Antimicrobial lipopeptide production by *Bacillus* spp. for post-harvest biocontrol

by

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Declaration

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Abstract

As overpopulation threatens the world's ability to feed itself, food has become an invaluable resource. Unfortunately, almost a third of the food produced for human consumption is lost annually. Pests including insects, phytopathogens and weeds are responsible for more than a third of the annual major crop losses suffered around the world.

The majority of current post-harvest control strategies employ synthetic agents. These compounds, however, have been found to be detrimental to the environment as well as human health, which has led researchers to investigate alternative strategies. Biocontrol agents are environmentally compatible, have a lower toxicity and are biodegradable, making them an attractive alternative to the synthetic control agents. The lipopeptides produced by *Bacillus* spp. in particular, have shown great potential as biocontrol agents against various post-harvest phytopathogens.

Most biocontrol strategies apply the biocontrol organism, for example *Bacillus*, directly, whereas this study focused on the use of the lipopeptide itself as an antifungal agent. This is advantageous as the lipopeptides are less sensitive to their surroundings, such as temperature and pH, compared to living organisms, allowing for the production of a standardized product.

This study investigated the production of the *Bacillus* lipopeptides surfactin, fengycin and iturin under controlled batch conditions. Parameters increasing lipopeptide production were quantified, focussing on antifungal lipopeptides (iturin and fengycin), and lipopeptide production was optimized. Experiments were performed in a fully instrumented 1.3 L bench-top bioreactor and lipopeptide analyses were performed *via* high pressure liquid chromatography (HPLC) and liquid chromatography-mass spectroscopy (LC-MS).

After screening four *Bacillus* spp., *Bacillus amyloliquefaciens* DSM 23117 was found to be the best antifungal candidate. This was based on it outperforming other candidates in terms of maximum antifungals produced, $Y_{p/x,antifungals}$ (yield per cells), and antifungal productivity.

Nitrate, in the form of NH_4NO_3, was critical for lipopeptide production and an optimum concentration was observed above which the CDW (cell dry weight) no longer increased significantly and both μ_{max} (maximum specific growth rate, h^{-1}) and lipopeptide production decreased. For μ_{max}, the optimum NH_3NO_4 concentration was 10 g/L and for lipopeptides it was 8 g/L. At these respective NH_4NO_3 concentrations μ_{max} = 0.58 (h^{-1}), the maximum antifungals (fengycin and iturin) were 285.7 mAU*min and the maximum surfactin concentration was 302 mg/L.

The lipopeptides produced by *B. amyloliquefaciens*, the antifungals (fengycin and iturin) and surfactin, are secondary metabolites, regardless of the optimization treatment, i.e. increased NH_4NO_3 concentrations.

Using 30% enriched air extended the nitrate utilization period, suggesting that when increasing supply concentration, more oxygen was available to act as electron acceptors, allowing nitrate to be used for lipopeptide production.

The number of iturin and fengycin homologues generally increased with an increase in nitrate concentration. This suggested that process conditions, such as nitrate concentration, can be used to manipulate homologue ratios, allowing for the possibility to tailor-make biocontrol-agent upstream, during the production process, and possibly increase the efficacy of the biocontrol strategy.

The lipopeptides produced by *B. amyloliquefaciens* showed complete inhibition against *Botryotinia fuckeliana* and diminished the growth capabilities of *Botrytis cinerea*. No inhibition was observed against *Penicillium digitatum.* These results indicate potential of the biocontrol strategy, although scale-up and fed-batch studies are recommended, especially when considering commercial implementation. Studies regarding the lipopeptide application method, i.e. a single application or multiple applications, should also be investigated as this will influence the efficacy of the lipopeptides against the target organisms.

Samevatting

Met oorbevolking wat die wêreld se vermoë om die groeiende bevolking te onderhou belemmer, het dit noodsaaklik geword om huidige voedselbronne te beskerm. Daar word beraam dat een derde van die voedsel wat wêreldwyd geproduseer word vir menslike verbruik verlore gaan elke jaar. Verder is insekte, plantpatogene en onkruide verantwoordelik vir meer as ‘n derde van die verliese rakend jaarlikse oeste.

Meeste bestaande na-oes beheermetodes maak gebruik van sintetiese stowwe. Ongelukkig kan hierdie verbindings nadelig wees vir die omgewing sowel as menlike gesondheid. Navorsers het hulsef dus toespits daarop om alternatiewe beheermetodes te ondersoek. Bio-beheermetodes is omgewingsvriendelik sowel as bio-afbreekbaar, wat hulle ideale alternatiewe maak vir die sintetiese stowwe. *Bacillus* spp. lipopeptiede het veral hoë potensiaal getoon as bio-beheermiddels teen verskeie na-oes plantsiektes.

Meeste bio-beheermetodes wend die biobeheer organisme, soos *Bacillus*, direk aan, waar hierdie studie op die gebruik van lipopeptiede as ‘n beheermiddel gefokus het. Die voordeel is dat lipopeptidiede minder sensitief is vir hul omgewings, soos temperatuur en pH, i.v.m. organismes en die moontlikheid bied van ‘n gestandardiseerde produk.

Hierdie studie het die produksie van spesifieke *Bacillus* lipopeptide, naamlik surfactin, fengycin en iturin, onder beheerde lottoestande ondersoek. Parameters wat lipopeptied produksie verhoog is gekwantifiseer, spesifiek antifungiese middels (iturin en fengycin) en lipopeptied produksie is geoptimeer. Eksperimente is uitgevoer in ‘n 1.3 L bioreaktor en lipopeptiedanaliese is met behulp van hoë druk vloeistof chromatografie en vloeistofchromatografie-massa spektroskopie uitgevoer.

Van die vier moontlike *Bacillus* spp., was *Bacillus amyloliquefaciens* DSM 23117 die mees belowende antifungus-produserende kandidaat. Dit het beter resultate gelewer in terme van maksimale antifungiese produksie, $Y_{p/x,antifungies}$ (opbrengs per sel) asook antifungiese produktiwiteit.

Nitraat, in hierdie geval NH_4NO_3, was noodsaaklik vir lipopeptied produksie en ‘n optimale konsentrasie is waargeneem waarbo die seldigtheid nie meer beduidend toegeneem het nie en beide die μ_{max} (maksimale spesifieke groei tempo, h^{-1}) en lipopeptied produksie afgeneem het. Die optimale NH_4NO_3 konsentrasie vir μ_{max} was 10 g/L en vir lipopeptiedproduksie was 8 g/L. By 10 g/L NH_4NO_3 was μ_{max} = 0.58 (h^{-1}) en by 8 g/L was die maksimale antifungiese produksie (fengycin en iturin) 285.7 mAU*min en die maksimale surfactin produksie 302 mg/L onderskeidelik.

Die lipopeptide, die antifungiese middels (fengycin en iturin) en surfactin, geproduseer deur *B. amyloliquefaciens* is sekondêre metaboliete, ongeag van die optimerings-behandelinge wat toegepas word, soos ‘n verhoging in NH_4NO_3 konsentrasie.

Die gebruik van 30% verrykte suurstof het die nitraat verbruikingsperiode verleng, wat voorgestel het dat met die verryking, meer suurstof beskikbaar was om te dien as finale elektron ontvanger en sodoende die nitraat beskikbaar te stel vir lipopeptied produksie.

Iturin en fengycin homoloë, oor die algemeen, het toegeneem soos wat die nitraat konsentrasie verhoog is. Hierdie resultate dui daarop dat prosestoestande, soos nitraat konsentrasie, gebruik kan word om die verhouding waarin lipopeptied homoloë geproduseer word te manipuleer. Hierdie resultate dui op die potensiaal vir die stroomop produksie van ‘n unieke bio-beheermiddel, wat die effektiwiteit van die bio-beheermetode moontlik sal verhoog.

Die geproduseerde lipopeptiede het totale inhibisie getoon teen *Botryotinia fuckeliana* en ook fungiese aktiwiteit belemmer met *Botrytis cinerea*. Geen inhibisie is getoon teen *Penicillium digitatum* nie. Hierdie resultate toon die potensiaal van die bio-beheermetode, maar ‘n opskalerings-studie asook ‘n voerlot studie word aanbeveel, veral met die oog op moontlike kommersiële implementering van die strategie. Verdere studies met betrekking tot die aanwendingsmetode van die lipopeptiede moet ook verder ondersoek word, m.a.w. enkel teenoor menigte aanwendigs, aangesien dit die effektiwiteit van die lipopeptiede teen die teikenorganismes sal beïnvloed.

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Glossary and Nomenclature

$Al_2(SO_4)_3$	Aluminium sulphate
ATCC 21332	Strain of *Bacillus subtilis*
aq	Aqueous
$CaCl_2.4H_2O$	Calcium chloride tetrahydrate
CDW	Cell dry weight (g/L)
$CoSO_4$	Cobalt sulphate
D	Dilution rate (h^{-1})
DO	Dissolved oxygen
DSM 23117	Strain of *Bacillus amyloliquefaciens*
DSM 347	Strain of *B. subtilis* subs. *spizizenii*
DSM 13	Strain of *B. licheniformis*
F	Flow rate
$FeSO_4.7H_2O$	Ferrous sulphate heptahydrate
g	Gram
h	Hour
HPLC	High pressure liquid chromatography
KH_2PO_4	Potassium dihydrogen phosphate
K_La	Volumetric oxygen transfer coefficient
KNO_3	Potassium nitrate
L	Litre
M	Molar
m	Mass
$MgSO_4.H_2O$	Hydrous magnesium sulphate
$MnSO_4.H_2O$	Hydrous manganese sulphate
min	Minute
mL	Millilitre
mL/min	Millilitre per minute
mg/L	Milligram per litre
mg/L/h	Milligram per litre per hour
mM	Millimolar
MSM	Minimal salts medium
mV	Millivolt
N	Newton
Na_2HPO_4	Disodium phosphate
$NaNO_3$	Sodium nitrate

NaOH	Sodium hydroxide
NH_4HCO_3	Ammonium hydrogen carbonate
NH_4NO_3	Ammonium nitrate
$NiSO_4$	Nickel sulphate
nm	Nanometer
p_i	Product concentration at time "i"
p_0	Initial product concentration
rpm	Revolutions per minute
s_i	Substrate concentration at time "i"
s_0	Initial substrate concentration
t	Time (h)
UV	Ultraviolet
μ	Specific growth rate (h^{-1})
μ_{max}	Maximum specific growth rate (h^{-1})
μL	Microlitre
μM	Micromolar
vvm	Volume per volume per minute
x	Cell concentration
x_i	Cell concentration at time "i"
x_0	Initial cell concentration
$Y_{p/s}$	Yield of product from substrate
$Y_{x/s}$	Yield of biomass from substrate
$Y_{p/x}$	Yield of product from biomass
$ZnSO_4$	Zinc sulphate
$ZrOCl_2$	Zirconyl chloride

Introduction

With overpopulation threatening the world's ability to feed itself, food production and protection has become a major issue world-wide. Recent studies (Thomas, 2011) have estimated that almost one-third of the food produced for human consumption, around 1.3 billion tonnes, is either wasted or lost annually. Furthermore, 27 – 42% of major crop losses around the world are attributed to pests, including invertebrates, pathogens and weeds (Glare *et al.*, 2012:250).

In the past, the control of post-harvest diseases has primarily been dependent on synthetic pesticides (Pimentel *et al.*, 1993:273; Pimentel, 2005:229), however these substances have been shown to be harmful to both the environment as well as human health (Pimentel *et al.*, 1993:273; Pimentel, 2005:229). Due to the undesired effects associated with synthetic pesticides, stricter rules and regulations are being enforced with regards to their maximum residue limits (Holzmann, 2010:89). In addition, the high costs associated with the discovery and development of new synthetic pesticides (Glare *et al.*, 2012:250) have created a need for a viable alternative control method. Biocontrol is a promising alternative due to its biodegradability (Coutte *et al.*, 2010:499-507; Desai and Banat, 1997:47-64; Nitschke and Costa, 2007:252), low toxicity (Cayuela *et al.*, 1993:383; Desai and Banat, 1997:47-64; Nitschke and Costa, 2007:252), environmental compatibility (Desai and Banat, 1997:47-64; Nitschke and Costa, 2007:252; Ongena and Jacques, 2008:115), as well as the fact that government regulations favour their use (Holzmann, 2010:89). Biological surfactants, more commonly known as biosurfactants, are one of the examples of biocontrol that have shown great potential in various fields of study, including pharmaceutical, cosmetic and remediation.

Biological surfactants have been found to be extremely effective in controlling phytopathogens, but the majority of biocontrol strategies currently available employ the micro-organism itself directly as a countermeasure to the pest. This is demonstrated by the use of products like Serenade (Marrone, 2002:193) and Bioshield™ (Jackson, 2007), where both products contain either living cells or endospores. Much of the research being carried out also includes the use of cells in the biocontrol agent (Yanez-Mendizabal *et al.*, 2012:954), making the current study very valuable, as it focused on the use of the lipopeptides, i.e. the products produced by the micro-organisms. These are less sensitive to changes in pH or temperature compared to the organisms themselves (1.3.2), allowing for the production of a standardized product. An additional advantage to the use of lipopeptides instead of products containing living organisms is that there are fewer restrictions on importation and quarantine associated with these products.

The aim of this project was to study biosurfactant production, specifically the antifungal lipopeptides fengycin and iturin. Parameters associated with lipopeptide production were quantified and the production of the antifungal lipopeptides was optimized and the efficacy of the lipopeptides was tested against specific phytopathogens. Important factors in the study included the choice of organism to be used for biosurfactant production, growth medium composition as well as the process conditions under which production would be optimal. An in-depth literature review was carried out to acquire the background on these topics as well as to determine the way forward for future work.

This thesis will present the background on crop spoilage control, biosurfactants and process conditions in the literature review (Chapter 1), discuss the hypotheses and objectives derived from the literature review (Chapter 2), describe the materials and methods used (Chapter 3), document and evaluate the results obtained (Chapter 4). Finally, the conclusion will evaluate the importance and the implications of the study (Chapter 5), followed by the recommendations.

