsorption rates.

The pseudo second-order model was better at modelling adsorption kinetics as it had R^2 values of approximately 0.99 for both adsorption with 0% (v/v) methanol and 30% (v/v) methanol. The pseudo second-order model also accurately predicted q_e for kinetics at 30% (v/v) methanol as the predicted q_e (0.0373 g_s/g_r) was similar to the observed q_e (0.036 g_s/g_r) (Figure 5-50). The adsorption rate of surfactin at 30% (v/v) methanol in the adsorption liquid was therefore 54.7 $g_s/g_r/h$, determined by the pseudo second-order model (Table 5-12).

Although the pseudo second-order model achieved an R^2 value of 0.999 when modelling adsorption kinetics at 0% (v/v) methanol, it was not able to accurately determine q_e at 0% (v/v) methanol. The predicted q_e at 0% (v/v) methanol (0.0547 g_s/g_r) was much larger (1.82 fold) than the observed q_e (0.03 g_s/g_r). The adsorption kinetics at 0% (v/v) methanol were therefore appropriately modelled by the pseudo first-order model, where an R^2 value of 0.983, and a q_e value of 0.03 g_s/g_r was achieved. The surfactin adsorption rate at 0% (v/v) methanol in the adsorption liquid was therefore 0.001 h^{-1}, determined by the pseudo first-order model (Table 5-12).

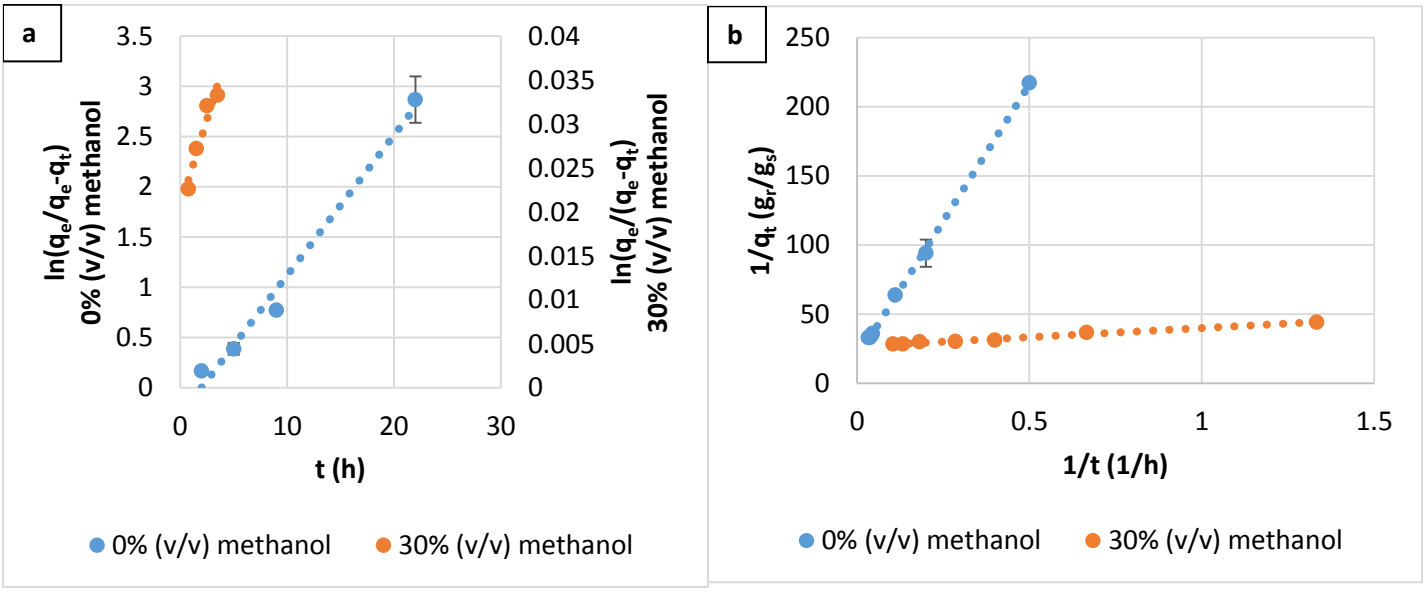

Figure 5-51: Modelling of adsorption kinetics using (a) the pseudo first-order and (b) the pseudo second-order models at an initial pH, operating temperature and RC/SC ratio of 11.5, 45 °C and 27 g_r/g_s respectively. The error bars indicate the standard

deviation of two replicates, and some error bars do not show on the graph as they are too small. q_e and q_t represent the adsorption capacity at equilibrium as well as at different time intervals during adsorption respectively, while t represents time.

Table 5-12: K and R^2 values obtained from modelling the experimental data using the pseudo first-order and pseudo-second order mode, where q_e represent the predicted equilibrium adsorption capacity while k1 and k2 represent the rate constants of the pseudo first-order model and pseudo second-order model respectively.

Methanol concentration	**0% methanol**	**30% methanol**
Pseudo first-order model		
k_1 (1/h)	0.001	0.021
q_e	0.0300	1.01
R^2	0.983	0.928
Pseudo second order-model		
k_2 [g_s/g_r/h]	0.844	54.7
q_e	0.0547	0.0373
R^2	0.999	0.986

The pseudo first-order and second-order models were used assuming that adsorption is controlled by the direct adsorption surfactin onto resins. The theoretical of the pseudo second-order ground is actually based on fundamental theories of surface reactions (Plazinski, et al., 2013). However, adsorption depends on other mechanisms (transport in the bulk solution, diffusion across the film surrounding the adsorbent particles and diffusion in the pores of the sorbent) in addition to direct adsorption on the solid surface (Fierro, et al., 2008). Intraparticle diffusion can be studied using the intra-particle diffusion model. The intraparticle diffusion model also gives useful information on the relative thickness of the boundary later, hence information of boundary layer diffusion (Fierro, et al., 2008).

5.5.5 Batch adsorption isotherms

Batch Isotherms were done to compare the effect of a change in the initial surfactin concentration on q_e (determined according to Equation 4-17) and % SA (determined according to Equation 4-11). Batch optimisation studies were carried out using % SA as the dependent variable, rather than q_e. This is

because high q_e can be associated with low recoveries. Figure 5-52 shows that q_e continues increasing while % SA, which is the percentage of surfactin in the adsorption liquid adsorbed onto resins, decreases. This means that q_e increased with decrease in surfactin recovery, thus q_e was not suitable for optimisation studies as maximum recoveries were desirable in this study. The adsorption capacity increases with increase in initial surfactin concentration before reaching a saturation plateau, after which the adsorption remains constant with increase in initial surfactin concentration as in the study by Dhanarajan et al. (2015).

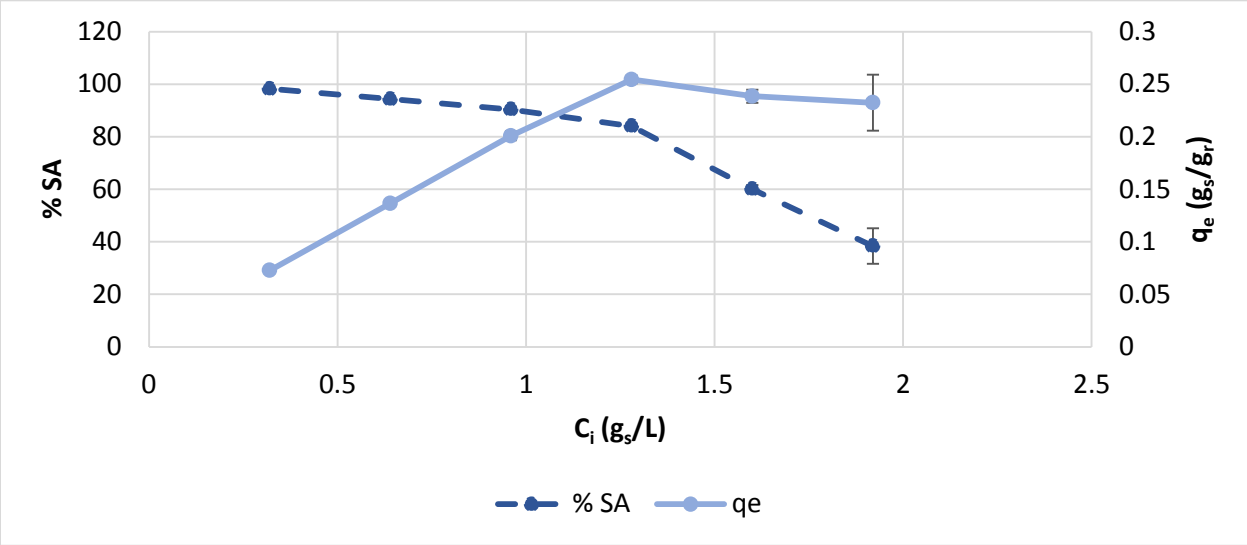

Figure 5-52: % SA and q_e obtained at an initial pH, operating temperature, resin concentration and methanol concentration of 11.5, 45 °C, 4 g_r/L and 30% (v/v) respectively. The error bars indicate the standard deviation of two replicates, and some error bars do not show on the graph as they are too small. C_i is the initial surfactin concentration.

Batch isotherms were also carried out to determine a suitable model for surfactin adsorption. The Langmuir model (which describes monolayer adsorption) and the Freundlich model (which describes both monolayer and multilayer adsorption) were tested. The Langmuir and Freundlich model are shown in Equation 4-18 and Equation 4-19 respectively, and they were used to model the surfactin adsorption isotherms as shown in Figure 5-53a and Figure 5-53b respectively. The model parameters and correlation factors are shown in Table 5-13. The Freundlich model (R^2> 0.99 for both adsorption at 0% methanol and 30% methanol) was better at modelling surfactin adsorption isotherms in comparison to the Langmuir model (R^2< 0.99 for both adsorption at 0% methanol and 30% methanol). The Langmuir model can also be used to model surfactin adsorption as the R^2 is approximately 0.98 for both, which is close to 0.99.

Since the Freundlich model was the best for surfactin adsorption, surfactin adsorption is likely a multilayer adsorption. The Freundlich constant (K_F) and the theoretical maximum surfactin adsorption capacity (q_m), which indicate the adsorption capacity, were higher at 30% (v/v) methanol in comparison to 0% (v/v) methanol in the adsorption liquid. The presence of methanol in adsorption liquids thus improves the surfactin adsorption capacities. The addition of methanol was therefore seen to improve the adsorption capacity. The Langmuir constant (K_L) was also higher in the presence of methanol indicating that an increase in the affinity of binding sites for surfactin (Wu, 2007), hence a higher stability of the combination between surfactin and resins (Zhang, et al., 2008).

The driving force of surfactin adsorption is described by 1/n, where the magnitude of the driving force is inversely proportional to 1/n (Ming, et al., 2006). There was therefore a lower driving force for adsorption at 30% (v/v) methanol in the adsorption liquid (1/n=0.573) compared to when there was no methanol in the adsorption liquid (1/n=0.404). Since methanol disperses surfactin micelles, it was expected that this would allow surfactin molecules to more easily adsorb with HP-20 resin active sites resulting in a relatively higher driving force for adsorption at 30% (v/v) methanol in the adsorption liquid. However, since high methanol concentration favours surfactin desorption, it is not surprising that the presence of methanol in the adsorption liquid resulted in reduced driving force for adsorption.

According to Jiang et al. (2002), if the 1/n is greater than 10, adsorption can be defined as chemical, and physical if 1/n is below 10. Surfactin adsorption was therefore physical in this study. Thermodynamic analyses would, however, be more appropriate to determine if surfactin adsorption was a physisorption or a chemisorption process. These were however not done as they were beyond the scope of this study, but they are recommended for future studies.