Chapter 1

Literature review

1.1 Control of crop spoilage organisms

Different crop spoilage control methods can be enforced during different stages of the food production process, i.e. pre-harvest and post-harvest. During the pre-harvest stage the produce is usually treated to prevent the growth of weeds and to deter insects (Glare *et al.*, 2012:250). The post-harvest stage of the process includes cooling, cleaning, sorting and the packing of the produce. The control methods used during this stage of production include temperature manipulations (cooling-chain), food coatings such as chitosan that activate defence mechanisms in the host tissue (Devlieghere *et al.*, 2004:703; Tripathi and Dubey, 2004:235) and the use of sulphur dioxide in packing houses to prevent the occurrence of oxidative and enzymatic reactions (Lund *et al.*, 2000).

The methods used for the control of crop spoilage organisms, both pre- and post-harvest, can be divided into three main groups, namely physical, chemical and biological control strategies. Physical strategies include temperature and pH manipulations, irradiation as well as moisture reduction or drying (Lund *et al.*, 2000). Pesticides such as DDT (dichloro-diphenyl-trichloroethane) (U.S. EPA, 2012) and heptachlor (U.S. EPA, 2007) are examples of synthetic chemical control compounds, whereas biological strategies include the use of living organisms to control the target organisms. Commercial examples include Serenade, based on *Bacillus subtilis* QST-713, which produces three lipopeptides (see 1.2.1.2) and is effective against fungal diseases (Marrone, 2002) as well as Bioshield™ which utilizes *Serratia entomaphila* against the grass grub *Costelytra zealandica* (Jackson, 2007). Yanez-Mendizabal *et al.* 2012 have also researched and developed the spray-drying of a mixture of vegetative cells, endospores and antifungal metabolites from *Bacillus subtilis* CPA-8, with effective control capabilities after 6 months storage. The majority of research currently available is based on the direct application of living organisms as a control method against the relevant pest. There are very few detailed reports with respect to metabolite application, however, Yanez-Mendizabal *et al.* (2011), conducted *in vitro* studies on various pathogens, including *Botrytis cinerea*, *Monilinia fructicola* and *Penicillium digitatum.* These studies showed that the cell-free supernatant, i.e. the biosurfactant itself, performed better than the samples containing cells or endospores. *In vitro* studies showed that the antifungal inhibition on all fungi tested, including *P. digitatum* and *M. laxa*, was between 40-73% for the samples containing cells and spores as opposed to the 89-100% inhibition demonstrated by the cell-free supernatant samples.

Further studies have shown that the lipopeptides are less sensitive to changes in their surroundings, i.e. temperature or pH, compared to the organisms themselves (Ohno *et al.*, 1995:517-519). These changes in temperature for example have also been shown to affect homologue production, allowing for less control over which metabolite is being applied when utilizing the organism directly as a biocontrol strategy.

The remainder of the section (section 1.1) will focus on biocontrol, looking at its advantages, limitations and the possible target organisms that will be considered for this project.

1.1.1 Advantages of the biocontrol strategy

The fact that biocontrol is based on the utilization of living organisms and their products to control the targeted organisms (micro-organisms, insects and weeds for example) allows for a number of advantages compared to synthetic control methods. The major advantages include its biodegradability (Coutte *et al.*, 2010:499-507; Nitschke and Costa, 2007:252; Yeh *et al.*, 2006:1799), low toxicity (Cayuela *et al.*, 1993:383; Nitschke and Costa, 2007:252; Yeh *et al.*, 2006:1799), environmental compatibility (Nitschke and Costa, 2007:252; Ongena and Jacques, 2008:115) along with the fact that government regulations favour their use (Holzmann, 2010:89). With the reduction in synthetic chemical use, the accumulation of chemicals in and on the produce is reduced or eliminated.

1.1.2 Limitations of the biocontrol strategy

In general the development and implementation of biocontrol strategies, whether organism- or product-based, are primarily hampered by high production costs (Abushady *et al.*, 2005:337-344; Akpa *et al.*, 2001:551-561; Davis *et al.*, 2001:346; Glare *et al.*, 2012:250). These high production costs are due to expensive substrates, product variability as well as complex and expensive downstream processing, including recovery and purification processes. In the past, these production costs were much higher than those associated with the development of a synthetic agent, which rather stimulated the production of synthetic agents instead of biological ones. An estimated cost associated with the development of a biocontrol agent in the USA is in the order of $ 3 – 5 million and takes about 3 years to finalize (Marrone, 2009). Furthermore, restrictions in some areas exist on the movement of products containing living organisms, i.e. importation and quarantine restrictions (Glare *et al.,*

2012:250). This problem, however, can be eliminated by using the lipopeptide itself instead of a mixture containing cells or spores. Other process limitations are excessive foaming during production, which is linked to aeration rate. This has been addressed by utilizing the foaming characteristics of the process, through foam fractionation for example, to increase product recovery (Chen *et al.*, 2006: 1915). Some of the other problems, i.e. high production costs for example, need to be addressed and researched further to ensure the economic viability of biosurfactant production on an industrial scale.

1.1.3 Organisms targeted by the biocontrol strategy

Some of the most prevalent plant pathogens that contribute to the major harvest losses include *Botrytis cinerea, Plasmopara viticola*, *Monilinia fructicola and Penicillium digitatum*. *B. cinerea* is a common pathogen in the grape and apple industry (Ongena and Jacques, 2008:115; Touré *et al.*, 2004:1151; Yanez-Mendizabal *et al.*, 2011:409) and is also known as “noble rot”. *P. viticola* is a grape downy mildew (Zezlina *et al.*, 2010:220), *M. fructicola* is the cause of brown rot on stone fruit such as apples while *P. digitatum* is the pathogen that leads to green mould on citrus (Yanez-Mendizabal *et al.*, 2011:409).

1.2 Biosurfactants

1.2.1 Classification, origin and structure

Biosurfactants are primarily classified according to their microbial origin as well as chemical structure. Specific organisms are known to produce specific biosurfactants that have unique properties depending on the structure of the biosurfactant molecule (Table 1-1).

In general, biosurfactant molecules consist of both a hydrophobic and a hydrophilic moiety. The hydrophobic functional group consists of either fatty-, saturated- or unsaturated acids, whereas the hydrophilic groups can consist of either amino acids or peptides, cations or anions, or polysaccharides (Desai and Banat, 1997:47-64). Biosurfactants can be classified into glycolipids, phospholipids, polymeric surfactants or lipopeptides and lipoproteins (Desai and Banat, 1997:47-64).

Table 1-1: Biosurfactants and their common producers

Biosurfactant	Organisms	References
Glycolipids		
Rhamnolipids	*Pseudomonas aeruginosa*	(Abdel-Mawgoud *et al.*, 2010:1323–1336; Guerra-Santos *et al.*, 1986:443-448)
	Pseudomonas spp.	(Parra *et al.*, 1989)
Trehalolipids	*Rhodococcus erythropolis*	(Rapp *et al.*, 1979:491-503)
	Nocardia erythropolis	(Desai and Banat, 1997:47-64)
Sophorolipids	*Torulopis bombicola*	(Gobbert *et al.*, 1984:225-230)
	T. apicola	(Hommel *et al.*, 1987:199-205)
	T. pertophilum	(Cooper and Paddock, 1983:1426-1429)
Lipopeptides and lipoproteins*		
Surfactin	*Bacillus subtilis*	(Arguelles-Arias *et al.*, 2009; Besson and Michel, 1992:1013-1018; Coutte *et al.*, 2010:499-507; Stein, 2005:845)
	B. amyloliquefaciens	(Arguelles-Arias *et al.*, 2009; Yu *et al.*, 2002:955)
Fengycin	*B. subtilis*	(Arguelles-Arias *et al.*, 2009; Coutte *et al.*, 2010:499-507; Stein, 2005:845)
	B. amyloliquefaciens	(Arguelles-Arias *et al.*, 2009)
Iturin A	*B. subtilis*	(Aranda *et al.*, 2005:51; Besson and Michel, 1992:1013-1018; Stein, 2005:845)
	B. amyloliquefaciens	(Arguelles-Arias *et al.*, 2009; Yu *et al.*, 2002:955)
Subtilisin	*B. subtilis*	(Stein, 2005:845)
Peptide-lipid	*B. licheniformis*	(Javaheri *et al.*, 1985:698-700)
Gramicidins	*B. brevis*	(Marahiel *et al.*, 1979:49-55)
Polymeric surfactants		
Emulsan	*Acinetobacter calcoaceticus*	(Rosenburg *et al.*, 1979:402-408)
Biodispersan	*A. calcoaceticus*	(Rosenburg *et al.*, 1988:323-326)
Liposan	*Candida lipolytica*	(Cirigliano and Carman, 1984:747-750)
Carbohydrate-protein-lipid	*P. fluorescens*	(Persson *et al.*, 1988:1-4)
Phospholipids	*Thiobacillus thioxidans*	(Beebe and Umbreit, 1971:612-614)

*Lipopeptides are generally smaller molecules compared to lipoproteins, comprised of a lipid and peptide as opposed to a lipid and protein

1.2.1.1 Glycolipids

Glycolipids are lipids which consist of sugars combined with long-chain aliphatic or hydroxyaliphatic acids and are some of the best characterized surfactants (Desai and Banat, 1997:47-64). Some of the best known glycolipids include rhamnolipids (Abdel-Mawgoud *et al.*, 2010:1323–1336; Lang and Wullbrandt, 1999:22-32), trehalolipids (Rapp *et al.*,

1979:491-503) and sophorolipids (Gobbert *et al.*, 1984:225-230; Hommel *et al.*, 1987:199-205).

1.2.1.1.1 Rhamnolipids

Rhamnolipids are commonly produced by *Pseudomonas aeruginosa* and are probably the best characterized of the glycolipids. *Pseudomonas aeruginosa* is a Gram-negative bacterium and a well-known pathogen. It has been known to cause lung infections and can infect any part of the human body in people suffering from immunosuppression (Giamarellou, 2000:103). Thus, it is not an ideal antimicrobial producer when considering large-scale production.

Rhamnolipids are synthesised by linking one or two fatty acid molecules, which can be either saturated or unsaturated C_8-C_{12} alkyl chains, with one or two rhamnose molecules (Haba *et al.*, 2003:316-322). The type of rhamnolipid produced by *P. aeruginosa* depends on the strain, the carbon source (glucose, glycerol, *n*-alkanes and triglycerides) and the production method used, i.e. batch, fed-batch or continuous strategies (Abdel-Mawgoud *et al.*, 2010:1323–1336; Lang and Wullbrandt, 1999:22-32). The four predominant rhamnolipid homologues are commonly known as R1 – R4 (Figure 1-1). Both R1 and R2 are mono-rhamnolipids, having only one rhamnose group, while R3 and R4 are di-rhamnolipids, with two rhamnose groups linked through an O-glycosidic bond.

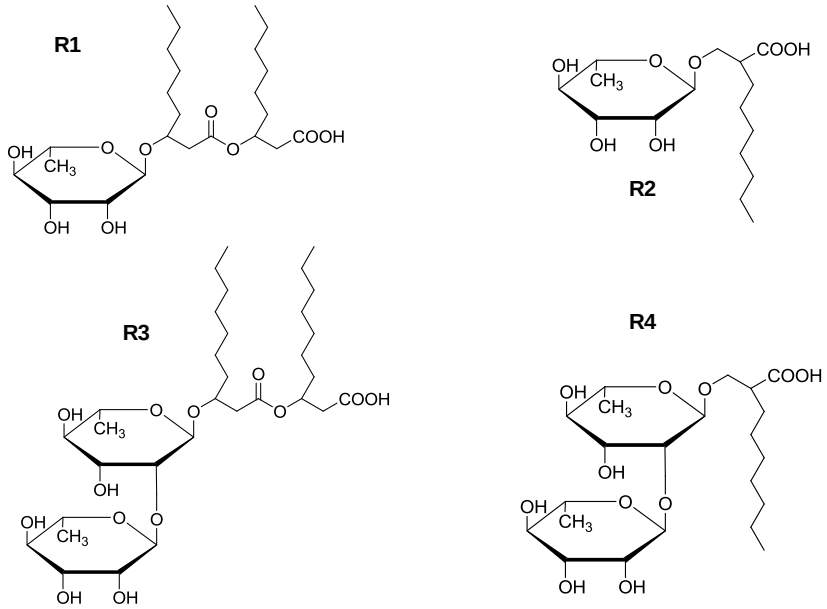

Figure 1-1: Rhamnolipid homologues (Lang and Wullbrandt, 1999)

1.2.1.1.2 Trehalolipids and sophorolipids

Trehalolipids and sophorolipids have similar structures to that of rhamnolipids. In trehalolipids, a disaccharide trehalose is linked at the C_6 position to two β-hydroxy-α-branched fatty acids (also known as mycolic acids) (Cooper and Zajic, 1980:229). Trehalolipids have been known to be produced by several species of *Mycobacterium, Nocardia* and strains of *Rhodococcus erythropolis* (Desai and Banat, 1997:47-64; Rapp *et al.*, 1979:491-503). The primary difference in trehalolipids produced by different organisms is the structure of the fatty acids and the degree of unsaturation (Desai and Banat, 1997:47-64).

Sophorolipids consist of a dimeric carbohydrate sophoros molecule that is linked to a long-chain hydroxyl fatty acid (Desai and Banat, 1997:47-64). These compounds are primarily produced by yeasts such *as Torulopis bombicola* (Gobbert *et al.*, 1984:225-230), *T. petrophilum* (Cooper and Paddock, 1983:1426-1429) and *T. apicola* (Hommel *et al.*, 1987:199-205).

1.2.1.2 Lipopeptides

The majority of known lipopeptides are produced by *Bacillus* spp. including *B. subtilis*, *B. licheniformis, B. amyloliquefaciens* and *B. brevis* (Besson and Michel, 1992:1013-1018; Deleu *et al.*, 2008:2667; Javaheri *et al.*, 1985:698-700; Marahiel *et al.*, 1979:49-55; Razafindralambo *et al.*, 1997:6026-6031; Stein, 2005:845; Arguelles-Arias et al., 2009). *B. subtilis,* a rhizobacterium, is one of the most frequently used and best-studied of this family of organisms (Ongena and Jacques, 2008:115).

There are a number of advantages linked to the use of *B. subtilis* for lipopeptide production. These include its GRAS (generally recognised as safe) status, its ability to form spores, making it one of the best candidates in the development of biotechnology, as it can endure extreme environments, as well as its abundance and variety found in nature.

Lipopeptides are characterized by a fatty acid linked to an amino acid chain, which is generally hydrophilic, also known as a peptide. Lipopeptides have large, complex structures, as is the case with surfactin, iturin and fengycin, cyclic peptides primarily produced by *B. subtilis, B. amyloliquefaciens* and *B. licheniformis* (Coutte *et al.*, 2010:499-507; Deleu *et al.*, 2008:2667; Vanittanakom *et al.*, 1986:888-901; Arguelles-Arias et al., 2009) (Figures 1-2 to 1-4).

Even though there are structural variations within the surfactin family, the basic structure (Figure 1-2) is made up of heptapeptides that are interlinked with a β-hydroxy fatty acid, forming a cyclic lactone ring (Peypoux *et al.*, 1999:553-563). The fatty acid chain varies in length from C_{13} – C_{15}. Surfactins or closely related variants, such as lichenysin have been isolated from *B. subtilis* and *B. licheniformis* (Ongena and Jacques, 2008:115).

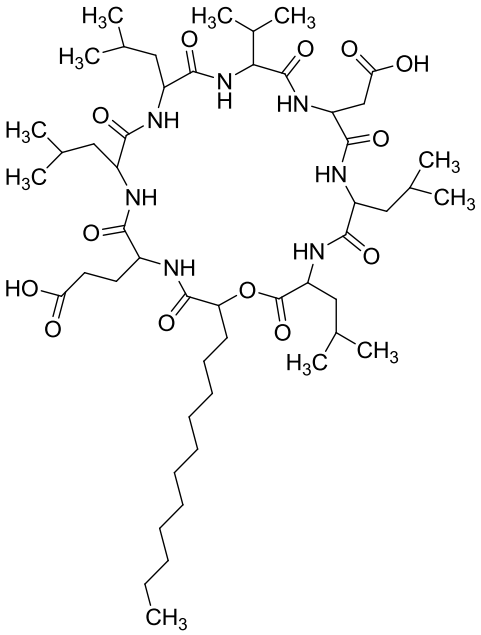

Figure 1-2: Surfactin molecule. Redrawn from Ongena and Jacques (2008) with modifications (ChemBioDraw 2008)

Apart from surfactin, *Bacillus* spp. are also known to produce two other lipopeptides which are of great interest, namely iturin and fengycin (Besson and Michel, 1992:1013-1018; Coutte *et al.*, 2010:499-507; Deleu *et al.*, 2008:2667; Razafindralambo *et al.*, 1997:6026-6031). The general structure of the iturin family consists of heptapeptides linked with a β-amino fatty acid chain of which the length can vary between 14 – 17 carbons (Figure 1-3) (Ongena and Jacques, 2008:115).

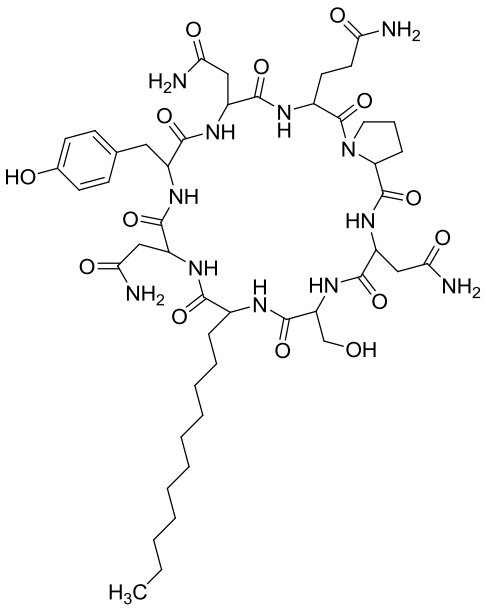

Figure 1-3: Iturin family molecule. Redrawn from Ongena and Jacques (2008) with modifications (ChemBioDraw 2008)

Fengycin molecules are larger than surfactins or iturins, with a general structure (Figure 1-4) consisting of a lipodecapeptide with an internal lactone ring in the peptidic moiety and with a β-hydroxy fatty acid chain (C_{14} – C_{17}) which can be either saturated or unsaturated (Ongena and Jacques, 2008:115). *B. amyloliquefaciens* has been shown to be a strong producer of fengycins and iturins (Arguelles-Arias et al., 2009; Caldeira *et al*., 2011).

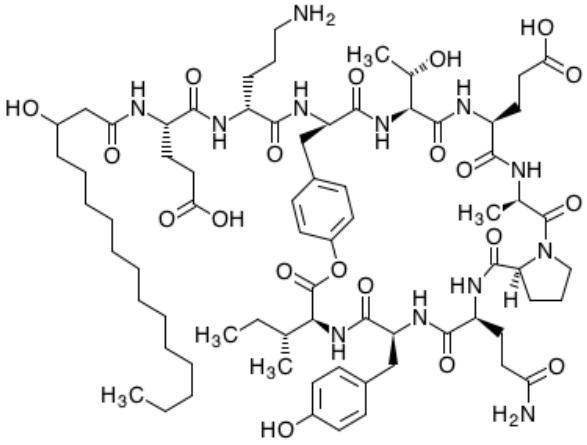

Figure 1-4: Fengycin family molecule. Redrawn from Ongena and Jacques (2008) with modifications (ChemBioDraw 2008)

The lipopeptide homologue produced, i.e. a C_{14} versus a C_{17} compound for example, usually depends on the organism being used, as seen from the several iturins and surfactins produced by *Bacillus amyloliquefaciens* CCMI 1051 (Caldeira *et al.*, 2011) as well as the 8

fengycins, ranging between C_{14} and C_{17} produced by *B. licheniformis* V9T14 (Pecci *et al*., 2010)

1.2.1.3 Phospholipids and polymeric surfactants

Phospholipids are well known as a major constituent of cell membranes and allow the formation of lipid bilayers. These lipids are made up of a fatty acid molecule which is linked to a cationic phosphate group. One of the best known producers of phospholipids is *Thiobacillus thiooxidans* (Beebe and Umbreit, 1971:612-614), while others include *Acinetobacter* spp. and *Aspergillus* spp. (Desai and Banat, 1997:47-64).

Polymeric biosurfactants are biological polymers consisting of a polysaccharide backbone, linked with fatty acid side chains (Desai and Banat, 1997:47-64). Emulsan, liposan and biodispersan are the best-studied members of this group (Rosenburg *et al.*, 1988:323-326) and are commonly produced by *Acinetobacter calcoaceticus* and *Candida lipolytica* (Cirigliano and Carman, 1984:747-750; Rosenburg *et al.*, 1979:402-408; Rosenburg *et al.*, 1988:323-326).

1.2.2 Antimicrobial biosurfactant mechanisms and applications

Some of the most sought after antimicrobial biosurfactants are glycolipids, especially rhamnolipids, and lipopeptides for their extremely effective activity against various target organisms, including *Staphylococcus* spp., *Mycobacterium* spp., *Botrytis cinerea*, *Plasmopara viticola* (Abdel-Mawgoud *et al.*, 2010:1323–1336; Stein, 2005:845).

1.2.2.1 Glycolipids

Of the three main glycolipids produced (section 1.2.1.1), rhamnolipids are of special interest. Studies have suggested that the rhamnolipid antimicrobial mechanism of action is similar to that of synthetic surfactants, i.e. targeting the cell envelope (Sotirova *et al.*, 2008:639-644). Here, the rhamnolipids insert themselves into the biological membrane and destroy it through their permeabilizing properties. Rhamnolipids have been shown to be very effective against both Gram-positive and Gram-negative organisms (Sotirova *et al.*, 2008:639-644)

1.2.2.2 Lipopeptides

Although *B. subtilis* has been stated to have the ability to produce more than two dozen antimicrobial compounds with a diverse range of structures (Stein, 2005:845), the cyclic lipopeptides are the ones that have demonstrated the most potential due to their strong surfactant properties (Ongena and Jacques, 2008:115). These cyclic lipopeptides include the well-documented surfactin, iturin and fengycin families. The production of these families and the ratios in which they are produced, depend on the type of *Bacillus* used as well as the strain thereof, for example *B. subtilis* ATCC 21332 can produce both surfactin and fengycin (Chtioui *et al.*, 2010:1795; Gancel *et al.*, 2009:975). The reported ratios of the different lipopeptides do however contradict one another, which is probably due to the different process strategies employed in the different studies.

As previously mentioned (section 1.2.1.2), other *Bacillus* strains are also known for producing lipopeptides. These include *B. licheniformis, B. amyloliquefaciens* and *B. brevis*.

1.2.2.2.1 Surfactin

Surfactins are the most active surfactant of the three cyclic lipopeptides, based on their ability to lower the surface tension of water from 72 to 27 nM/m (Stein, 2005:845). It is this surface activity that makes surfactins exceptional emulsifiers and foaming agents. They also display antibacterial, antiviral, antibiotic and anti-tumoral properties, along with being haemolytic, i.e. they induce the rupturing of red blood cells (Ongena and Jacques, 2008:115; Stein, 2005:845; Vollenbroich *et al.*, 1996:44-49). Notably, antifungal activity has not been investigated.

Cell membranes are composed of lipid bilayers. Lipid bilayers are arranged in such a way that the hydrophilic phosphate heads are on the outside, known as the outer leaflet, while the hydrophobic tails are pointed inward, forming the inner leaflet (Figure 1-5 a). Surfactin's amphiphilic nature allows it to associate readily with and securely anchor into lipid layers leading to interference with the membrane integrity in a dose-dependent way (Figure 1-5 b) (Ongena and Jacques, 2008:115). Studies (Heerklotz and Seelig, 2007:305-314; Ongena and Jacques, 2008:115) have shown that at low concentrations (surfactin-to-lipid mole ratio (R_b) < 0.04 in the membrane), surfactin only inserts into the outer leaflet of the membrane, leading to partial perturbation. At intermediate concentrations (0.05 < (R_b) < 0.1), transient membrane failure and permeabilization is induced, but the membrane appears to

re-anneal itself as the dosage is not high enough to penetrate the membrane far enough. Higher concentrations ($0.1 < (R_b) < 0.2$) lead to irreversible pore formation, caused by the insertion of surfactin-rich clusters into the membrane. The critical micelle concentration (CMC) is reached with a further increase in the surfactin concentration ($(R_b) > 0.22$), leading to total disruption and solubilisation of the lipid bilayer (Carrillo *et al.*, 2003:91; Heerklotz and Seelig, 2007:305-314; Ongena and Jacques, 2008:115).

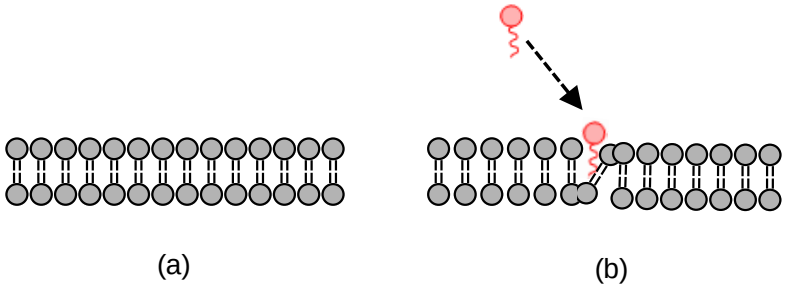

Figure 1-5: (a) Lipid bilayer and (b) how it is affected by surfactin

1.2.2.2.2 Iturin

Iturins demonstrate strong *in vitro* antifungal properties against various yeasts and fungi, with limited antibacterial abilities relative to surfactin (Moyne *et al.*, 2001:622; Phae *et al.*, 1990:1; Stein, 2005:845). The iturin bio-mechanism differs from that of surfactins. The fungi-toxicity demonstrated by iturins has been linked to their membrane permeabilization characteristics (Ongena and Jacques, 2008:115). It has been suggested that the underlying mechanism is based on osmotic perturbation caused by the formation of ion-conducting pores, unlike the membrane disruption and solubilisation caused by surfactins (Aranda *et al.*, 2005:51). Dosage levels weren't specified. Furthermore, the iturin family has also been found to have strong haemolytic properties (Stein, 2005:845).

1.2.2.2.3 Fengycin

Fengycins are less haemolytic than surfactins or iturins and have a strong fungi-toxicity against, specifically, filamentous fungi (Deleu *et al.*, 2008:2667; Stein, 2005:845; Vanittanakom *et al.*, 1986:888-901). The fengycin mechanism of action is less well-known than those of the other two lipopeptides discussed, but they have been found to interact readily with lipid layers and do, to a certain degree, have the potential to alter the cell

membrane structure and permeability in a dose-dependent way (Deleu *et al.*, 2005:358). It appears that fengycin interferes with the structural packing of the membranes during interaction. Deleu *et al.* (2005), using fengycin with a dipalmitoylphosphatidylcholine (DPPC) monolayer, reported that a fengycin molar ratio surpassing 0.66 dissolved the ordered phase of the lipid.

Both mechanisms associated with the iturin family (section 1.2.2.2.2) and fengycin family (section 1.2.2.2.3) are what afford these compounds their efficacy against fungi, making them ideal candidates for biocontrol strategies against phytopathogens such as *Botrytis* or *Penicillium digitatum* (section 1.1.3). This is especially true for the fengycin family with its efficacy against filamentous fungi (Ongena and Jacques, 2008:115; Caldeira *et al.,* 2011).