No study on surfactin adsorption isotherms has been carried out in literature. There is, however, a study on lipopeptide adsorption isotherms by Dhanarajan et al. (2015). In the study by Dhanarajan et al. (2015), total lipopeptide adsorption of surfactin, iturin and fengycin from solubilised acid precipitates of *B. megaterium* cultures was studied using HP-20 resinis at 0% (v/v) methanol in adsorption liquids. Dhanarajan et al. (2015) found that the Redlich–Peterson model was best for modelling surfactin adsorption isotherms (R^2=0.998) followed by the Langmuir model (R^2=0.987). Unlike the finding of this study, Dhanarajan et al. (2015) found that the Freundlich model was poor of modelling surfactin adsorption isotherms (R^2=0.948).

K_F and q_m, which were 0.257 $g_s/g_r(L/g_s)^{1/n}$ and 0.25 g_s/g_r respectively, were higher in the study by Dhanarajan et al. (2015) than those in this study, indicating higher adsorption capacities. This is possibly due to that only surfactin was considered in this study, thus less mass is adsorbed per unit of resin compared to when total lipopeptide mass is considered. K_L (11.72 L/g) was higher while 1/n (0.33) was lower in the study by Dhanarajan et al. (2015) compared to this study, indicating a higher affinity of binding sites for lipopeptides and a better driving force for adsorption in the study by Dhanarajan et al. (2015).

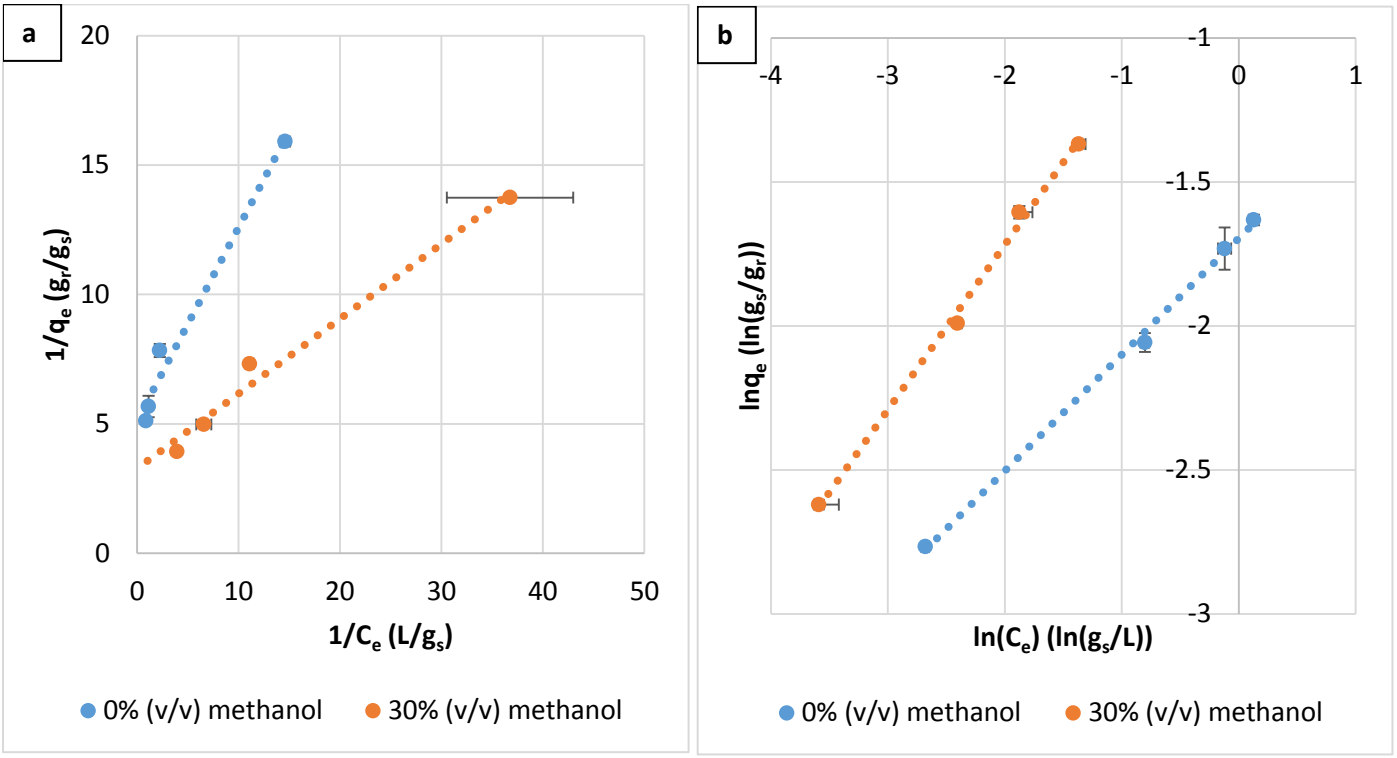

Figure 5-53: Modelling adsorption isotherms using the Langmuir model (a) and the Freundlich model (b) for adsorption carried out at an initial pH, operating temperature, methanol concentration and resin concentration of 11.5, 45 °C, 30% (v/v) and 4 g_r/L respectively. The error bars indicate the standard deviation of two replicates, and some error bars do not show on the graph as they are too small. q_e represents the equilibrium adsorption capacity, while C_e represent the equilibrium surfactin concentration.

Table 5-13: Model parameters and R^2 values obtained from modelling the experimental data using the Langmuir and Freundlich models. K_L and K_F are the Langmuir and Freundlich constants respectively, q_m is the theoretical maximum surfactin adsorption capacity and 1/n is an empirical constant.

Langmuir model		
Methanol concentration	0% (v/v)	30% (v/v)
K_L (L/g_s)	6.85	11.3
q_m (g_s/g_r)	0.195	0.306
R^2	0.978	0.983
Freundlich model		
Methanol concentration	0% (v/v)	30% (v/v)
K_F [$(g_s/g_r)(L/g_s)^{1/n}$]	0.183	0.564
1/n	0.4035	0.573
R^2	0.998	0.996

5.6 Repeatability

This section discusses the repeatability of the experiments done in this study. The reproducibility was calculated according to Equation 5-5, where σ and χ are the standard deviation and average values obtained from experimental runs.

$$Repeatibility = 100\left(1 - \frac{\sigma}{\chi}\right) \qquad \textit{Equation 5-5}$$

The repeatability of experiments for analysis of concentrations of products and selectivity from surfactin production was determined according to Equation 5-5 (Figure 5-54). The repeatability in surfactin production experiments was tested between 29 and 66 hours during surfactin production. The experiments had a high repeatability (≥80%), hence they were repeatable.

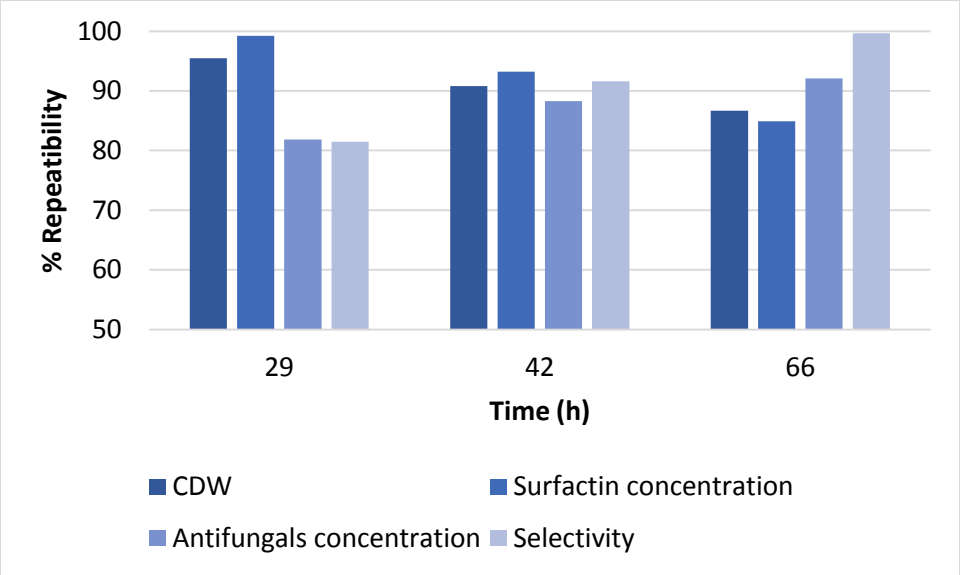

Figure 5-54: Repeatability of experiments for analysis of concentrations of products and selectivity during surfactin production

The repeatability of experiments for analysis of concentrations of nutrients (glucose, ammonium and nitrate) during surfactin production was also determined according to Equation 5-5 (Figure 5-55). The repeatability was tested between 0 and 29 during surfactin production. The experiments had a high repeatability (≥80%), hence they were repeatable.

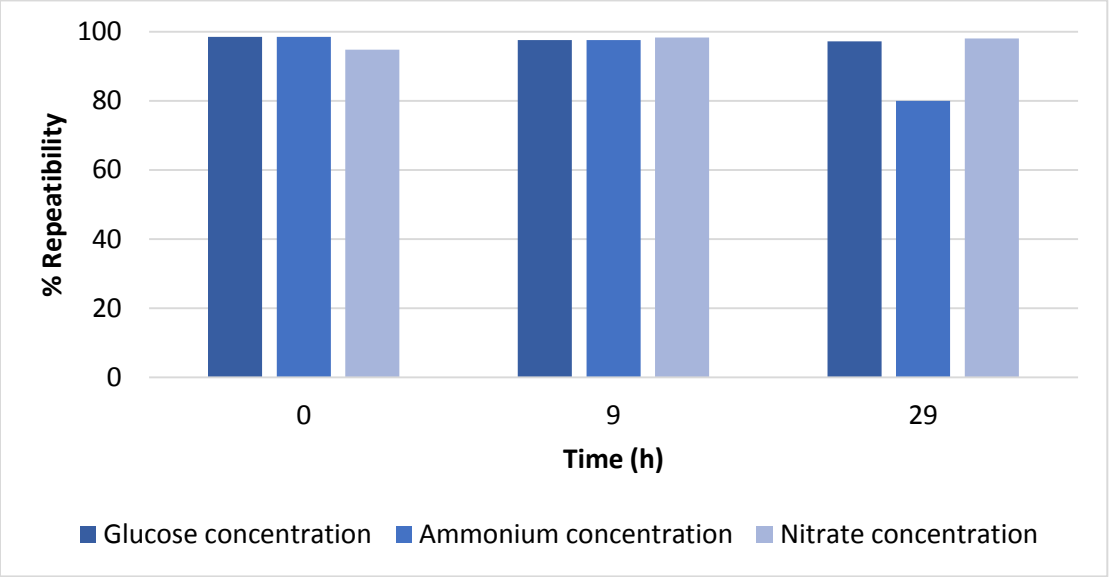

Figure 5-55: Repeatability of experiments for analysis of concentrations of nutrients during surfactin production

The repeatability of the experiments in development of the analytical technique was not discussed in this section, as the reproducibility of TLC analysis was discussed during validation of the TLC analytical technique (section 5.2.2.4.1). This reproducibility was determined through intraday error analysis, and TLC analysis was found to give reproducible results as it gave high accuracy and precision (98% and ≥84% respectively) when analysis was carried out in different days.

The repeatability of experiments for determining recoveries, purities, selectivity and improvement in selectivity was determined according to Equation 5-5 (Figure 5-56). The repeatability of these experiments was tested at pH 2 and pH 4, and a high repeatability (>85%) was obtained. Acid precipitation experiments were therefore repeatable.