1.2.3 Lipopeptide initiation phase

Biosurfactant production kinetics differs greatly depending on the system parameters, such as the micro-organism and reactor geometry employed during production. This has led to controversies in literature regarding the phase with which lipopeptide production is associated, i.e. whether lipopeptides are primary (growth-associated) or secondary metabolites. Primary metabolites are produced during the growth or exponential phase, whereas secondary metabolites tend to be produced toward the end of the exponential/start stationary phase. Secondary metabolite production is also usually associated with nutrient limitation (Desai and Banat, 1997:47-64).

As lipopeptide production is generally considered to be a defence mechanism, the more apparent deduction is that they would be secondary metabolites. Researchers, on the other hand, have stated growth-associated production with *B. subtilis* C9 (Kim *et al.*, 1997:41), *B. subtilis* ATCC 6633 (Reis *et al.*, 2004:899) as well as *Bacillus* sp. IAF 343 and *B. cereus* IAF 346 (Cooper and Goldenburg, 1987:224). Production of lipopeptides as secondary metabolites have been shown with *B. subtilis* ATCC 21332 (Davis *et al.*, 1999:322) as well as *B. amyloliquefaciens* GA1 (Arguelles-Arias *et al.*, 2009). Further investigation is required into these contradictory results as these results will influence the optimum production method, i.e. batch, fed-batch or continuous culture, that will be employed for lipopeptide production (section 1.4.1.1).

1.3 Process conditions

Biosurfactant production is influenced by the environment in which organism growth and product synthesis occurs. The primary conditions influencing growth and production are the nutrients, which include the substrate, nitrogen source and metal ions and physiological conditions, which include temperature, pH, dissolved oxygen and agitation.

1.3.1 Nutrients

1.3.1.1 Substrate

One of the key components required in biosurfactant production is an appropriate carbon source. Primary carbon sources that can be utilized include simple carbohydrates and vegetable oils, however hydrocarbons have also been used (Kim *et al.*, 1997:41). One of the primary differences between simple carbohydrates and hydrocarbons is their solubility in water, i.e. simple carbohydrates are soluble while hydrocarbons and oils are not. It has been suggested that hydrocarbon utilization is more energy intensive and subsequently results in less energy being available for cell growth and surfactant production (Kim *et al.*, 1997:41).

High cell- and biosurfactant concentrations from *B. subtilis* are generally (Yeh *et al.*, 2006:1799) associated with carbohydrate substrates, such as glucose, sucrose and starch (Abushady *et al.*, 2005:337-344; Kim *et al.*, 1997:41; Makkar and Cameotra, 1997:37). Glucose is one of the most frequently used carbon sources, especially for the production of surfactin, fengycin and iturin A (see Table 1-2). Even though a wide range of glucose concentrations has been used, from 2 – 60 g/L (Abushady *et al.*, 2005:337-344; Chen *et al.*, 2006:1923), 40 g/L has been found to be the optimum concentration for the production of surfactin (Abushady *et al.*, 2005:337-344; Kim *et al.*, 1997:41). The literature suggests that this optimum concentration is not strain specific. The initial glucose has a notable effect on the maximum surfactant concentration as seen from 136 mg/L surfactin produced with glucose 2 g/L by Chen *et al*. (2006) compared to the other studies that achieved more than 4 times the amount of surfactin using higher concentrations (Fernandes *et al.,* 2007:704; Sen and Swaminathan, 1997:358) (Table 1-2).

The utilization of various vegetable oils by some *B. subtilis* strains (Abushady *et al.*, 2005:337-344; Kim *et al.*, 1997:41) has shown that the subsequent growth and surfactin concentrations obtained from these oils were notably lower than the corresponding values obtained from carbohydrate utilization. Abushady *et al.* (2005) reported surfactin

concentrations ranging from 1000 – 1500 mg/L, obtained from vegetable oils, as opposed to the 2000 – 2750 mg/L obtained from carbohydrates under the same conditions. This study was not strain specific, as two different strains of *B. subtilis* BBk1 were used (AB01335-1 and AB02238-1).

Glycerol, a waste product from soap-making and biodiesel production, has shown promise as an alternative carbon source to those mentioned above. One of the major advantages of using glycerol is actually twofold. Firstly, substrate costs could be decreased, depending on the grade of the glycerol and secondly, by using a waste-product, the process creates feed-stock opportunities. It has been reported that certain *B. subtilis* strains can utilize glycerol and, in fact, prefers glycerol to other sources such as glucose or maltose (Jamil *et al.*, 2007:26-31; Wiame *et al.*, 1953:627 - 628). Both strains MH-4 (Jamil *et al.*, 2007:26-31) and ATCC 9524 (ATCC, 2012) have been reported to utilize glycerol. Unfortunately neither utilization nor the actual surfactant concentrations have been reported.

Even with identical carbon (and accompanying nitrogen) concentrations, there can be a prominent difference in the maximum biosurfactant- and cell growth concentrations as seen from the 2600 mg/L surfactin reported by Wei and Chu (2002), the 3500 mg/L reported by Wei *et al.* (2003) and the 6450 mg/L reported by Yeh *et al.* (2006) (Table 1-2), all using the same organism strain (ATCC 21332). These differing results are due to a difference in the other nutrients added to the growth media as well as the production methods employed, which will be discussed in the subsequent sections (see sections 0 and 1.4.1.1).

1.3.1.2 Nitrogen source

Both inorganic and organic nitrogen sources can be used for synthesis of *B. subtilis* biosurfactants, but it has been found that inorganic nitrogen sources yield higher biosurfactant concentrations compared to organic sources (Makkar and Cameotra, 1997:37). Biosurfactant concentrations of 731 mg/L and 724 mg/L were produced from KNO_3 and $NaNO_3$ respectively, compared to the 327 mg/L and 458 mg/L biosurfactant concentrations obtained from peptone and yeast extract respectively (Makkar and Cameotra, 1997:37). Furthermore, studies have shown that NH_4NO_3 and $NaNO_3$ resulted in higher yields compared to other inorganic sources such as NH_4Cl and NH_4HCO_3 (Abushady *et al.*, 2005:337-344). Biosurfactant concentrations of around 2750 mg/L and 1950 mg/L were obtained from NH_4NO_3 and $NaNO_3$ respectively compared to other inorganic sources (like

NH_4Cl and NH_4HCO_3) that yielded concentrations of around 1500 mg/L and lower from equivalent amounts of the various nitrogen sources (Abushady *et al.*, 2005:337-344).

NH_4NO_3 is one of the inorganic nitrogen sources used most frequently for *B. subtilis* (Table 1-2). Abushady *et al*. (2005) stated that surfactin concentration increased from approximately 1500 to a maximum of 2200 mg/L as the NH_4NO_3 concentration increased from 1.6 g/L to 9 g/L, with production reaching its maximum at 4.6 g/L.

1.3.1.2.1 Anaerobic growth of *Bacillus* spp. in the presence of nitrate

B. subtilis was believed to be a strict aerobe up to 1993, when Priest found it could in fact grow under anaerobic conditions (Nakano and Zuber, 1998:165 - 190). Previous studies have found that *B. subtilis* can grow *via* anaerobic respiration, where nitrate is utilized as the terminal electron acceptor instead of oxygen, as is the case with aerobic respiration (Glaser *et al.*, 1998:1112 - 1115; Hoffmann *et al.*, 1998:186 - 189; Nakano and Hulett, 1997:1-7; Nakano *et al.*, 1997:6749-6755). When operating in the absence of oxygen, it has been reported that nitrate is the preferred terminal electron acceptor because of its high midpoint redox potential (E'^0 = 430mV) (Nakano and Zuber, 1998:165 - 190). Nitrate respiration has also been stated as one of only two known possible forms of anaerobic respiration, the other being nitrite respiration (Nakano and Zuber, 1998:165 - 190).

Micro-organisms can utilize nitrate in two very distinct ways, through either assimilation or dissimilation (Painter, 1970:393). Assimilation (Figure 1-6) is the process where nitrate is reduced to ammonia, *via* nitrite, with subsequent nitrogenous cell constituents being produced (Glaser *et al.*, 1998:1112 - 1115; Painter, 1970:393). Dissimilation, also known as nitrate respiration, is an oxidative process where nitrate is used as the terminal hydrogen acceptor instead of oxygen (Painter, 1970:393). With dissimilation, the nitrogen is not incorporated into the cell constituents and the reduction end product yields any of the following (depending on the organism): nitrate, ammonia, nitrous oxide or nitrogen (Hoffmann *et al.*, 1998:186 - 189; Painter, 1970:393). A further distinction can be made between the two special cases of dissimilation, depending on the end product. In cases where nitrous oxide or nitrogen is formed, the process is known as denitrification (Painter, 1970:393) and in cases where ammonia is formed, the process is known as ammonificaiton (Hoffmann *et al.*, 1998:186 - 189). It has been suggested that *B. subtilis* is an ammonifying facultative aerobe, as studies have reported that during anaerobic growth no nitrous oxide or nitrogen was observed in the end products (Hoffmann *et al.*, 1998:186 - 189).

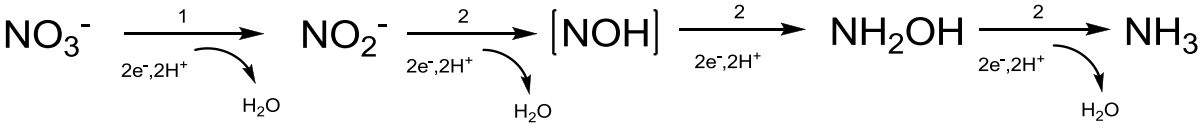

Figure 1-6: Assimilatory nitrate reduction pathway. Present in all bacteria reducing nitrate to ammonia for further conversion to nitrogenous cell constituents. Enzymes: 1 - nitrate reductase; 2 -nitrite reductase (Redrawn from White, 2007)

1.3.1.3 Metal ions

Certain metal cations, such as those from iron, manganese, magnesium and potassium have a notable effect on *B. subtilis* based biosurfactant production (Cooper *et al.*, 1981:408-412; Gancel *et al.*, 2009:975; Sheppard and Cooper, 1991:72 - 76; Wei *et al.*, 2007:40).

Adding iron to mineral salt mediums, for example $FeSO_4$, has been found to be a simple and very effective way of over-producing surfactin (concentrations increased almost 10-fold, compared to those deficient in iron) as well as increasing biomass concentrations (Wei and Chu, 1998:724). Unfortunately, the addition of the iron affects the pH of the medium, decreasing it, causing surfactin precipitation and the subsequent decrease of its concentration in the broth (Wei and Chu, 1998:724; Wei *et al.*, 2003:174). By adjusting the pH accordingly, keeping it above 6, this problem can be avoided (Wei *et al.*, 2003:174).

Manganese, Mn^{2+}, affects nitrogen utilization (by *B. subtilis*) by acting as a cofactor for several of the enzymes involved in nitrogen metabolism, including the reaction of glutamate and ammonia, catalysed by the glutamine synthetase enzyme (Sheppard and Cooper, 1991:72 - 76). Mn^{2+} addition has been found to increase surfactin production by almost 10-fold (Wei and Chu, 2002:479-482) as well as reduce the nitrogen requirement (Sheppard and Cooper, 1991:72 - 76) for ATCC 21332.

It has been suggested (Cooper *et al.*, 1981:408-412) that certain metal ions, specifically those from salt, such as $MgSO_4$, $CaCl_2$ and $ZrOCl_2$, had little to no effect on biomass or surfactin concentrations, while $ZnSO_4$ suppressed *B. subtilis* growth and others including $CuSO_4$, $CoSO_4$, $NiSO_4$ and $Al_2(SO_4)_3$ completely inhibited growth. This was most likely due to the toxic properties associated with some of these heavy metals, as demonstrated by Baek and An (2011). However, more recent studies (Wei and Chu, 2002:479-482; Wei *et al.*, 2007:40) have found that the surfactin concentration is notably decreased (by almost 75% compared to the control medium) in mediums that are Mg^{2+} and K^+ deficient, i.e. containing less than 10 mM K^+. The addition of Ca^{2+} has been found to have insignificant impact on cell

growth and surfactin production (by ATCC 21332) (Cooper *et al.*, 1981:408-412; Wei *et al.*, 2007:40).

Growth media compositions differ widely depending on the purpose of the study as well as the requirements of the organism and specific strain. These sometimes small differences have notable effects on the maximum biosurfactant concentrations, as seen by the surfactin concentrations reported by Wei and Chu (2002) (2600 mg/L) and Wei *et al.* (2003) (3500 mg/L). The experiments in both studies were performed under the same conditions (temperature, shaker speed, culture time, etc.) and with the same strain but with slightly different medium salt compositions (Table 1-2) in an attempt to optimize the growth medium. Wei and Chu (2002) investigated the effect of different metal salts ($CuSO_4$, $MnSO_4$, $MgSO_4$, $CoSO_4$ and $NiSO_4$) of which Mn^{2+} resulted in the highest increase in surfactin concentration. The optimum Mn^{2+} concentration for cell growth and maximum surfactin concentration was found to be 0.01 mM. Wei *et al*. (2003) on the other hand investigated the effect of iron-enrichment on ATCC 21332 cultures during surfactant production. Here Fe^{2+} was omitted from the defined medium (Table 1-2) to study its acidification behaviour and how it correlated with biosurfactant production. It was found that Fe^{2+} severely affects the pH of the broth, lowering it, causing surfactin to precipitate from the broth, in turn lowering its concentration. The maximum Fe^{2+} concentration allowable before precipitation occurs was reported to be 5 mM, which also yielded the highest surfactin concentration. The authors stated that the Fe^{2+} concentrations reported were much higher than expected, leading them to believe that the ATCC 21332 strain has a defect in iron transport, due to the production of sequestering compounds, subsequently making iron in the bulk unavailable.

1.3.1.4 Amino acids

Amino acid addition has been shown to have an effect on both the type of surfactant produced, i.e. surfactin or iturin for example (Besson and Michel, 1992:1013-1018) as well as the homologues that are produced (Youssef *et al*. 2005: 7690–7695). Activity has also been shown to be dependent on the fatty acid composition of the lipopeptide, allowing for the production of tailor-made product, by manipulating the structure of the lipopeptide.