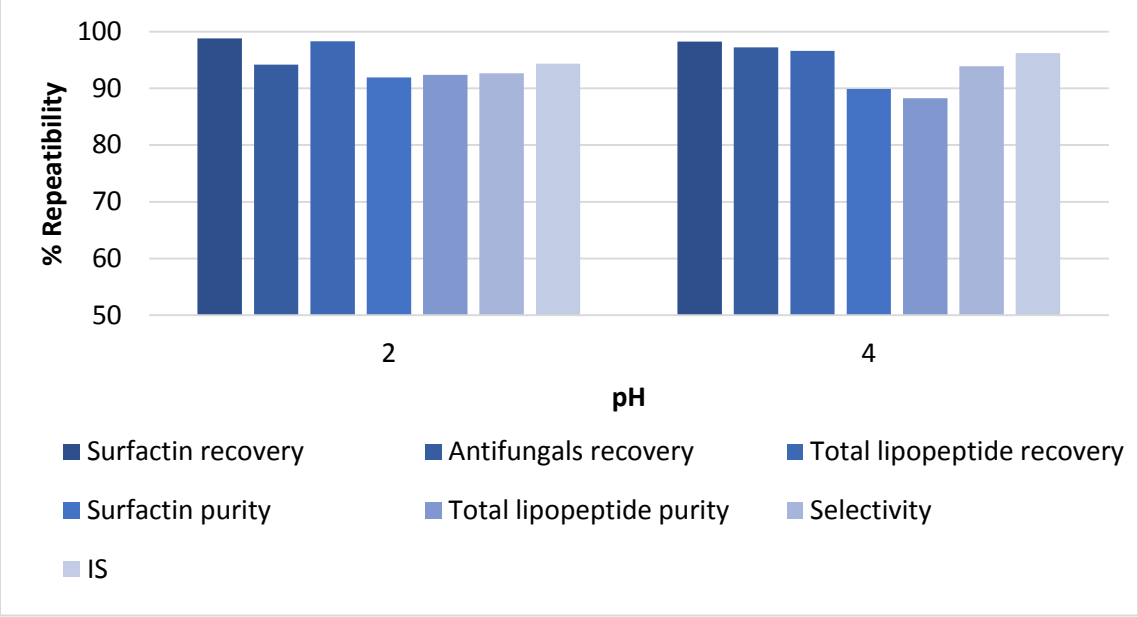

Figure 5-56: Repeatability of experiments for determining recoveries, purities, selectivity and IS after surfactin purification by acid precipitation

The repeatability of experiments for determining surfactin recovery and purity after surfactin purification by solvent extraction was determined according to Equation 5-5 (Figure 5-57) for all solvents used in the extraction experiments. A high repeatability (>80%) was obtained for extraction using methanol, *i*-propanol, C/M (1:1), C/M (2:1), acetone, chloroform, ethyl acetate and MTBE as solvents. The experiments for determining the purity and recovery of extraction using acetonitrile were 78% (which is close to 80%). Purity experiments for extraction using *n*-hexane showed a high repeatability (87%), while recoveries showed a repeatability of 75%. Extraction experiments using petroleum ether showed a low repeatability (up to 45%). This low repeatability could be due to low surfactin

concentrations in the solvents after extraction and due to the high volatility of hexane and petroleum ether. Petroleum ether has the lowest boiling point (36 °C) among the solvents used in this study (Table 8-1 in the appendix section).

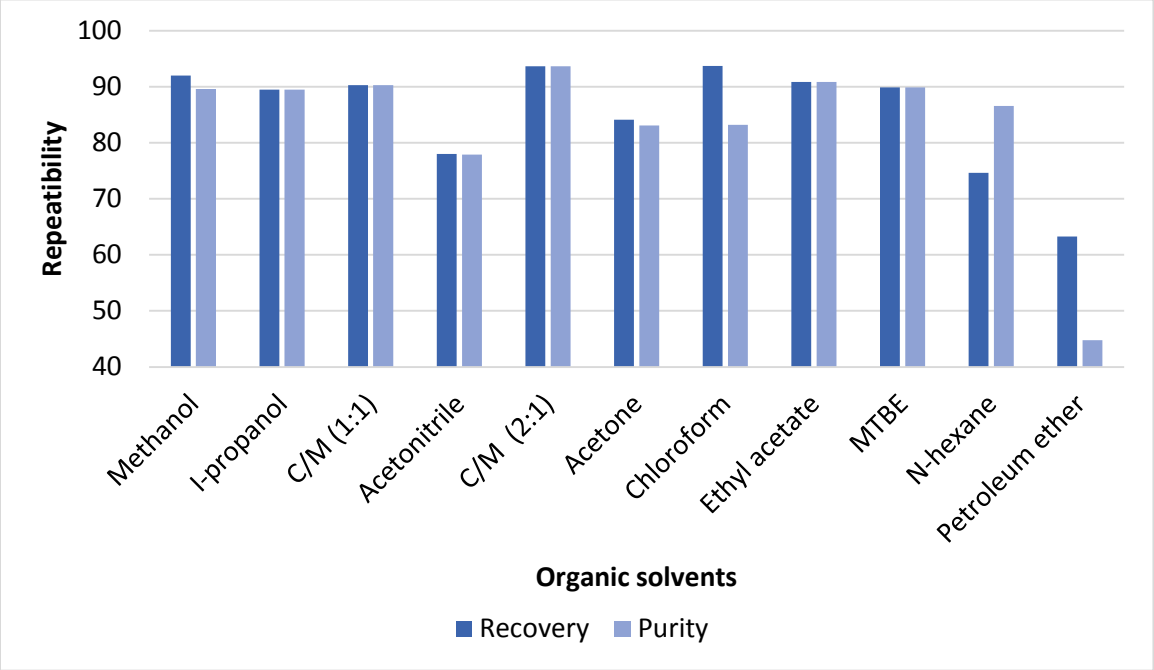

Figure 5-57: Repeatability of experiments for determining surfactin recovery and purity after surfactin purification by solvent extraction

The repeatability of experiments conducted in surfactin adsorption studies was determined according to Equation 5-5 (Figure 5-58). The repeatability of experiments for determining % SA, q_e and IS were tested at an initial pH, operating temperature, RC/SC ratio and methanol concentration of 6.5, 35 °C, 11 g_r/g_s and 30% (v/v) respectively. The repeatability of experiments for determining surfactin purity and recovery was tested at an initial pH, operating temperature, RC/SC ratio and methanol concentration of 11.5, 45 °C, 5 g_r/g_s and 30% (v/v) respectively. The repeatability of the experiments for determining % SA, q_e and IS were approximately 98%, while the repeatability of the experiments for determining recovery and purity were approximately 90%. Adsorption experiments were therefore repeatable.

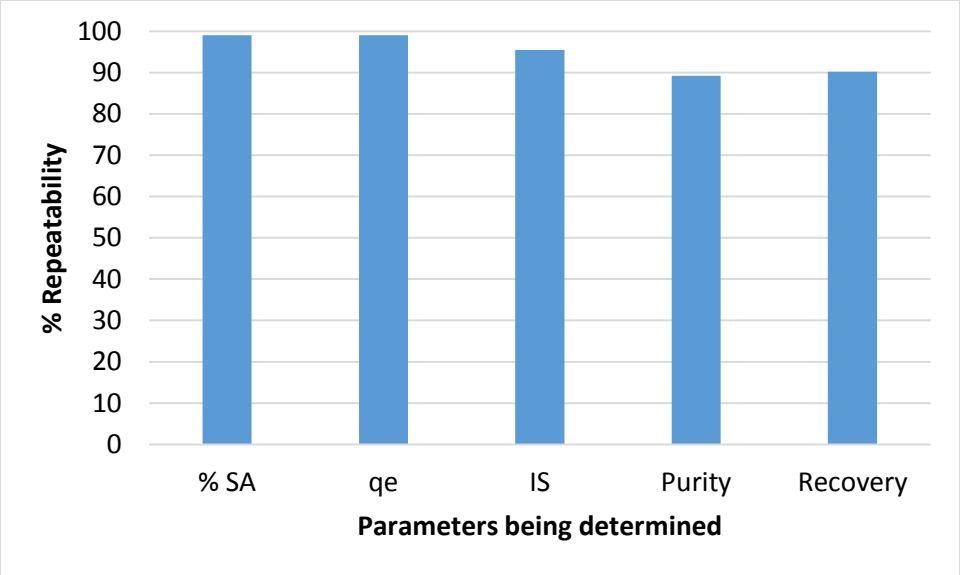

Figure 5-58: Repeatability of experiments for determining % SA, q_e, IS as well as surfactin purity and recovery. Repeatability of experiments for determining % SA, q_e and IS were tested at an initial pH, operating temperature, RC/SC ratio and methanol concentration of 6.5, 35 °C, 11 g_n/g_s and 30% (v/v) respectively, while the repeatability of experiments for determining surfactin purity and recovery was tested at an initial pH, operating temperature, RC/SC ratio and methanol concentration of 11.5, 45 °C, 5 g_n/g_s and 30% (v/v) respectively.

6 Conclusions and recommendations

This section provides the conclusions of this study as well as recommendations for future studies based on the results and discussion (chapter 5).

6.1 Conclusions

The production and purification of surfactin is necessary due to the potential effectiveness of surfactin against organisms causing TB. The acid precipitation, solvent extraction and adsorption purification techniques were proposed for surfactin purification from *B. subtilis* cultures. Prior to surfactin purification studies, surfactin was produced and a technique for quantification of surfactin concentration was developed. Surfactin was successfully produced batch-wise using *B. subtilis* to a concentration of 1109 mg/L.

The TLC analytical technique was successfully developed and validated for the quantification of surfactin concentration. A linear relationship was observed between surfactin concentration and the area formed by surfactin bands on TLC plates after analysis between surfactin concentrations of 0.5 g/L and 3 g/L. The TLC analytical technique was suitable for surfactin analysis when surfactin was dissolved in different solvents and when surfactin was spotted by multiple spotting on TLC plates to an accuracy and precision of up to 98% and 95% respectively. Based on intraday error analysis, surfactin gave reproducible results as a high accuracy and precision (98% and ≥84% respectively) were obtained when analysis was carried out in different days (two weeks apart). TLC analysis was also found to be specific for surfactin analysis, and selective for surfactin analysis in the presence of other lipopeptides (fengycin and iturin).

TLC analysis also allowed the qualitative analysis of impurities with lipid and peptide parts in *B. subtilis* cultures, which are the major impurities in these cultures. Impurities with lipid parts were determined using primuline as a detector while impurities with peptide parts could be determined using ninhydrin as a detector.

After surfactin production and development of the TLC analytical technique, surfactin was successfully recovered from cell-free supernatants by acid precipitation, with a purity and recovery of 43% and 97% respectively. Based on literature, surfactin recoveries greater than 97% were expected, with a total lipopeptide purity of approximately 55%. The surfactin recoveries in this study (97%) were therefore comparable to the literature values, while the total lipopeptide purity (50%) was marginally lower. This

is possibly due to a higher than expected concentration of impurities precipitating with surfactin during acid precipitation.

The surfactin purity and recovery obtained after surfactin purification by acid precipitation were seen to be independent of operating pH for operating pHs lower than 4. Surfactin recovery by acid precipitation reported in literature has been carried out at pH 4 and pH 2. However, no study has been done to optimise the operating pH between these two pH (pH 2 and pH 4). In this study, surfactin recovery by acid precipitation was therefore examined in the range between pH 2 and pH 4, and the surfactin recovery and purity were as consistent with pH (at 97% and 43% respectively) in this range. An operating pH of 4 was recommended as the operating pH as it requires less acid to achieve the precipitation, and the precipitates formed require less sodium hydroxide to re-solubilise.

Like the purity and recovery, the surfactin selectivity was found to be independent of operating pH for operating pHs below 4 during surfactin purification by acid precipitation. Surfactin selectivity after acid precipitation was found to be 5.7 g_s/g_a, which was comparable to the surfactin selectivity in supernatants after production (5.5 g_s/g_a). This was in line with the hypothesis which stated that surfactin selectivity does not alter after surfactin purification by acid precipitation

The precipitates obtained after surfactin purification by acid precipitation were further purified by solvent extraction. Solvent extraction successfully purified surfactin in acid precipitates, and a maximum surfactin recovery and purity of 100% and 80% respectively was obtained. The highest surfactin recovery and purity was achieved when MTBE was used as a solvent. MTBE was thus the best solvent for solvent extraction as it gave the highest surfactin recovery and purity. The high surfactin purity is due the high selectivity of MTBE for surfactin extraction in the presence of impurities. In comparison to surfactin purification by adsorption, surfactin purification by solvent extraction is superior as it gave a higher surfactin recovery and purity.

Maximum surfactin recoveries were obtained when polar solvents were used for solvent extraction. The most polar solvent (methanol) achieved a 100% surfactin recovery. The non-polar solvents *n*-hexane and petroleum ether achieved the lowest surfactin recoveries (16% and 8% respectively). This was in line with our hypothesis which stated that polar solvents recover more surfactin in comparison to non-polar solvents.

In addition to high recoveries, maximum surfactin purities were also obtained when polar solvents were used for surfactin purification by solvent extraction. The least polar solvents, *n*-hexane and petroleum

ether, achieved the lowest surfactin purities (30%). This was not in line with our hypothesis as it was expected that non-polar solvents would give the highest surfactin purities. The major impurity in non-polar solvents after solvent extraction was expected to be lipids. The low purity in non-polar solvents was possibly due to a higher recovery of lipid impurities into the solvents during solvent extraction in comparison to surfactin.

Besides purification by solvent extraction, the precipitates from acid precipitation were also further purified by adsorption. Surfactin was successfully purified by adsorption, with a recovery and purity of 91% and 58% respectively. Based on literature, it was expected that surfactin precipitates from acid precipitation could be purified by adsorption with a recovery and purity of 90% and 80% respectively. The low purities in surfactin adsorption means that more impurities were adsorbed than expected. This is possibly because static rather than dynamic adsorption was used in this study. Additionally, resin washing and gradient elution were not done in this study.

During surfactin purification adsorption, the surfactin selectivity was improved, which was in line with the hypothesis of this study. The highest improvement in selectivity was 4. The improvement in selectivity improved with an increase in methanol concentration and initial pH, while it decreased with an increase in RC/SC ratio. The highest improvement in selectivity was observed at a methanol concentration, initial pH and RC/SC ratio of 30% (v/v), 11.5 and 4 g_r/g_s.

Since it was hypothesised that surfactin adsorption is dependent on the operating temperature as well as the initial pH, RC/SC ratio and methanol concentration in the adsorption liquid, the effect and interactions of these independent variables was determined using a face-centred central factorial design. The factorial design was successful in determining the effects and interactions of the independent variables on surfactin adsorption. The surface design, however, had a limitation in quantifying the effects of RC/SC ratio on surfactin adsorption as it uses a quadratic model to model the change in surfactin adsorption with RC/SC ratio, while this change would be appropriately modelled using the Langmuir of Freundlich models. Surfactin adsorption isotherms studies showed that the Freundlich model was best for modelling surfactin adsorption.

Since the methanol bases adsorption liquids has never been studied before, adsorption was optimised separately in adsorption liquids with methanol and adsorption liquids without methanol for comparison. In the absence of methanol, an increase in operating temperature was seen to improve surfactin adsorption. At low RC/SC ratios (4 g_r/g_s), an increase in temperature from 25 °C to 45 °C improved

surfactin adsorption from approximately 25% to 43%. This was expected as some studies in literature stated that surfactin adsorption is an endothermic process. The recommended operating temperature for surfactin adsorption was 45 °C.

The initial pH did not affect surfactin adsorption in the absence of methanol. Additionally the effects of interactions between initial pH with operating temperature or RC/SC ratio also had no effect surfactin adsorption at a 95% confidence interval. The initial pH was, however, expected to improve surfactin adsorption as it affects surfactin charge. The selected pH for surfactin adsorption in the absence of methanol was 11.5, based on the fact that bacterial growth in the adsorption liquid during adsorption is likely to be negligible, compared to that at the lower pHs.

The RC/SC ratio was had the most significant effect on surfactin adsorption in the absence of methanol. An increase in RC/SC ratio was expected to improve the surfactin concentration until a 100% adsorption is reached, where the % adsorption would then remain constant. In this study, the % SA increased from 25% (when RC/SC ratio was 4 g_r/g_s) to a turning point at 105% (when the ratio was 31 g_r/g_s) before decreasing again to approximately 100% (when the RC/SC ratio was 40 g_r/g_s). The quadratic behaviour is due to the fact that a quadratic model was used to experimental data on % SA optimisation in this study. The optimum RC/SC ratio obtained for studies in the absence of methanol was 31 g_r/g_s.

The factorial design used for studying the effect of operating temperature, initial pH and RC/SC ratio was then extended to study the effect of methanol concentration on surfactin adsorption as well as the effects of the interteractions of methanol concentration with operating temperature, initial pH and RC/SC ratio on adsorption. Surfactin adsorption improved with increase in methanol concentration in the adsorption liquid. The addition of methanol in the adsorption was studied as methanol disperses surfactin micelles and was thus expected to improve adsorption. The addition of methanol was seen to improve surfactin adsorption rates, and adsorption kinetics studies showed that adsorption equilibrium could be achieved 5 times faster when methanol was added into the adsorption liquid. The change in initial pH was seen to have a more significant on % adsorption as well as IS surfactin adsorption when adsorption was carried out in the presence of methanol. IS of up to 4 times was achieved when adsorption was carried out in the presence of methanol. Methanol addition also improved adsorption capacities, and the optimum RC/SC ratio was 5 g_r/g_s in the presence of methanol. The optimum initial pH remained 11.5, as an increase in pH was seen to improve both % adsorption and surfactin selectivity.

The proposed surfactin purification techniques in this study, which were acid precipitation, solvent extraction and adsorption were successful in purifying surfactin from *B. subtilis* cultures. The best method for purifying surfactin from cell-free supernatants of *B. subtilis* cultures in this study is to pre-purify the surfactin by acid precipitation and dry the precipitate. Thereafter, purify the dry precipitate by solvent extraction using MTBE as a solvent. This is of major significance as surfactin can be used in the fight against TB, which is among the top five major causes of death globally.

6.2 Recommendations

Based on the results and discussion (Chapter 5), the following recommendations were made:

6.2.1 Further studies should be studied to determine the optimum pH in the range between 4 and 6 during surfactin purification by acid precipitation

The surfactin purity, recovery and selectivity were consistent for pH below pH 4. The actual optimum pH for acid precipitation could therefore be higher pH 4, thus it is recommended that more acid precipitation studies be done between pH 4 and pH 6 to determine the optimum pH. Acid precipitation studies between pH 4 and pH 6 are recommended since surfactin starts precipitating at pH below 6 according to literature.

6.2.2 A two-stage extraction should be considered for purification by solvent extraction

The surfactin purity after solvent extraction can be improved by a two-stage extraction. Since non-polar solvents (hexane and petroleum ether) had poor recoveries of surfactin, but were better at recovering lipids, these solvents can be used for removal of lipid impurities in the first extraction stage (leaving surfactin in the solid precipitate). The second extraction stage would be extraction using MTBE, which mostly recovers surfactin in comparison to protein.

6.2.3 Multi-fold extraction should be considered to improve surfactin recovery when non-polar solvents are used as solvents in solvent extraction studies

A low recovery was attained by solvent extraction using hexane and petroleum ether. Surfactin is known to be partially soluble in hexane and petroleum ether. Therefore, there is a possibility that the solvents were saturated with surfactin after solvent extraction. Fresh solvent can therefore be used to further extract surfactin in the surfactin in the remaining precipitate after extraction to improve the recovery.

6.2.4 Optimisation of initial temperature and optimisation using temperatures greater than 45 °C should be considered in surfactin adsorption studies

Adsorption studies were optimised by varying the operating temperature, while the initial temperature was room temperature. Since % SA studies increased with operating temperature, there is a possibility that a high initial temperature will improve the % SA. Maximum surfactin adsorption was obtained at 45 °C. This was the upper range at which temperature was studied, chosen as high temperatures could negatively affect the efficacy of surfactin. Higher temperatures should be considered for future adsorption studies, while the efficacy of surfactin is tested to ensure that optimisation is done without negatively affecting the efficacy.

6.2.5 Methanol concentration higher 30% (v/v) should be considered in surfactin adsorption studies

An increase in methanol concentration was seen to improve surfactin adsorption. The maximum methanol concentration that was studied in this study was 30% (v/v), since this concentration is best for micelle destabilisation based on literature. Future studies can be studied for methanol concentrations greater than 30% (v/v) in the adsorption liquid. This would particularly important as high methanol concentrations are likely to improve surfactin selectivity.

6.2.6 Resin washing should be considered to improve surfactin purities after surfactin purification by adsorption

After adsorption or prior to desorption, some of the adsorption liquid (which contains impurities) may be trapped in the resins when the resins are recovered. This liquid can be washed off using deionised water. Surfactin is also harder to elute than other impurities, thus methanol can also be added to the deionised water used for resin washing without affecting surfactin recovery. This could be used to wash off some adsorbed impurities.

6.2.7 Column studies should be considered to improve the surfactin adsorption efficiency

In static adsorption, surfactin resins need to be recovered after adsorption in order to desorb the surfactin. The resins are recovered by filtration. Dynamic adsorption in columns is however more efficient as it allows easy desorption since resins do not have to be filtered out prior to desorption. Dynamic adsorption also improves adsorption capacities compared to static adsorption.

6.2.8 Carrying out solvent extraction and adsorption purification steps sequentially should be considered to improve surfactin purity, without affecting the surfactin recovery

In this technique, surfactin would be extracted from dry precipitates using methanol. This would improve the surfactin purity, while 100% of the surfactin is recovered. The methanol with surfactin can then be mixed with alkaline water to form an adsorption liquid with a methanol concentration of 30% (v/v) at a pH of 11.5. This adsorption liquid can then be purified by adsorption with an RC/SC ratio and operating temperature of 45 °C and 5 g_r/g_s respectively to further improve the purity. Higher adsorption capacities and purities are expected as there are less there would be less impurities in this adsorption liquid.

6.2.9 A quantitative technique should be used for efficacy studies of surfactin after purification

Since the surfactin produced in this study is for use in the fight against TB, antimicrobial tests of the purified surfactin against *M. tuberculosis* are necessary. Purified surfactin is expected to improve the antibacterial activity of surfactin (Snook, et al., 2009 and Mukherjee, et al., 2009). Razafindralambo et al. (1993), on the other hand, stated that acid precipitation and exposure of surfactin to organic solvents may result in hydrolysis or esterification of lipopeptide functional groups, and this may reduce the antibacterial activity of surfactin. Surfactin efficacy studies should therefore be quantitatively done to test the efficacy after surfactin purification by acid precipitation, solvent extraction and adsorption. These tests can be done using *M. aurum*, a surrogate of *M. tuberculosis*, which is much safer to work with. A suitable test might be the agar well diffusion test on Mueller-Hinton agar medium, which was used by Mukherjee et al. (2009) to show that antibacterial activity of surfactin is improved by surfactin purification.

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8 Appendix

8.1 Lipopeptide analysis

This section provides HPLC chromatograms for surfactin, iturin and fengycin. These are shown in Figure 8-1 to Figure 8-3.