Table 1-2: Growth media used for the production of biosurfactants from *Bacillus subtilis*

Reference	Carbon source	Inorganic nitrogen source	Organic nitrogen source	Other nutrients	C/N ratio	Max. biosurfactant concentration	Yield (Yp/x)
Abushady *et al.*, 2005:337-344	Glucose: 5 - 60 g/L	(In g/L) $(NH_4)_2SO_4$: 2 - 10, NH_4NO_3: 4.5 (optimum) , NH_4Cl, $(NH_4)_2S_2O_8$, NH_4Mo, $(NH4)_2HPO_4$, $NH_4H_2PO_4$, NH_4HCO_3, $NaNO_3$ (Concentrations not specified)	Urea, Beef extract, yeast extract, casein hydrolysate (Concentrations not specified)	$FeSO_4.7H_2O$: 0.5 – 8 mM/L (optimum:6), $MnSO_4.H_2O$: 10 – 60 mg/L (optimum 40)	11.8 (40g/L glucose, 4.5 g/L NH_4NO_3)	2750 mg/L (from glucose)	Not specified
Cooper *et al.*, 1981:408-412	Glucose: 40 g/L	NH_4NO3: 50 mM	None	KH_2PO4: 30 mM, Na_2HPO_4: 40 mM, $MgSO_4$: 0.8 mM, $CaCl_2$: 7µM, Na_2-EDTA: 4 µM, $FeSO_4$: 4 µM	13.3	Not specified	Not specified
Chen *et al.*, 2006:1923	Glucose: 2 g/L	(In g/L) NH_4Cl: 1	None	KH_2PO_4: 3 g/L, NaCl: 5 g/L, $MgSO_4$: 1 mM, $CaCl_2$: 0.1 mM	3.6	136 mg/L	0.262 g/g
Davis *et al.*, 1999:322	Glucose: 40 g/L	(In g/L) NH_4NO_3: 4	None	Not specified	4.4	439 mg/L	0.075 g/g
Fernandes *et al.*, 2007:704-709	Glucose: 40 g/L	(In g/L) $(NH_4)_2SO_4$: 8.5, $NaNO_3$: 8.5	None	KH_2PO_4: 4 g/L, K_2HPO_4: 13.6 g/L, $MgSO_4.7H_2O$: 0.5 g/L, Trace element solution: 10 mL - ($CaCl_2$: 0.42 g/L), ($FeSO_4.7H_2O$: 2.29 g/L), ($MnCl_2.4H_2O$: 0.10 g/L), ($ZnCl_2$: 0.17 g/L), ($CuCl_2$: 0.03 g/L), ($CoCl_2.6H_2O$: 0.06 g/L), ($Na_2MoO_4.2H_2O$: 0.06 g/L)	5.8	2000 mg/L	Not specified
Jamil *et al.*, 2007:26-31	Glycerol: not specified	(In g/L) L-glutamic acid: 5	None	KH_2PO_4: 0.5 g/L, K_2HPO_4: 0.5 g/L, $MgSO_4.7H_2O$: 0.2 g/L, $MnSO_4.H_2O$: 0.01 g/L, NaCl: 0.01 g/L, $FeSO_4.7H_2O$: 0.01 g/L, $CuSO_4.7H_2O$: 0.01 g/L,$CaCl_2.2H_2O$: 0.015 g/L	-	Not specified	Not specified

Table 1-2: Growth media used for the production of biosurfactants from *Bacillus subtilis* (continued)

Reference	Carbon source	Inorganic nitrogen source	Organic nitrogen source	Other nutrients	C/N ratio	Max. biosurfactant concentration	Yield (Yp/x)
Kim *et al.*, 1997:41	Glucose: 40 g/L, Soybean oil: NS, Hexadecane: NS, Glucose+SO: NS, Glucose+Hex: NS	NH_4NO_3: 50 mM	(In g/L) Yeast extract: 0.5	KH_2PO_4: 30 mM, Na_2HPO_4: 40mM, $MgSO_4$: 0.8 mM, $CaCl_2$: 7 µM, $FeSO_4$: 4 µM	< 13.3	4500 mg/L (from glucose)	Not specified
Makkar and Cameotra, 1997:37	Glucose: 20 g/L, Sucrose: 20 g/L, Sodium pyruvate: 20 g/L, Sodium acetate: 20 g/L, Trisodium citrate: 20 g/L, Dodecane: 20 (mL/L), Hexadecane: 20 (mL/L), Pristane: 20 (mL/L), Decane: 20 (mL/L), Kerosene: 20 (mL/L)	(In g/L) NH_4NO_3: 3, $(NH_4)_2SO_4$: 3, $NaNO_3$: 3, Other: 3	(In g/L) Urea: 3, Yeast extract: 20, Beef extract: 20	KNO_3: 3 g/L, NaCl: 0.01 g/L, $MgSO_4$: 0.6 g/L, $CaCl_2$: 0.04 g/L, $FeSO_4$: 0.02 g/L,Trace element solution: 0.1 mL - ($ZnSO_4.7H_2O$: 2.32 g/L), ($MnSO_4.4H_2O$: 1.78 g/L), (H_3BO_3: 0.56 g/L), (KI: 0.66 g/L), ($CuSO_4.5H_2O$: 1 g/L), ($Na_2MoO_4.2H_2O$: 0.39 g/L), ($CoCl_2.6H_2O$: 0.42 g/L), (EDTA: 1 g/L)	8.9 (glucose and NH_4NO_3)	744 mg/L (from sucrose)	0.352 g/g (from glucose)
Sen and Swaminathan, 1997:358 - 363	Glucose: 36.5	(In g/L) NH_4NO_3: 4.5	None	KH_2PO_4: 30 mM Na_2HPO_4: 40 mM, $MgSO_4$: 0.8 mM, $CaCl_2$: 7 µM, EDTA: 4 µM, $FeSO_4$: 14.5 µM, $MnSO_4$: 1.63 mM	10.8	1100 mg/L	Not specified
Wei and Chu, 2002:479-482	Glucose: 40	NH_4NO_3: 50 mM	None	KH_2PO_4: 30mM, Na_2HPO_4: 40 mM, $CaCl_2$: 7 µM, Na_2-EDTA: 4 µM, $FeSO_4.7H_2O$: 4 µM Optimum: 0.01 mM Mn^{2+} ($MnSO_4$)	13.3	2600 mg/L	Not specified
Wei *et al.*, 2003:174	Glucose: 40	NH_4NO_3: 50 mM	None	KH_2PO_4: 30 mM, Na_2HPO_4: 40mM, $CaCl_2$: 7 µM, Na_2-EDTA: 4 µM, $MgSO_4$: 7 µM	13.3	3500 mg/L	Not specified
Yeh *et al.*, 2006:1799	Glucose: 40	NH_4NO_3: 50 mM	None	KH_2PO_4: 30 mM, Na_2HPO_4: 30mM, $MgSO_4.7H_2O$: 0.8mM, $CaCl_2$: 7µM, Na_2-EDTA: 4 µM, $FeSO_4.7H_2O$: 2 mM	13.3	6450 mg/L	Not specified

**NS* indicates that amount was not specified

1.3.2 Physiological

The amount of dissolved oxygen (DO) available during growth and biosurfactant production, the geometry of the experimental setup as well as the agitation rate are important factors to consider. The oxygen transfer rate is a critical factor to ensure that the process does not become transport limited. Other important physiological factors to consider are the temperature and the pH of the system.

1.3.2.1 Dissolved oxygen

The fact that *B. subtilis* is a facultative anaerobe (section 1.3.1.2.1) has led to many studies on aerobic versus anaerobic growth and biosurfactant production. Unfortunately, these studies and their conclusions show strong contradictions on the importance of oxygen availability.

Cell growth is generally thought to be more favourable under aerobic rather than anaerobic conditions, as demonstrated by the maximum optical density of 3.00 under aerobic, compared to the 0.12 under anaerobic conditions reported by Clements *et al*. (2002). Studies (Abushady *et al.*, 2005:337-344) have shown that surfactin production is also enhanced as the volumetric oxygen concentration increases. A comparative study showed a surfactin concentration increase from 1200 mg/L at a volumetric oxygen percentage of 30% to about 2100 mg/L at 90% volumetric oxygen.

Contradictory studies (Davis *et al.*, 1999:322; Kim *et al.*, 1997:41) have found that cell growth and high surfactant concentrations are favoured by oxygen limitation. Davis *et al.* (1999) reported an almost 10-fold increase in surfactin concentration, from 45.3 to 439 mg/L, when the dissolved oxygen became depleted. Kim *et al*. (1997) (Table 1-3) also observed an increase in the maximum surfactin concentration, from 1300 mg/L under O_2-sufficient conditions compared to the 4500 mg/L under O_2-limited conditions. Unfortunately, no apparent reason for these contradictory studies has been proposed, creating the need for further investigation. The authors do not supply enough quantitative data regarding the effect of oxygen or oxygen limitation on antifungal production, which would allow for a kinetic comparison, necessitating further investigation.

Dissolved oxygen concentrations can be controlled more effectively and easily when using a reactor than in shake flasks. The majority of reactor studies in literature were performed under O_2-sufficient conditions (Chen *et al*. 2006: 1923; Davis *et al.*, 1999:322; Kim *et al.*, 1997:41) (Table 1-3), where the DO concentrations are maintained above the 20% saturation level. DO concentrations can be controlled, to an extent, by agitation.

1.3.2.2 Agitation

Increasing the agitation has been known to increase the K_La (volumetric oxygen transfer coefficient) (Yeh *et al.*, 2006:1799). Studies have found that the agitation rate, by influencing the oxygen transfer in the system, also influences the production of biosurfactant (Abushady *et al.*, 2005:337-344; Chtioui *et al.*, 2013). The effect of agitation speed was investigated (Abushady *et al.*, 2005:337-344) between 0 – 200 rpm. It was found that surfactin concentrations increased linearly (from 1000 mg/L to 1600 mg/L) only up to 150 rpm, followed by a smaller increase afterwards (to 1700 mg/L). The proposed reason for this was the increase in shear on the organisms at higher speeds. Yeh *et al.* (2006) also investigated the effect of agitation speed on surfactant production, between 200 – 350 rpm. The surfactin concentrations increased to 6450 mg/L (Table 1-3) with an increase in speed up to 300 rpm, after which the surfactin concentrations decreased to 1010 mg/L at 350 rpm. This decrease could be due to the extreme foaming caused by the high degree of agitation.

Chtioui *et al.* (2013) addressed the foaming issue by modifying a disc bioreactor. With this modified configuration, they were able to achieve similar K_La values (around 48 h^{-1}) to those reported by Yeh *et al.* (2006) with a maximum fengycin concentration of 787 mg/L and a maximum surfactin concentration of 108 mg/L. Even with the reduction in surfactin concentration compared to the 6450 mg/L achieved by Yeh *et al.* (2006), the modification to the bioreactor seemingly favours the production of fengycin, further supported by the 87.9% fengycin selectivity reported by Chtioui *et al.* (2013).

1.3.2.3 Temperature and pH considerations

Environmental conditions such as temperature and pH influence cell growth and biosurfactant production. Most experiments are performed at temperatures between 30°C and 37°C, with 30°C being the most common (Akpa *et al*., 2001:551; Chenikher *et al*., 2010:1800; Fernandes *et al*., 2007:704) (Table 1-3). Temperature optimization, with temperatures ranging between 23 - 48°C, for the strain RB14 has shown that the optimum temperature for surfactin production is 37°C and 25°C for iturin (Ohno *et al.*, 1995:517-519). This study found that surfactin concentrations increased with increasing temperature up to 37°C after which a decrease was observed, while iturin concentrations decreased as temperature increased above 25°C.

The pH of the broth has a major effect on the maximum biosurfactant concentration, as precipitation can ensue when the pH level drops below 5 (Makkar and Cameotra, 1997:37; Wei *et al.*, 2003:174). For this reason, the pH in most studies (Table 1-3) is kept above 6. The effect of pH variations (ranging from 4.5 – 10.5) has been investigated (Makkar and Cameotra, 1997:37) and found that pH = 7 is the optimum for biosurfactant production. As the pH increased from 4.5 to 7, the biosurfactant, similar to surfactin, concentration increased from 108 to 840 mg/L respectively. Further pH increases lead to decreased concentrations of 648 mg/L at pH = 10.5. This trend corresponds with that found in other studies (Wei *et al.*, 2003:174) (Table 1-3) where surfactin concentrations were reported to be 40 mg/L at pH = 4 and 1500 mg/L at pH = 6.1.

Table 1-3: Process conditions and modes of operation for *B. subtilis* cultures

Reference	Strain	Product	Carbon source concentration (g/L)	Method	Temperature (°C)	pH	Agitation (rpm)	Aeration (vvm)	Max. biosurfactant concentration (mg/L)	Yield (Yp/x) (g/g)
Akpa *et al.*, 2001:551-561	S 499	Iturins, Fengycin, Surfactin	20	Shake flask	30	7	130	Not specified	Not specified	Not specified
Chen *et al.*, 2006:1923	BBK 006	Surfactin	2	Shake flask/ Reactor (Batch)	30	7	200 (flask)	Not specified	92/ 136	0.071/ 0.262
Chen *et al.*, 2006:1915	BBK 006	Surfactin	0.25 - 2	Reactor (Continuous)	30	7	200	0.4	18	0.041
Chenikher *et al.*, 2010:1800	BBG 100/ ATCC 6633	Mycosubtilin (lipopeptide)	20 (initial), 0.001 (minimum), 40 (feed stream)	Reactor (Fed-batch)	30	6.5	200-500	0.75 L/h	120	Not specified
Chitioui *et al.*, 2013	ATCC 21332	Fengycin, Surfactin	20	Reactor (Rotating disc)	30	7	6 - 42	100 L/h	787 (fengycin) 108 (surfactin)	0.296
Davis *et al.*, 1999:322	ATCC 21332	Surfactin	40	Reactor (Batch)	32	7	220	0.5	439 (O_2 limited conditions)	0.075
Fernandes *et al.*, 2007:704-709	R14	Lipopeptide biosurfactant (similar to surfactin)	40	Shake flask	30	7	150	Not specified	2000	Not specified
Kim *et al.*, 1997:41	C9 (KCTC)	Lipopeptide biosurfactant (similar to surfactin)	40	Reactor (Batch)	30	6.8	300	0.1 or 1	4500 (O_2 depleted conditions)	Not specified
Wei *et al.*, 2003:174	ATCC 21332	Surfactin	40	Shake flask	30	Above 6	200	Not specified	3500	Not specified
Yeh *et al.*, 2006:1799	ATCC 21332	Surfactin	40	Reactor (Batch)	30	Not specified	200-350	0.5-1.5	6450	Not specified

1.4 Biosurfactant production

The upstream biosurfactant production methods are batch, fed-batch and continuous culture production.

1.4.1.1 Batch culture

Batch culture experiments are widely employed (Table 1-3), especially on lab-scale. This is due to their relatively easy setup with no additional considerations in terms of feeding rates (as is the case with fed-batch and continuous cultures). Batch cultures are also very useful as preliminary tests when considering the possibility of running fed-batch or continuous cultures.

Unfortunately, batch cultures tend to become nutrient-limited during cell growth and biosurfactant production, which (depending on the cell growth-phase required) could hamper biosurfactant production. As stated in section 1.2.3, controversy exists regarding the phase with which lipopeptide production is associated, i.e. whether they are primary or secondary metabolites. In the case of growth-associated products (Ohno *et al.*, 1995:517-519; Peypoux *et al.*, 1999:553-563; Yeh *et al.*, 2006:1799), fed-batch or continuous operations might be more suited, whereas batch conditions may be suitable for the production of secondary metabolites being produced toward the stationary phase (Jacques *et al.*, 1999:223-233; Touré *et al.*, 2004:1151). Batch experiments can be conducted in shake flasks as well as in bioreactors.

1.4.1.1.1 Shake flask experiments

Shake flasks are usually employed because of their ease of use, as a preliminary test for further bioreactor tests or when a bioreactor is not available. Operating conditions can be regulated more effectively in a bioreactor than in shake flasks (aeration, for example, is less controllable in a flask than in a reactor). Therefore shake flasks tend to result in lower biosurfactant concentrations when compared to bioreactors (Table 1-3) under the same conditions. This is demonstrated by the results reported by Chen *et al*. (2006) with a surfactin concentration of 92 mg/L from a shake flask, compared to the 136 mg/L concentration from a controlled bioreactor.

There have however been shake flask experiments that have shown high biosurfactant concentrations (Table 1-3). These however are due to media optimization and operating

condition manipulation (pH for example) as seen from the 2000 mg/L reported by Fernandes *et al.* (2007) and the 3500 mg/L reported by Wei *et al*. (2003).

1.4.1.1.2 Reactor experiments

With bioreactors, the operating conditions (aeration and pH for example) are more controlled than with shake flask cultures. This allows for more accurate measurements and results, as demonstrated by the comparative study in shake flasks and a bioreactor, performed by Chen *et al.* (2006). Here the flask experiment yielded 92 mg/L surfactin compared with 136 mg/L surfactin in the bioreactor. Media optimization and the manipulation of the production process (addition of solid carrier particles for example) can further increase the potential for maximal biosurfactant production, demonstrated by the 4500 mg/L biosurfactant, similar to surfactin, obtained by Kim *et al.* (1997) using a batch reactor configuration (Table 1-3).

1.4.1.1.3 Addition of solid carrier particles

The addition of certain solid carrier particles has been shown to stimulate growth as well as the production of biosurfactants (Chtioui *et al.*, 2010:1795; Gancel *et al.*, 2009:975; Yeh *et al.*, 2006:1799). The exact mechanism behind this phenomenon has not been elucidated to date, however a few theories have been proposed. One such theory is that the addition of these support particles increases the available surface area within the reactor (or flask) along with offering additional surface roughness, which is optimal for cell attachment (Chtioui *et al.*, 2010:1795). The surface hydrophilicity of the particles, as well as the increase in roughness contribute to the adhesion of the cells (Chtioui *et al.*, 2010:1795). Chtioui *et al*. (2010) also reported that the colonized surfaces showed an increase in surface wettability and hydrophilicity, in turn accelerating colonization by the hydrophillic strain (ATCC 21332) even further.

Furthermore, rhizobacterial microcolonies are generally considered to be a type of biofilm and it is widely assumed that microbial populations evolve and behave as biofilm communities on root and soil particles (Ongena and Jacques, 2008:115). This suggests that the addition of solid particles mimics the environment in which these organisms are found naturally.

These mechanisms were observed in literature, however, no clear, definitive mechanism has been proven as of yet.

Table 1-4: Solid particle supports for cell immobilization

Reference	Strain	Method	Support particles added	Product	Maximum biosurfactant concentration (mg/L)
Chtioui *et al.*, 2010:1795	ATCC 21332	Reactor	Polypropylene, Polybutene, Polyethane sulfone (PES), Polystyrene with thin layer activated carbon, Polypropylene with thin layer activated carbon	Surfactin	310 (Polypropylene with activated carbon)
				Fengycin	310 (Polypropylene with activated carbon)
Gancel *et al.*, 2009:975	ATCC 21332	Reactor	Light polypropylene coated with Fe^{2+}	Surfactin	360
				Fengycin	680
Yeh *et al.*, 2005:1329	ATCC 21332	Shake flask	Activated carbon, Expanded clay, Agar cubes	Surfactin	3600 (From 25 g/L activated carbon)
Yeh *et al.*, 2006:1799	ATCC 21332	Reactor	Activated carbon	Surfactin	6450

Certain types of solids have been found to be more effective than others in increasing cell growth as well as biosurfactant production. Coated polymer particles and activated carbon (Table 1-4) are frequently used materials for cell immobilization. With cell immobilization the biosurfactant concentrations vary from 310 – 6450 mg/L. Carrier particle addition has shown definite increases in the surfactant concentrations, as seen from the reported 4-fold increase in surfactin and 15-fold increase in fengycin concentration (Chtioui *et al.*, 2010:1795).

The ratio between different biosurfactants produced, specifically fengycin and surfactin, has also been shown to be influenced by the addition of the solid support particles (Gancel *et al.*, 2009:975; Yeh *et al.*, 2006:1799). The results from the experiments under batch conditions have the potential to be very useful in continuous culture experiments, where actual cell retention may be required to increase the cell concentration in the reactor.

Furthermore, immobilization is said to reduce the costs associated with downstream processing and has also been known to increase the stability of the isolated products (Jeanfils *et al.*, 1993:369). This makes the addition of these particles an attractive option when considering downstream processing.

1.4.1.2 Fed-batch culture

Fed-batch strategies entail the addition of nutrients to the culture broth, with an exponential feed rate, to manipulate the specific biomass growth rate (μ) (Chenikher *et al.*, 2010:1800; Guez *et al.*, 2007:67). Some of the key reasons behind fed-batch employment include keeping the specific growth rate constant, preventing the accumulation of toxic substrates as well as obtaining high cell densities (Lee *et al.*, 1999:29; Riesenberg *et al.*, 1991:17; Seok Oh *et al.*, 2002:747), especially if the required product is growth-associated (see section 1.4.1.1).

Studies (Chenikher *et al.*, 2010:1800; Guez *et al.*, 2007:67) have shown that the optimum specific growth rate for *B. subtilis* BBG100 is 0.05 – 0.07 h^{-1}. Guez *et al*. (2007) related the optimum specific growth rate to the maximum yield of mycosubtilin (1.27 mg/g dry biomass).

1.4.1.3 Continuous culture

Operating under continuous conditions would provide well-defined conditions, allowing for stable biomass, substrate and biosurfactant concentrations. Under these conditions, steady-state metabolism can be achieved and the process parameters can be adjusted to ensure optimum production of the desired biosurfactants. This operation method has the potential for high productivity, uncomplicated process control and more reliable product quality (Chen *et al.*, 2006:1915).

It has been reported that the dilution rate (D, h^{-1}) should be kept between 0.2 – 0.4 h^{-1} to prevent growth limitations due to maintenance metabolism (D < 0.2 h^{-1}) or cell washout (D > 0.4 h^{-1}) (Chen *et al.*, 2006:1915; Tsuchiya and Kimura, 1982:794-799), irrespective of the *B. subtilis* strain used. The maximum growth rate (μ_{max}) has been reported to be between 0.4 h^{-1} (BBK006 strain) (Chen *et al.*, 2006:1915) and 0.45 h^{-1} (KYA 741 strain) (Tsuchiya and Kimura, 1982:794-799). Surfactin production has been found to be at its optimum at lower dilution rates (0.1 – 0.2 h^{-1}) (Chen *et al.*, 2006:1915).

With continuous cultures, surfactin production increases with carbon-source depletion, even though the bacterial concentration decreases (Chen *et al.*, 2006:1915). At a constant dilution rate (D = 0.2 h^{-1}) a decrease in glucose concentration from 0.54 g/L to 0.25 g/L resulted in a surfactin concentration increase from about 8 to 18 mg/L.

Batch cultures result in higher maximum cell- and biosurfactant concentrations compared to continuous cultures (Table 1-3). It has been suggested that the lower surfactant production is due to the comparatively lower cell density in continuous cultures (Chen *et al.*, 2006:1915). Using the same experimental setup and growth medium, continuous cultures resulted in a maximum cell concentration of 290 mg/L compared to the 520 mg/L from batch cultures (Chen *et al.*, 2006:1915). This comparison was made at a dilution rate (*D*) of 0.2 h^{-1} where surfactin production was at its maximum.

1.4.2 Conclusions

Most studies have been focussing on the use of or direct application of living organisms as a control method, as seen with Serenade for example. The potential of cell-free supernatant, i.e. the biosurfactants themselves, has been shown to surpass that of the mixtures containing vegetative cells or endospores, compelling the need for further investigation into the efficacy of cell-free supernatant as a biocontrol agent. Using cell-free supernatant also eliminates the restrictions associated with the importation and quarantine of living organisms or products containing living organisms (section 1.1.2).

Bacillus lipopeptides have been found to be effective against a wide variety of phytopathogens, including *Botrytis cinerea, Monilinia fructicola and Penicillium digitatum*. As an application against fungal pathogens, the iturin and fengycin lipopeptides would be most effective and these are produced by an organism like *B. amyloliquefaciens, B. licheniformis* (Table 1-1) or *B. subtilis* ATCC 21332 (Table 1-1 and Table 1-3), depending on the operating conditions. Unfortunately, in terms of process optimization, most studies focus on surfactin, as it is the most well-documented and well-characterized of the three lipopeptides. This however, makes comparative studies relating to fengycin and iturin difficult.

The minimum requirements for an effective growth medium include a high enough concentration of an accessible carbon source, such as glucose (optimum concentration: 40 g/L); an available nitrogen source, such as nitrate; a buffer to control the pH; Fe^{2+}, ideally between 0.002 to 0.008 mM; Mn^{2+}, having an optimum concentration of 0.01 mM; K^{+}, at least 10mM as well as Mg^{2+}, usually between 0.6 to 3 mM (Wei *et al.,* 2007:40)

The phase with which lipopeptide production is associated needs to be identified, as this will affect the optimum operation configuration, i.e. batch, fed-batch or continuous

(sections 1.2.3 and 1.4.1.1), that will be employed. This is especially important when considering the potential commercial implementation of a process.

An accessible nitrogen source is vital to growth and lipopeptide production, but unfortunately controversy exists in literature regarding the optimum concentration of this source (section 1.3.1.2). Similarly, contradictory information is given regarding the effect that oxygen concentration and availability has on growth and lipopeptide production (sections 1.3.1.2.1 and 1.3.2.1).

If process conditions, such as substrate concentration, can be altered to optimize lipopeptide production, it stands to reason that altering process conditions can possibly alter the ratio in which the lipopeptide homologues are produced. If this is the case, this information can be used to optimize the production process to favour the production of a specific homologue, potentially creating a specialized, highly effective biocontrol agent.

Lipopeptide-based products have been shown to remain stable and viable for extended periods of time, as proven by the commercial production of products including Serenade. This however could be attributed to their inclusion of endospores. The effectiveness of a cell-free lipopeptide product needs to be researched.

Chapter 2

Hypotheses and objectives

Can *Bacillus* spp. lipopeptides be implemented in biocontrol strategies against phytopathogens such as *Botrytis cinerea* and *Penicillium digitatum*?

2.1 Hypotheses

The following hypotheses were constructed from the conclusions drawn from the literature review (section 1.4.2):

1) ***B. amyloliquefaciens* is the strongest potential antifungal producing candidate.**
B. subtilis, *B. amyloliquefaciens*, *B. subtilis* subsp. *spizizenii* and *B. licheniformis* are all antifungal lipopeptide producers.

2) **An optimum nitrate concentration exists for antifungal lipopeptide production.**
A readily available nitrogen source, such as nitrate, is crucial for *Bacillus* spp. growth and lipopeptide production and optimum conditions exist.

3) **The lipopeptides produced by the selected *Bacillus* spp. are secondary metabolites.**
The primary function of the lipopeptides is thought to be that of a survival mechanism, when one or more nutrient becomes limiting.

4) **An increase in oxygen supply concentration will increase growth as well as antifungal lipopeptide production.**
Bacillus spp. are facultative aerobes and will grow and produce better under oxygen rich conditions.

5) **Homologue ratios can be manipulated by altering process conditions.**
This includes the composition of the growth medium or the availability of oxygen.

6) **Lipopeptides remain stable over extended periods of time.**
Lipopeptides are not volatile compounds and should remain in a stable condition during freezing.

7) *Bacillus* spp. lipopeptides are effective against phytopathogens such as *Botrytis cinerea* and *Penicillium digitatum*.

Fengycin, and to a lesser extent iturin, produced by *Bacillus* spp. are effective against filamentous fungi.

2.2 Objectives

1) Quantify maximum growth rates, biomass production, maximum lipopeptide production (focussing on antifungal lipopeptide), product yields and productivity for each *Bacillus* spp. to determine the best candidate for antifungal production and use in further experiments.