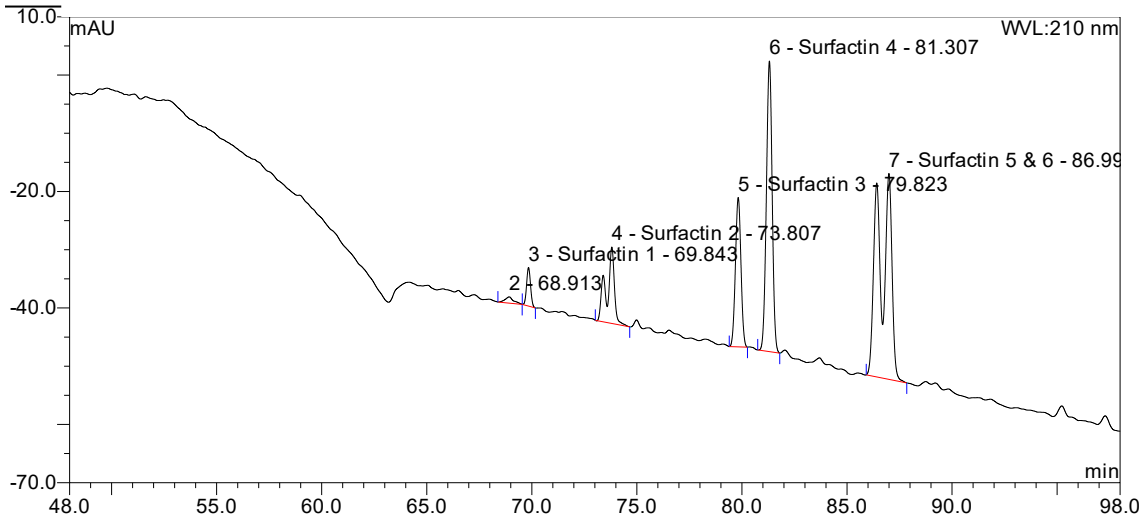

Figure 8-1: Surfactin HPLC chromatogram

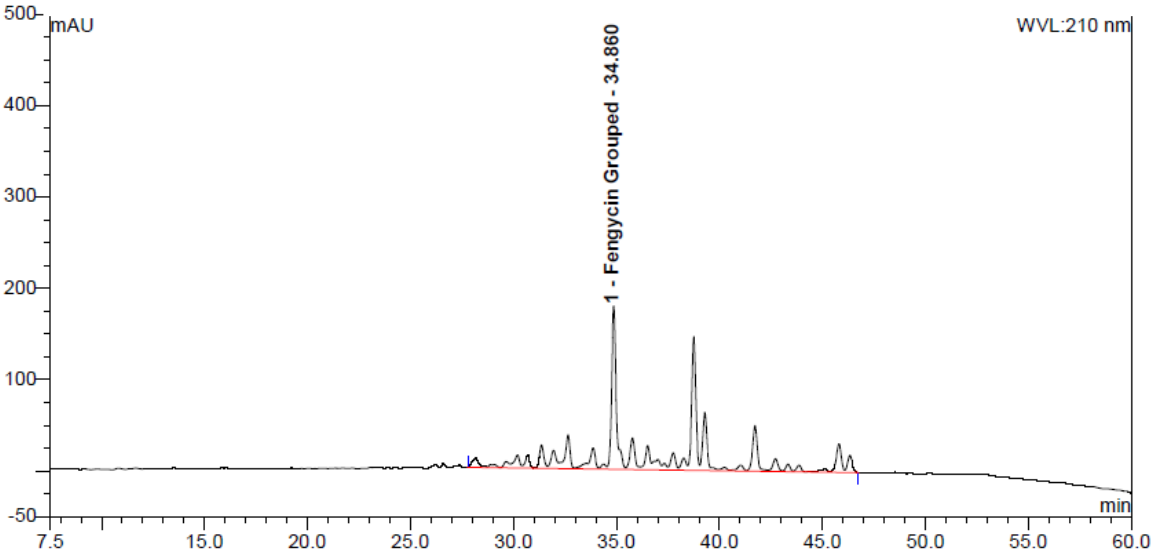

Figure 8-2: Fengycin HPLC chromatogram

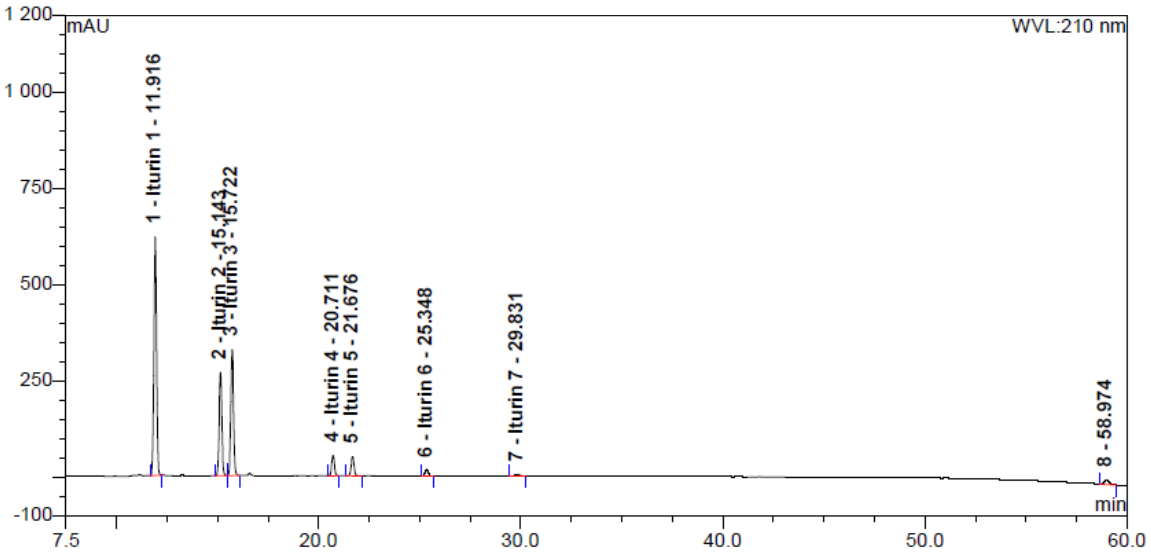

Figure 8-3: Iturin HPLC chromatogram

8.2 Solvent extraction

This section details how solvent polarities were determined in the solvent extractions study as well as boiling points of solvents used in solvent extraction studies. The solvent polarities were obtained from Bosch & Roses (1992) and Smallwood (2012), which use the ET polarity scale. The polarity of solvent mixtures was determined using Equation 8-1, where PI_{SM} is the polarity index of the solvent mixture. x_{SA}, x_{SB}, PI_{SA} and PI_{SB} the compositions and polarities of the solvents being mixed respectively, denoted as solvent A and solvent B.

$$PI_{SM} = x_{SA}PI_{SA} + x_{SB}PI_{SB} \qquad \textit{Equation 8-1}$$

The boiling points of solvents used in solvent extraction studies are shown in Table 8-1.

Table 8-1: Boiling points of solvents used in solvent extraction studies

Solvents	Boiling points (°C)
methanol	64
***I*-propanol**	82
chloroform: methanol (1:1)	54
Acetonitrile	81.6
chloroform: methanol (2:1)	53.5
Acetone	56
Chloroform	61
Ethyl acetate	77
MTBE	55
***N*-Hexane**	69
Petroleum ether	36

8.3 Adsorption

8.3.1 Effect of methanol addition on pH measurement and volume of adsorption liquid

The pH can be measured in water as well as in water-methanol solutions. The presence of methanol in aqueous solutions is different from the pH of aqueous solutions without methanol. The difference between the pH obtained from water and methanol-water solutions can be defined as delta (δ). δ changes with the methanol concentration in water according to Equation 8-2 and Figure 8-4. In this equation, υ is the methanol volume fraction in methanol-water solutions. From Figure 8-4, it can be seen that the δ is insignificant for methanol concentrations below 30%.

$$\delta = \frac{0.09\upsilon - 0.11\upsilon^2}{1 - 3.15\upsilon + 3.51\upsilon^2 - 1.35\upsilon^3}$$ *Equation 8-2*

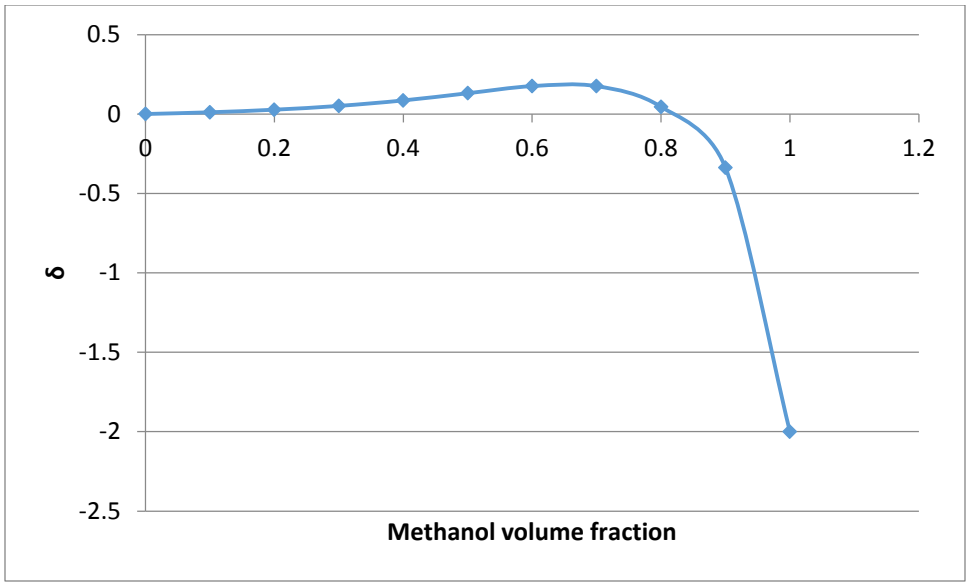

Figure 8-4: Difference between pH measured in water and pH measured in methanol-water solutions resulting from methanol addition in water

When methanol is added to aqueous mixtures, the sum of the volumes of methanol and aqueous solution is less than the final volume of the aqueous-methanol mixture. The difference is known as the volume contraction. The relationship between the volume contraction and the final methanol concentration in the aqueous phase is shown in Figure 8-5. At 30% (v/v) methanol, the volume contraction is approximately 2.4%. The surfactin and resin concentrations are therefore expected to be slightly higher than the actual concentrations in solutions with methanol compared to solutions without methanol. However, the volume contraction was not taken into account as surfactin is dependent on the resin concentration to surfactin concentration ratio is maintained despite the volume contraction. Additionally, a volume contraction of 2.4% will result in a negligible error in the concentrations.

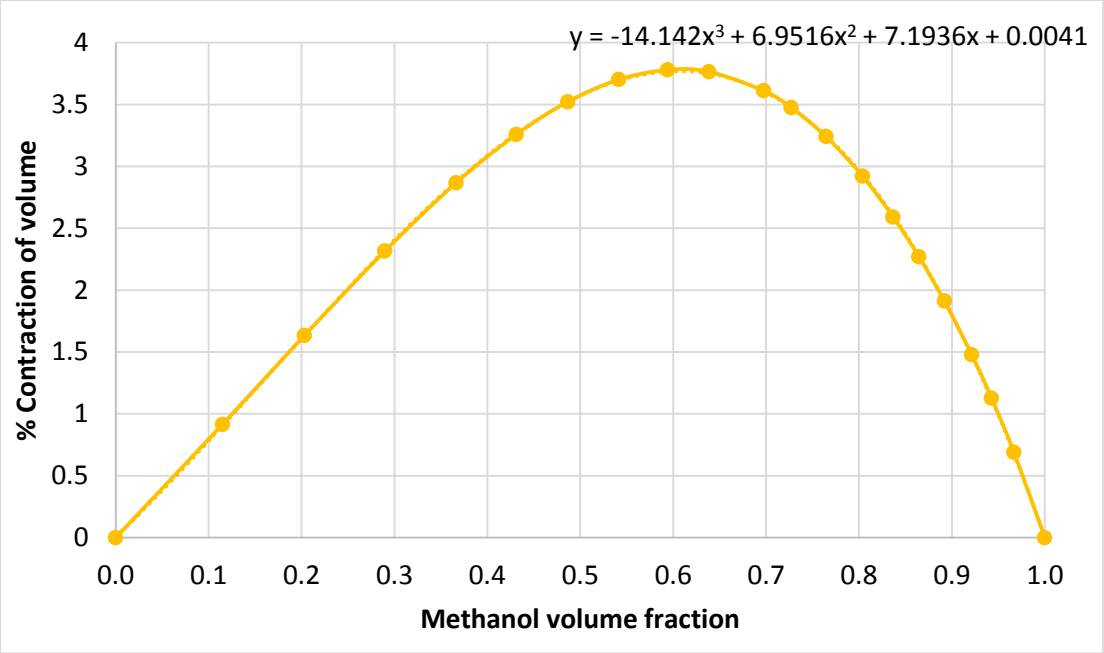

Figure 8-5: Volume contraction due to addition of methanol in water

8.3.2 Surfactin adsorption preliminary studies

Preliminary tests were carried out to determine if equilibrium was reached after 24 h during adsorption. The adsorption was carried out for 24 h. This was based on the study by Dhanarajan et al. (2015), who found that HP-20 resins were saturated with lipopeptides after 8 h during adsorption. Prior to adsorption studies, it was tested if equilibrium will be reached within 24 h. This was done by carrying out adsorption at a resin concentration and surfactin concentration of 0.4 g_r/L and 20 g_s/L. The temperature and pH were 45 °C and 11 respectively. This study was done in an incubator (labcon, 150 rpm, 48 h) using 100 mL non-baffled shake flasks and 25 mL of the adsorption liquid. The results (Figure 8-6) showed that equilibrium was reached after 24 h.