2) Evaluate the effect of nitrate, specifically NH_4NO_3, concentration on biomass production, maximum growth rates and antifungal lipopeptide production. Determine the optimum nitrate concentration for the kinetic parameters, including product yields and productivity.

3) Investigate the phase with which lipopeptide production is associated, i.e. whether the lipopeptides are primary or secondary metabolites, by assessing nutrient utilization profiles.

4) Evaluate the effect of oxygen availability on biomass production, antifungal lipopeptide production, growth rate, product yield and productivity under enriched air conditions.

5) Quantify the lipopeptides production from selected *Bacillus* spp., specifically regarding the ratios in which the different lipopeptides and their homologues are produced. Determine whether this information can be used to alter the process strategy to favour the production of a specific lipopeptide or homologue.

6) Determine the stability of lipopeptides after prolonged periods of being frozen. Quantify this in terms of lipopeptide (antifungal and surfactin) concentrations before and after freezing.

7) Evaluate the efficacy of the biosurfactants against the selected target organisms.

Chapter 3

Materials and methods

3.1 Micro-organisms and culture maintenance

Lipopeptide production experiments were performed using four different *Bacillus* strains, namely *B. subtilis* ATCC 21332, *B. amyloliquefaciens* DSM 23117, *B. subtilis* subsp. *spizizenii* DSM 347 and *B. licheniformis* DSM 13. Freeze-dried cultures were obtained from the American Type Culture Collection (ATCC) as well as the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

Strain revival was done by adding 1 mL nutrient broth media to the applicable freeze-dried cultures, followed by incubation at 30°C for 30 minutes. Nutrient agar plates were then streaked, aseptically, with 100 µL from the applicable culture tube and incubated for a further 24 hours. Following incubation, the plates were stored at 4°C.

Botrytis cinerea DSM 877 and *Penicillium digitatum* DSM 2751 were used and *Botryotinia fuckeliana* was isolated and characterized by Mr. J. van Rooyen (BSc. (Hons.) Biochemistry, University of Stellenbosch) from fruit collected from the surrounding area. The fungi were maintained on potato dextrose agar (PDA) plates and also stored at 4°C.

3.2 Growth medium

The growth medium for the *Bacillus* spp. was defined from the collated literature data (sections 1.3.1 and 1.4.2). Modifications were made with the addition of 0.5 g/L yeast extract and an increase in buffer capacity and K^+ concentration. Table 3-1 shows the resulting modified growth medium.

Table 3-1: Modified growth medium for *Bacillus* spp.

Component	Concentration (g/L)	Concentration (mM)
Glucose	40	
NH_4NO_3	4	50
Na_2HPO_4 (Buffer)	7.098	50
KH_2PO_4 (Buffer)	6.805	50
$MgSO_4.H_2O$	0.332	2.4
$MnSO_4.H_2O$	0.0017	0.01
$FeSO_4.7H_2O$	0.002	0.008
$CaCl_2.4H_2O$	0.001	0.007
Yeast extract	0.5	

The buffer capacity of the optimized medium (Table 3-1) was increased to 100 mM from 6.7 mM and the K^+ concentration was also increased to 50 mM compared to the 10 mM in the control medium. Na_2HPO_4 was used as a replacement buffer, instead of K_2HPO_4 due to the high buffer requirement and the detrimental effect that too high a K^+ concentration could have on both growth and surfactant production.

3.3 Experimental procedures

3.3.1 Inoculum development

A two-stage inoculum procedure was followed. Two loops of the relevant *Bacillus* spp. were transferred aseptically from nutrient agar plates to 50 mL of the optimized media (Table 3-1), prepared in 100 mL Erlenmeyer flasks. Inocula were incubated for 12 hours at 30°C and 150 rpm (Labcon Orbital Shaker). Subsequently, 15 mL (10% v/v) of the first stage was transferred aseptically to 135 mL of the optimized growth media in a 500 mL baffled Erlenmeyer flask. The second stage was incubated for a further 8 hours at 30°C and 150 rpm. For the bioreactor studies, the reactor was inoculated (with 6 mL of the second stage in 994 mL of sterilized optimized growth medium) to have a start-up OD of 0.1.

3.3.2 Shake flask experiments

Shake flask experiments were used prior to the bioreactor experiments that were performed. The shake flask experiments were conducted in 1 L Erlenmeyer flasks. These were carried out in duplicate in flasks containing 180 mL of the desired medium inoculated with 20 mL of the second stage (10% v/v). The flasks were incubated at 30°C and 150 rpm.

3.3.3 Bioreactor experiments

All reactor-based batch experiments were performed in a 1.3 L instrumented modular bench-top reactor (BioFlo 110, New Brunswick Scientific) with a maximum working volume of 1 L. The temperature was controlled at 30°C using a heating-jacket and cooling water flowing through an internal unit. The pH was initially set to a value of 7 and allowed to decrease no lower than 6.8. This was achieved through the controlled addition of sterile 2 M $NaOH_{(aq)}$, set

on an automatic peristaltic pump. The DO concentration was monitored throughout, an aeration rate of 0.8 vvm was used and agitation was set at 250 rpm.

Temperature, pH (Mettler Toledo) and DO probes (Mettler Toledo) were used for the experiments. A 10% (v/v) solution of sterile Antifoam A (30% aqueous polymer emulsion, Fluka) was added to the broth at intervals as required, by means of a peristaltic pump to reduce foaming.

3.4 Analytical methods

3.4.1 Cell concentration

The cell concentration was determined *via* two methods; cell dry weight (a direct method) as well as absorbance measurements (an indirect method). These methods are discussed in the following sections (section 3.4.1.1 and 3.4.1.2).

3.4.1.1 Cell dry weight (CDW)

Shake flasks were prepared as described in sections 3.3.1 and 3.3.2 with the exception of the growth time allowed for the second stage. To ensure that the culture's cell density was high enough to allow a dilution series with at least five data points, the second stage inoculum was incubated for 24 hours. A dilution series was then prepared. 15 mL samples were taken at each dilution and then filtered to separate the cell mass from the supernatant. A Buchner vacuum filter (Millipore®) lined with 0.2 µm (pore size) filter paper disks (Anatech®) was used for the filtration. Before use, the filter paper disks were dried in a vacuum oven (SHEL LAB) at 60°C for 24 hours, cooled in a desiccator and weighed to 4 decimal places (OHAUS adventurer®). After filtration, the wet filter paper was dried again in a vacuum oven at 60° for 24 hours. After drying and cooling (in a desiccator) the filter paper was weighed, as before. The difference in weight (before and after filtration) represents the CDW.

3.4.1.2 Absorbance

The CDW data (3.4.1.1) were then calibrated against the broth absorbance, using a UV-visible spectrophotometer (Varian®), as represented in Figure 3-1. This allows the indirect

method (absorbance) to be correlated with the direct method (CDW). This allowed for the use of the quicker, easier indirect method (absorbance) to determine cell concentration during sampling procedures, after which the absorbance could be related back to CDW.

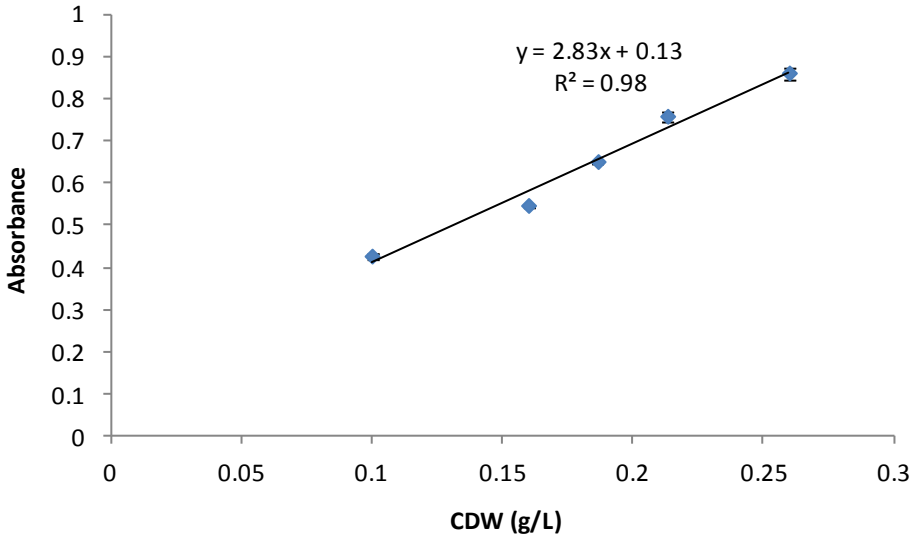

Figure 3-1: CDW calibration curve for *B. amyloliquefaciens*. Data points represent mean of the absorbance measurements taken for each CDW value. Error bars represent standard deviation

Absorbance measurements taken, in duplicate, at a wavelength of 621 nm (visible light region) were found to have the least amount of interference from the yellow-brown colouration of the growth medium. The following procedures were followed:

A 1 mL sample was withdrawn from the test flask (or bioreactor) and pipetted into a micro-centrifuge tube. The sample was centrifuged (Eppendorf® Minispin Plus) for 5 min at 14 500 rpm. The supernatant was separated from the cell pellet and the pellet was re-suspended in 1 mL physiological saline (0.85% m/v NaCl solution) *via* vortexing. A sterile physiological saline sample was used as the blank for the cell density measurements. The absorbance of the suspension was then measured with the spectrophotometer at 621 nm. The linear region of the absorbance versus cell concentration graph could therefore be determined. In the case of an absorbance value exceeding 0.80, the sample was diluted to ensure that the measurement remained within the linear region (Figure 3-1).

3.4.2 Glucose concentration

Glucose concentrations were determined by High Pressure Liquid Chromatography (HPLC). The HPLC specifications are seen in Table 3-2.

Table 3-2: HPLC specifications for glucose concentration determination

HPLC specifications	
Column	Aminex HPX-87H column equipped with a Cation-H Micro-Guard Cartridge (Bio-Rad, Johannesburg, South Africa). Column temperature was set to 65°C
Mobile phase	5 mM sulphuric acid
Sample volume	20 µL
Flow rate	0.6 mL/min
Detector	RI detector (Shodex, RI-101)

A pure D-Glucose (Sigma-Aldrich®) standard curve was constructed with standards ranging from 0.15 to 10 g/L in concentration (Figure 3-2).

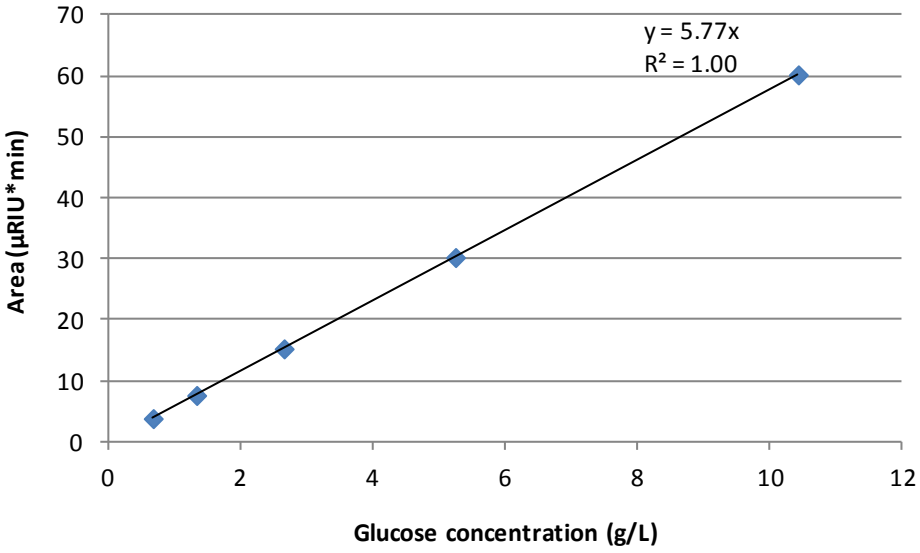

Figure 3-2: D-Glucose standard curve

The glucose samples taken from the shake flasks or the reactor were prepared as follows:

A 1 mL sample was transferred to an Eppendorf® micro-centrifuge tube *via* pipette. The sample was centrifuged at 14 500 rpm for 5 min to separate the cells from the supernatant. Then, the supernatant was filtered through a 0.22 µm syringe filter to ensure that all the cells were separated from the supernatant. 300 µL of the filtered supernatant were added to 900 µL milliQ water to ensure a 4-fold dilution. The sample was filtered through a 0.22 µm syringe filter and 20 µL was injected onto the HPLC column.

3.4.3 Nitrate concentration

Nitrate concentrations were analysed with an Ion Chromatograph (Dionex) equipped with a conductivity detector. The following settings were used for the chromatograph (Table 3-3):

Table 3-3: Ion chromatograph specifications

Separator column	IonPac AS4A-SC 4mm
Mobile phase	1.80 mM Na_2CO_3/ 1.70mM $NaHCO_3$
Flow rate	1.0 mL/min
Regenerant	25 mM H_2SO_4

Prior to the nitrate sample analyses, a nitrate standard curve was constructed (Figure 3-3).