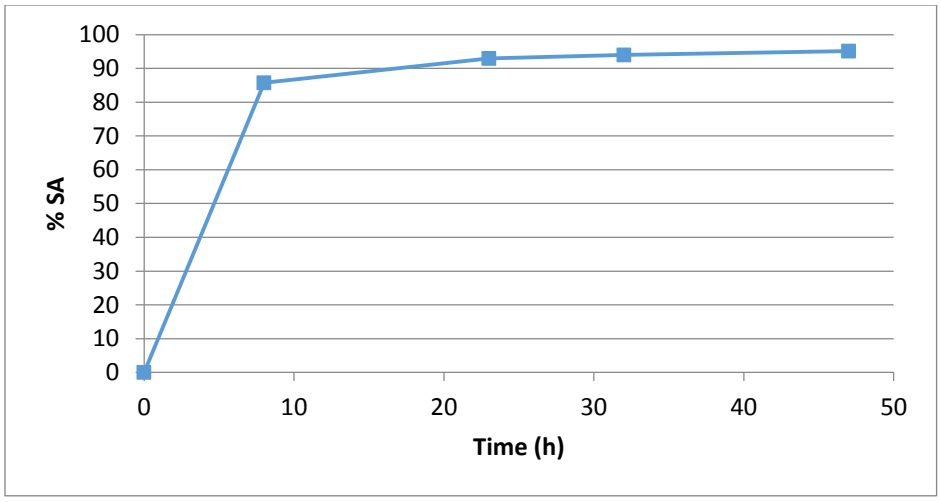

Figure 8-6: Change in % SA during adsorption (error bars are not shown as experimental repeatability was not studied)

Preliminary studies were also done to determine if surfactin adsorption is dependent on RC/SC ratio or on resin concentration and surfactin concentration independently. These studies were done at resin concentrations of 5.5 g/L and 11 g/L, while maintaining the RC/SC ratio at 40 g_r/g_s. The initial pH and operating temperature were held constant at 11.5 and 45 °C respectively, and the experiments wer carried out in an incubator (Labcon, 150 rpm, 24 h). The results are shown in Figure 8-7. The two means in Figure 8-7 were compared using a single factor ANOVA, which showed that they were similar at a 95% confidence interval.

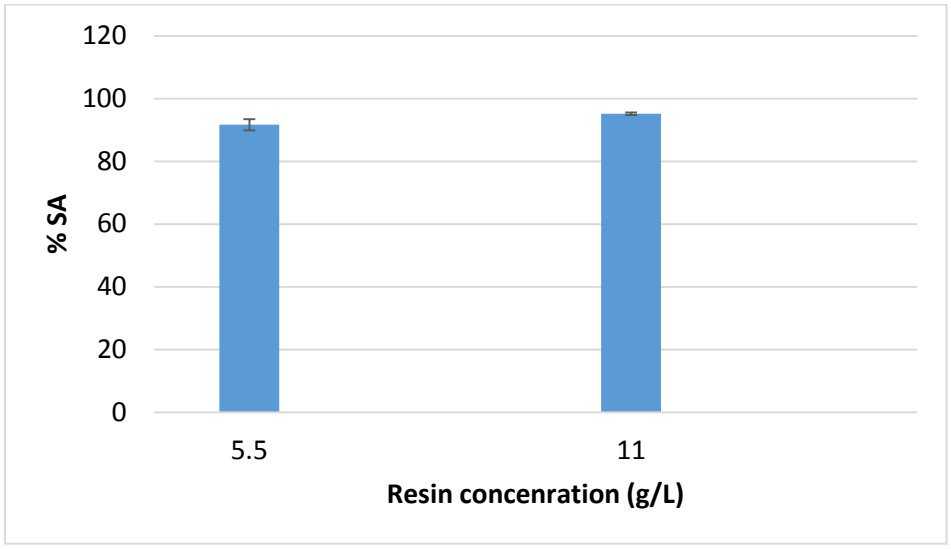

Figure 8-7: Plot to show that surfactin concentration is dependent on RC/SC ratio rather than surfactin concentration and resin concentration independently, by comparing the % SA at different resin concentrations at an RC/SC ratio of 40 g_r/g_s. The error bars indicate the standard deviation of two replicates

8.3.3 Equilibrium pH, % SA and IS surface after adsorption

8.3.3.1 *Equilibrium pH surface plots*

This section shows the change in equilibrium pH obtained after adsorption experimental runs. The equilibrium pH for studies at 0% (v/v) methanol is shown in Figure 8-8 to Figure 8-10, while equilibrium pH for studies in the presence of methanol is shown in Figure 8-11 to Figure 8-13

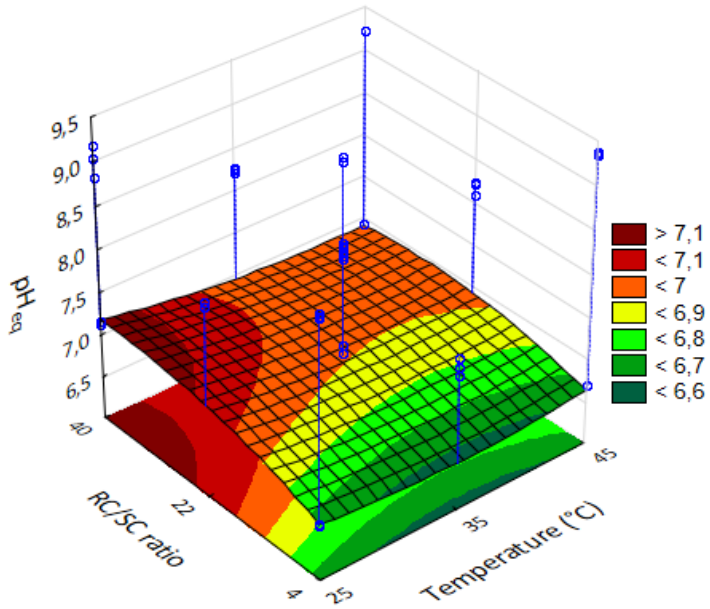

Figure 8-8: Equilibrium pH obtained after surfactin adsorption in the absence of methanol at an initial pH of 6.5

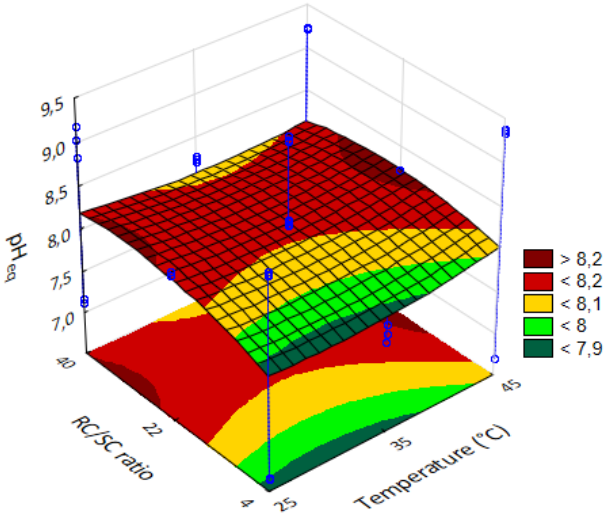

Figure 8-9: Equilibrium pH obtained after surfactin adsorption in the absence of methanol at an initial pH of 9

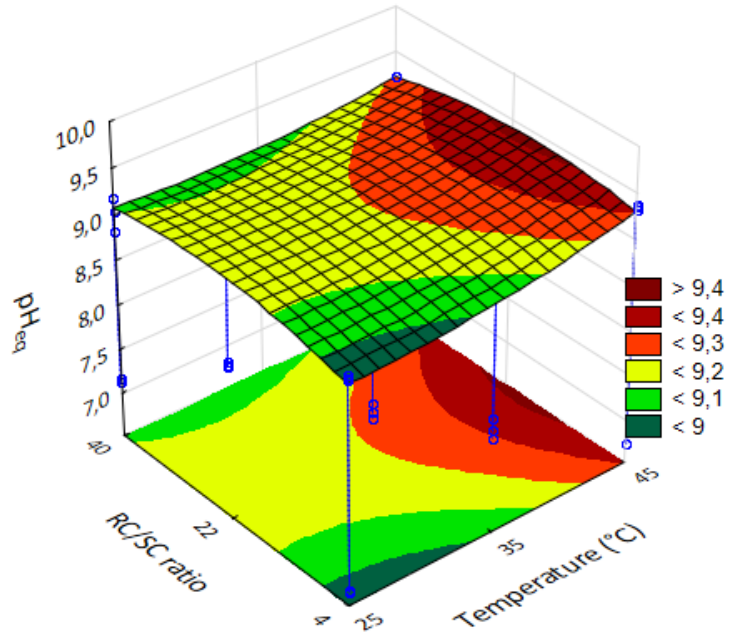

Figure 8-10: Equilibrium pH obtained after surfactin adsorption in the absence of methanol at an initial pH of 11.5

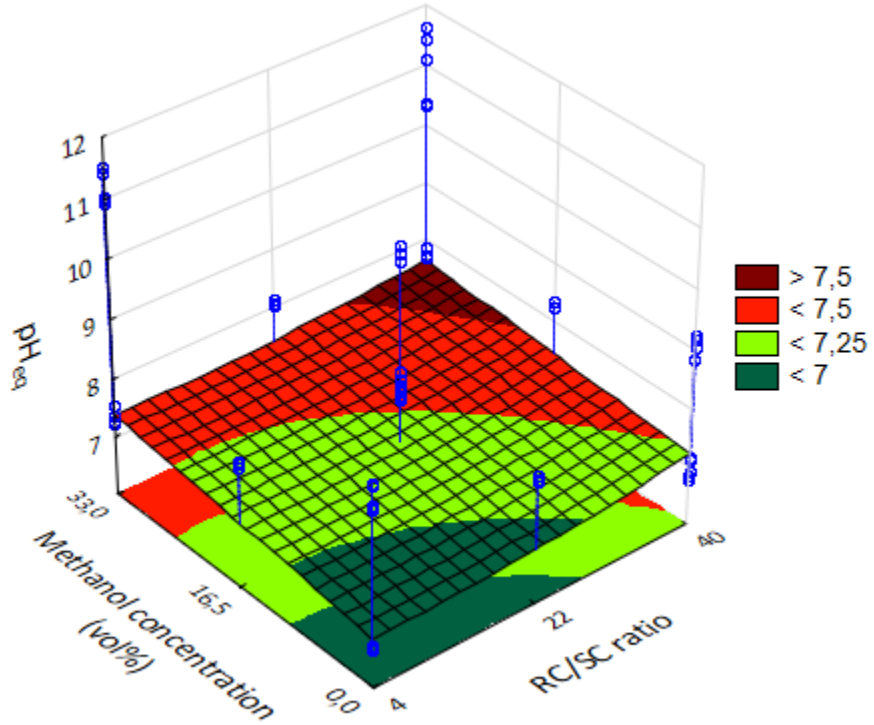

Figure 8-11: Equilibrium pH obtained after surfactin adsorption in the presence of methanol at an operating temperature and initial pH of 35 °C and 6.5 respectively

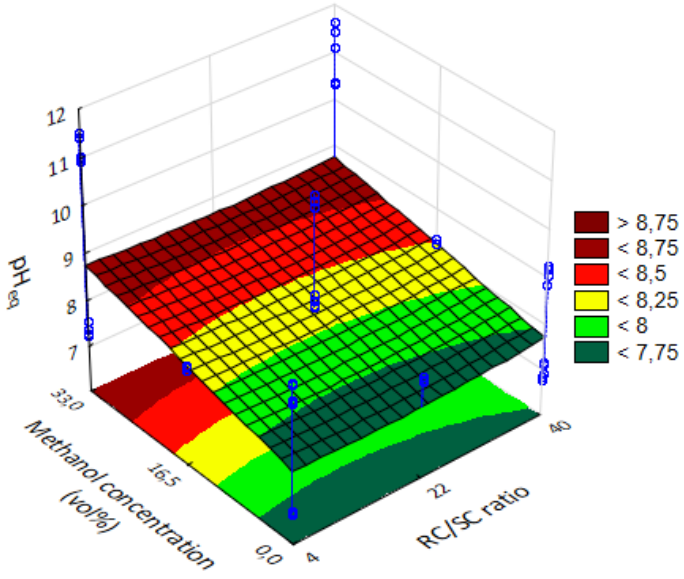

Figure 8-12: Equilibrium pH obtained after surfactin adsorption in the presence of methanol at an operating temperature and initial pH of 35 °C and 9 respectively

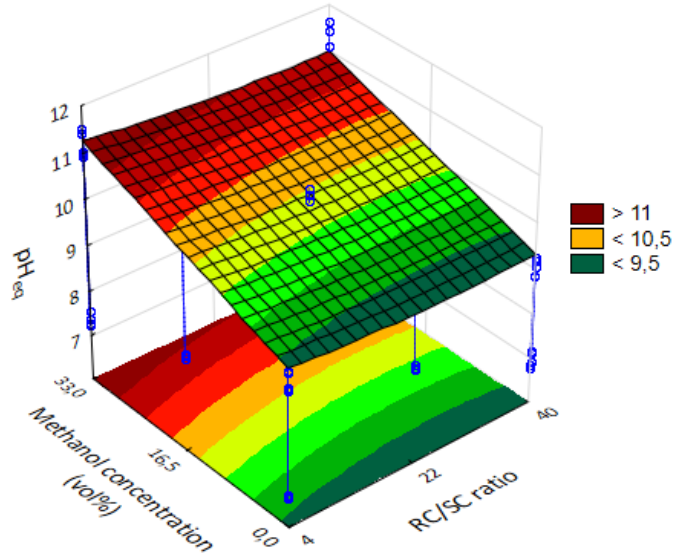

Figure 8-13: Equilibrium pH obtained after surfactin adsorption in the presence of methanol at an operating temperature and initial pH of 35 °C and 11.5 respectively

8.3.3.2 *% SA surface plots*

This section shows the change in % SA obtained after adsorption experimental runs. These are shown through surface plots (Figure 8-14 to Figure 8-19)

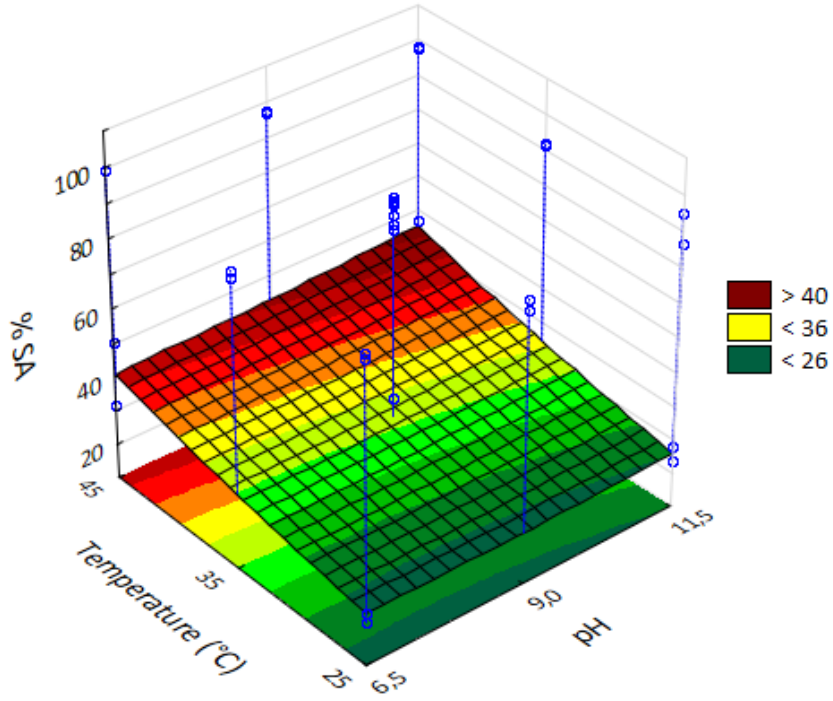

Figure 8-14: % SA obtained after surfactin adsorption in the absence of methanol at an RC/SC ratio of 4 g_r/g_s

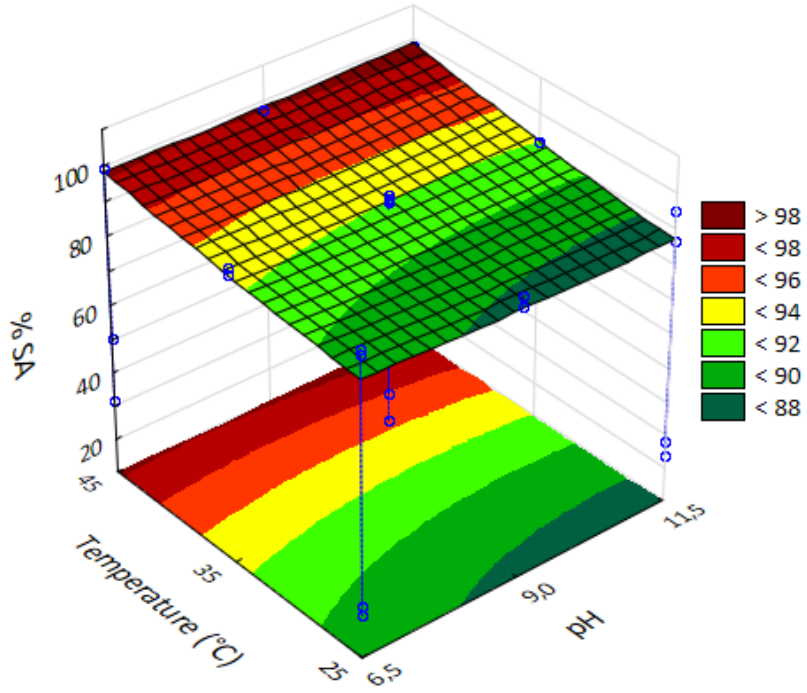

Figure 8-15: % SA obtained after surfactin adsorption in the absence of methanol at an RC/SC ratio of 22 g_r/g_s

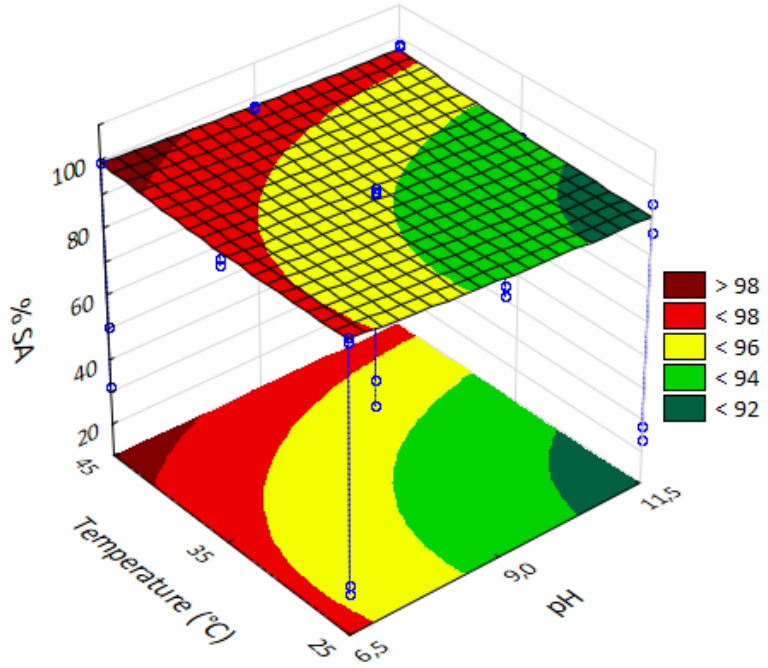

Figure 8-16: % SA obtained after surfactin adsorption in the absence of methanol at an RC/SC ratio of 40 g_r/g_s

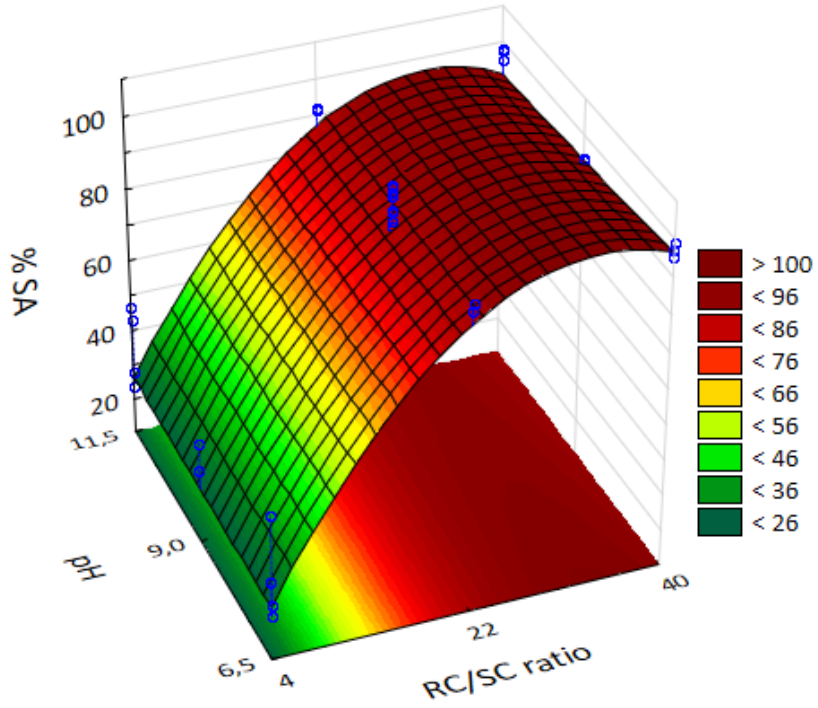

Figure 8-17: % SA obtained after surfactin adsorption in the absence of methanol at an operating temperature of 25 °C

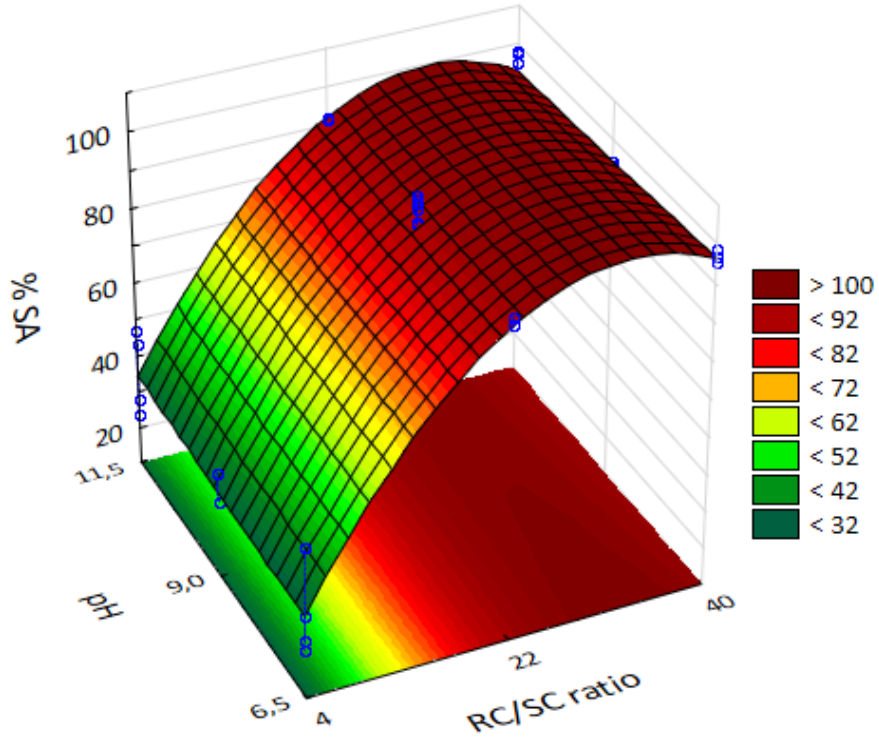

Figure 8-18: % SA obtained after surfactin adsorption in the absence of methanol at an operating temperature of 35 °C

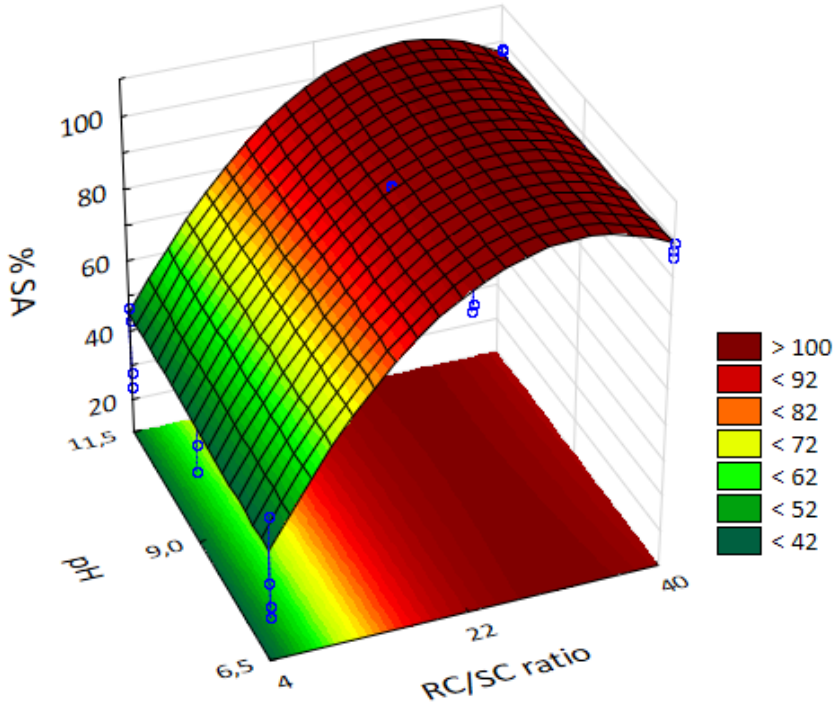

Figure 8-19: % SA obtained after surfactin adsorption in the absence of methanol at an operating temperature of 45 °C

8.3.3.3 *Improvement in selectivity surface plots*

This section shows the change in IS obtained after adsorption experimental runs. These are shown through surface plots (Figure 8-20 to Figure 8-25)

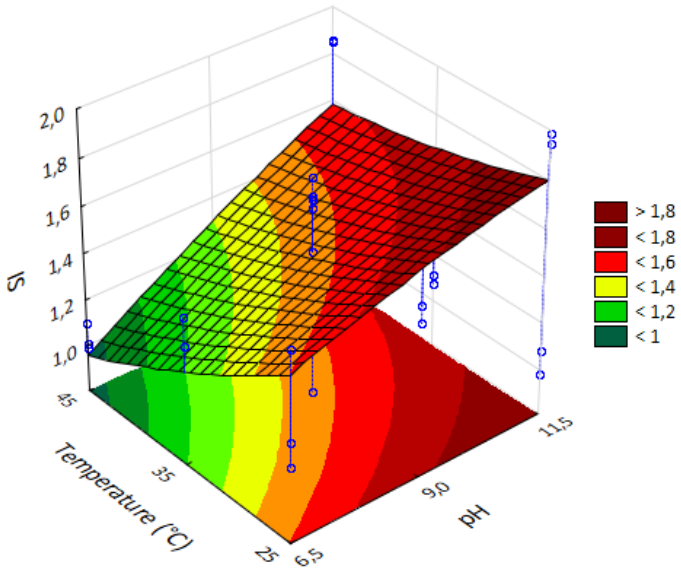

Figure 8-20: IS obtained after surfactin adsorption in the absence of methanol at an RC/SC ratio of 4 g_r/g_s

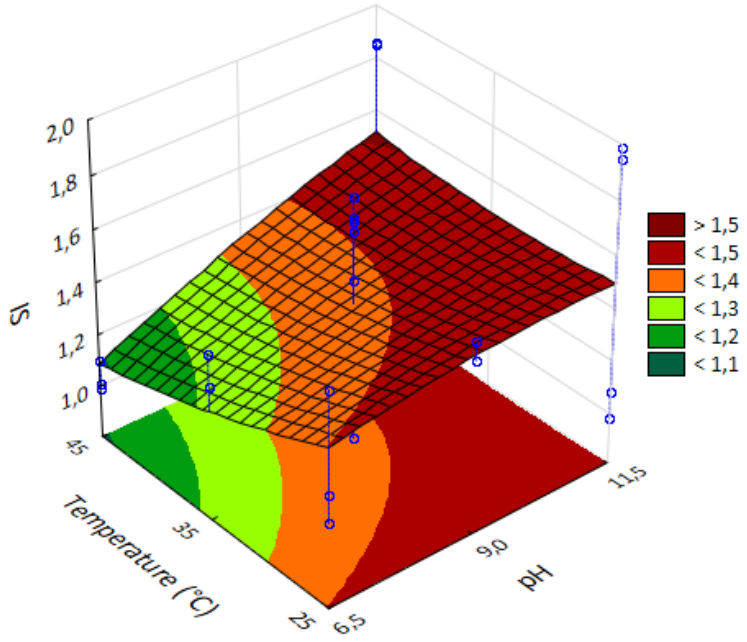

Figure 8-21: IS obtained after surfactin adsorption in the absence of methanol at an RC/SC ratio of 22 g_r/g_s

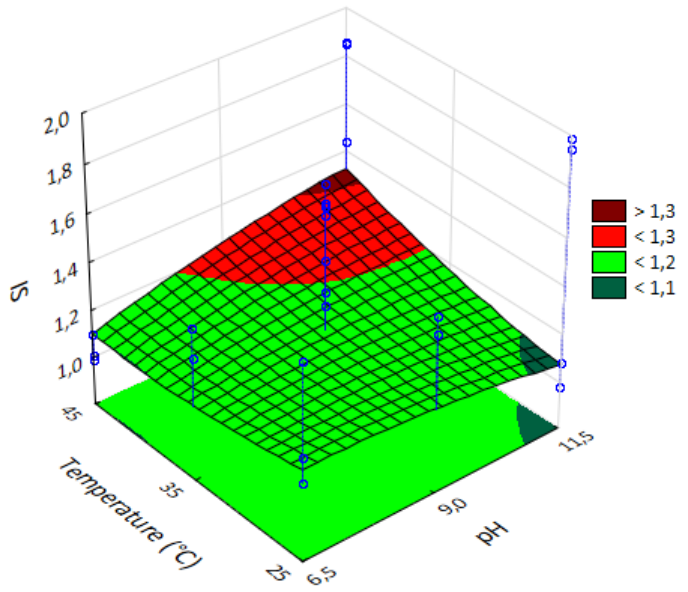

Figure 8-22: IS obtained after surfactin adsorption in the absence of methanol at an RC/SC ratio of 40 g_r/g_s

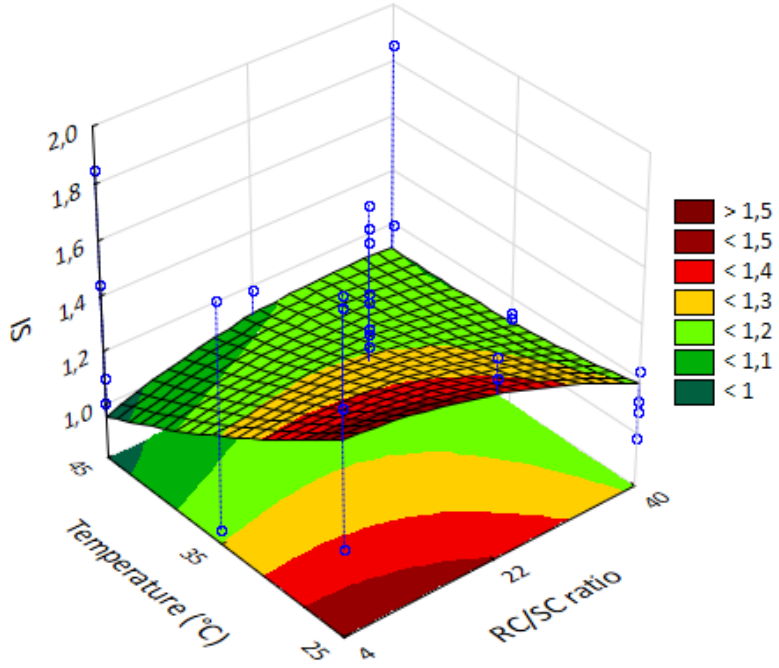

Figure 8-23: IS obtained after surfactin adsorption in the absence of methanol at an initial pH of 6.5

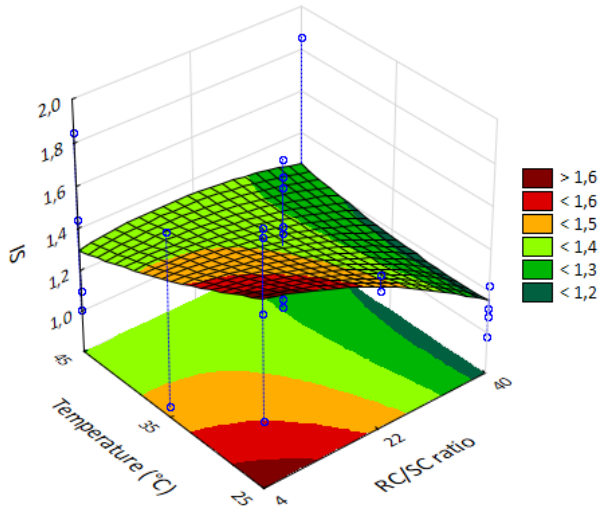

Figure 8-24: IS obtained after surfactin adsorption in the absence of methanol at an initial pH of 9

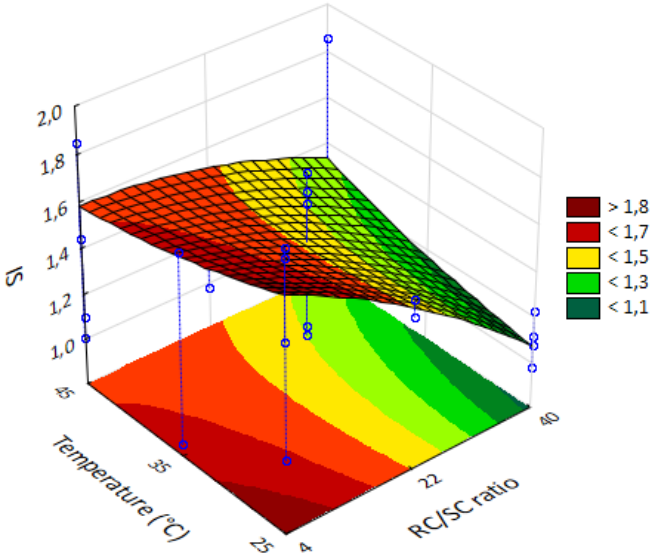

Figure 8-25: IS obtained after surfactin adsorption in the absence of methanol at an initial pH of 11.5