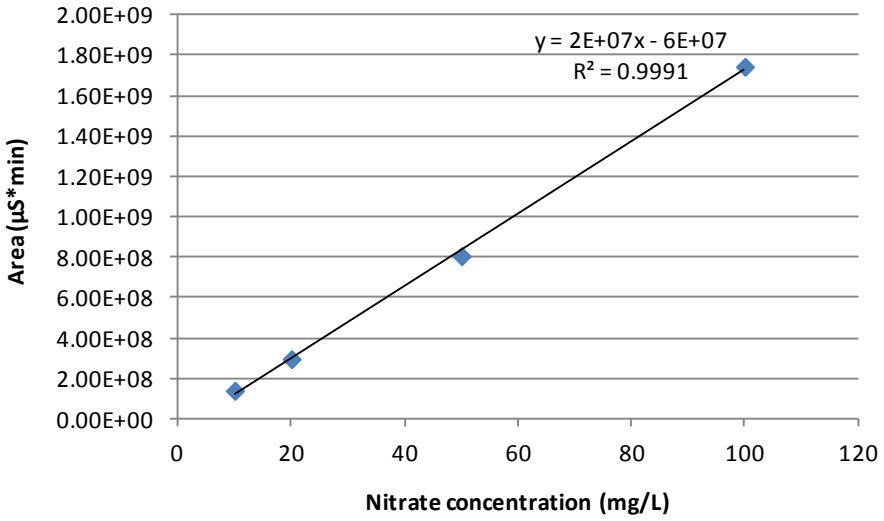

Figure 3-3: Nitrate standard curve

The procedures for the nitrate sample analyses were as follows:

Culture broth samples were centrifuged for 5 min at 14 500 rpm to separate the cells from the supernatant. The supernatant was then filtered through a 0.22 µm syringe filter to ensure that all the cells had been removed. To prevent the detector becoming saturated, the filtered supernatant was diluted 100-fold. A 50 µL sample of the diluted supernatant was injected onto the column. Sample peak areas were compared to those obtained from the calibration, using it as a concentration reference.

3.4.4 Lipopeptide concentration

3.4.4.1 Surfactin concentration

The surfactin concentrations were also determined *via* HPLC. The HPLC specifications are seen in Table 3-4.

Table 3-4: HPLC specifications for surfactin concentration determination

HPLC specifications	
Column	Phenomenex Luna 3µm C18 column (250 x 4.6 mm)
Detector	Dionex Ulltimate 3000 Diode-array detector
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
Absorbance	210 nm

Using pure surfactin obtained from Sigma-Aldrich®, standards were prepared and a standard concentration curve (Figure 3-5) could be constructed from the six main peaks observed. These peaks corresponded to the different surfactin homologues at their respective retention times. Note that peaks 5 and 6 were grouped together for analyses (Figure 3-4).

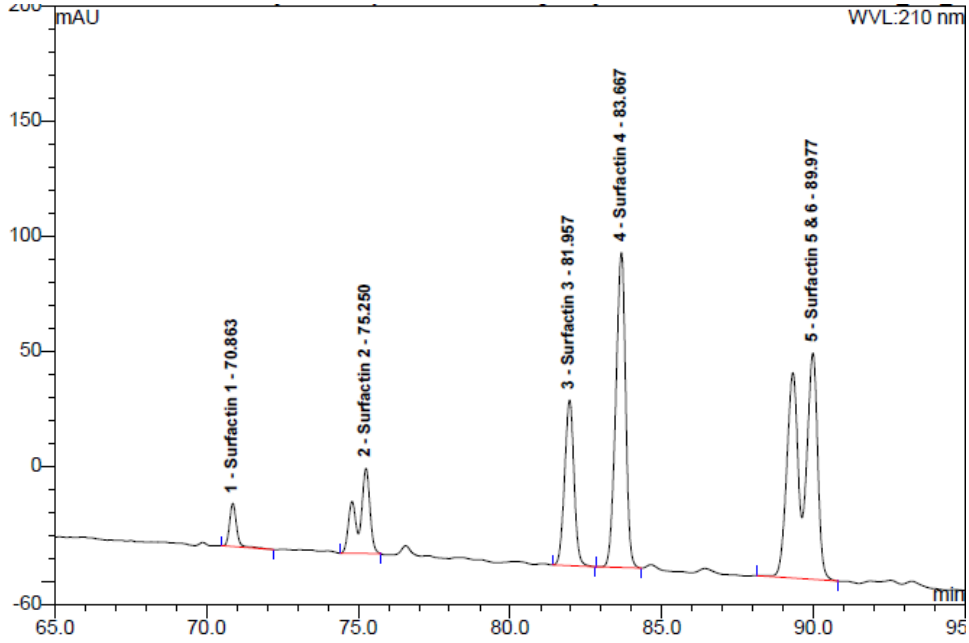

Figure 3-4: Surfactin standard chromatogram

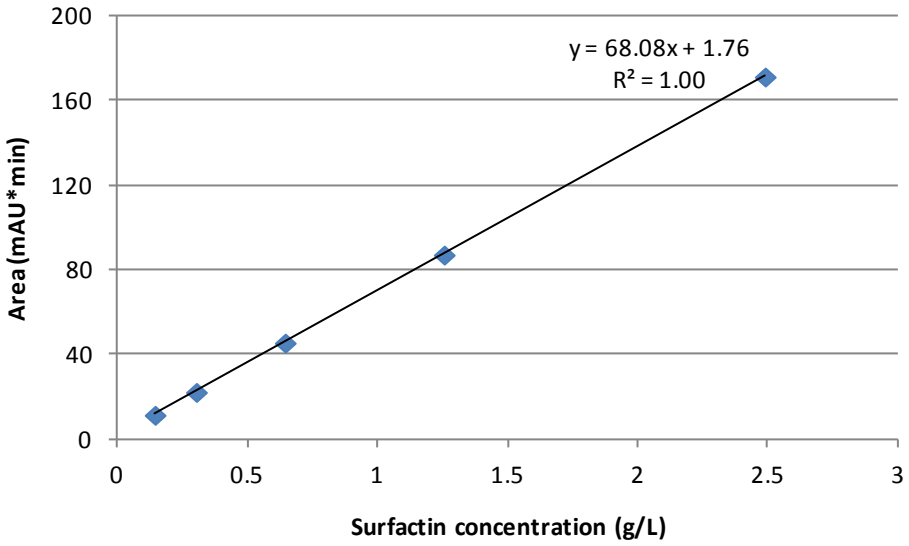

Figure 3-5: Surfactin standard curve

Surfactin concentration in the broth samples was determined as follows:

Samples were centrifuged for 5 min at 14 500 rpm to separate the cells from the supernatant. The supernatant was then filtered through a 0.22 µm syringe filter to ensure that all the cells had been removed. 500 µL of the filtered supernatant were added to 500 µL acetonitrile mixture (80% acetonitrile, 0.05% trifluoroacetic acid). The mixture was then filtered through a 0.22 µm syringe filter after which 50 µL of the supernatant/acetonitrile

mixture were injected onto the column. The eluent absorbance was monitored at 210 nm. The sample absorption peak areas could be compared to those obtained from the pure standards in order to determine the surfactin concentration.

3.4.4.2 Antifungal concentration

The antifungal concentrations were determined using the same sample preparation steps and HPLC method that were used for surfactin quantification (section 3.4.4.1). Standards were available for iturin (Sigma-Aldrich), but unfortunately none could be obtained for fengycin, either commercially or through research groups. Antifungal quantification was therefore done in terms of the peak areas produced in HPLC analyses. Once a pure fengycin standard is available, the area can be converted directly to a g/L quantity. It should be noted that even though an iturin standard (Figure 3-6) is available from Sigma-Aldrich, this cannot be used as it does not quantify the entire range of iturin homologues that are produced.

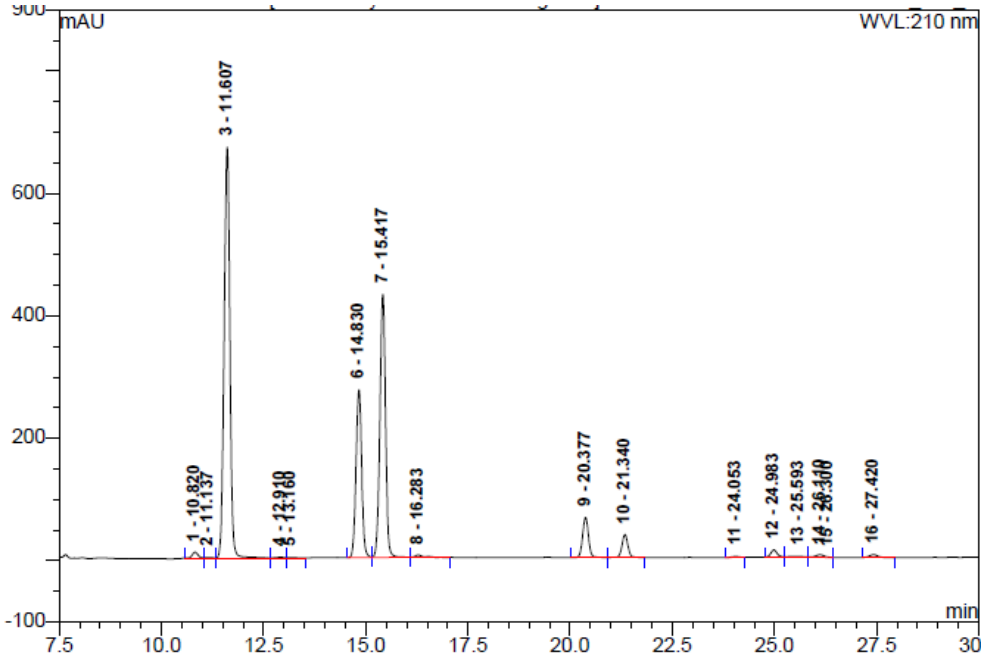

Figure 3-6: Iturin A standard chromatogram

3.4.5 Lipopeptide identification and classification *via* LC-MS

Liquid chromatography-mass spectroscopy (LC-MS) was used to distinguish between the different lipopeptide groups, i.e. surfactins, iturins and fengycins, produced. The LC-MS specifications are given in Table 3-5.

Table 3-5: LC-MC specifications for lipopeptide peak identification and classification

LC-MS specifications	
Column	Luna C18(2) reversed phase column (4.6 x 250 mm, 3 µm particle size) equipped with a Luna C18(2) precolumn (Phenomenex)
Detector	Waters Acquity UPLC LG 500 nm diode array detector
Mobile phase A	0.1% Formic acid in water
Mobile phase B	0.1% Formic acid in acetonitrile
Mobile phase gradient	Start at 30% B, increase to 60% B after 60 minutes, increase to 80% B after 10 minutes, increase to 90% B after 30 minutes, decrease to 30% B after 10 minutes, keep stable at 30% B for the next 5 minutes
Flow rate	0.8 mL/min
Absorbance	210 nm

Lipopeptides were analysed on a Waters Acquity ultraperformance liquid chromatograph connected to a Waters Synapt-G2 mass spectrometer. Chromatographic separation was achieved on a Luna C18(2) reversed phase column (4.6 x 250 mm, 3 µm particle size) equipped with a Luna C18(2) precolumn (Phenomenex). Column temperature was set to 40 °C and flow rate to 0.8 mL/min. The mobile phases used for elution were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Separation was carried out by gradient elution as shown in Table 3-5. Lipopeptide peaks were detected by UV detection at 210 nm using a Waters Acquity UPLC LG 500 nm diode array detector. Peak UV spectra were determined by scanning in the range 210-500 nm. Lipopeptide peaks were also detected by the Waters Synapt-G2 mass spectrometer. A capillary voltage of 2.5 kV was applied, with the source temperature set at 120°C and cone voltage at 15 V. Data acquisition was in the positive mode, scanning through m/z = 250 – 1800 (m/z is defined as the mass to charge ratio).

3.4.6 Antifungal efficacy

To determine the antifungal efficacy of the lipopeptides produced, radial diffusion plate assays were utilized.

Sterile potato dextrose agar (PDA) containing 100 mg/L ampicillin was poured into Petri-dishes and allowed to cool. Four 5 mm circular wells were cut into the agar, aseptically, at equal distances from one another with the back-ends of sterile yellow pipettes. A plug from the target fungus, either *Penicillium digitatum*, *Botrytis cinerea* or *Botryotinia fuckeliana* was placed in the centre of the agar plate and incubated at 25°C for 24 hours. 50 µL of the supernatant from the *B. amyloliquefaciens* were loaded aseptically into the wells. Each Petri-dish also had a control well containing 50 µL of sterile saline (0.85% m/v NaCl solution). The resulting clear halos, indicating antifungal activity, were monitored and recorded after 4 days or more (up to 21 days) if fungal growth was not sufficient. The wells were prepared in triplicate and distances were measured with a calliper.

3.5 Experimental design

Figure 3-7 gives a graphical representation of the experimental design, with three primary sections, i.e. the organism screening, lipopeptide production optimization and the testing of the antifungal efficacy of the lipopeptides produced. The optimization section is subdivided to represent the conditions at which the optimization was done. The results obtained under different conditions were also used to investigate the lipopeptide production phase initiation, to identify the lipopeptides and their possible homologues as well as the stability of the lipopeptides.

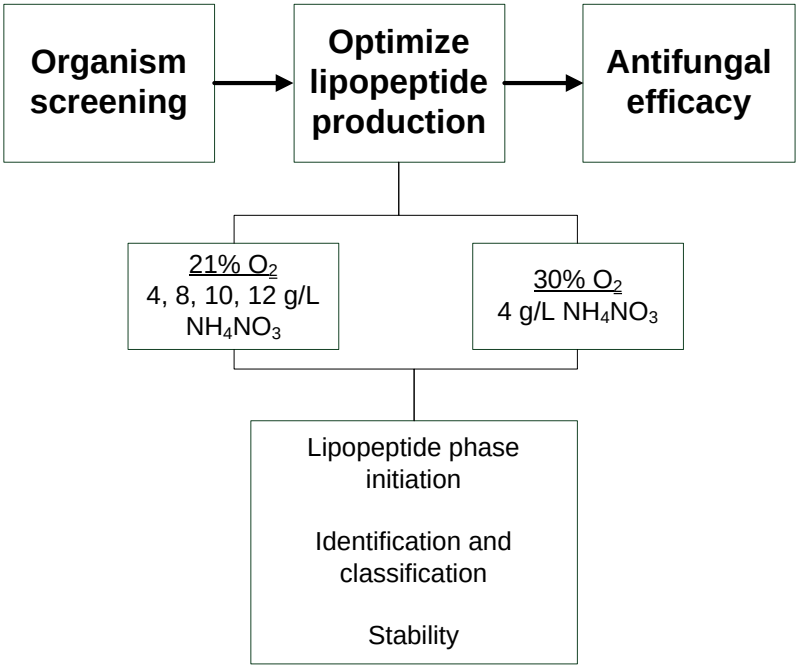

Figure 3-7: Experimental design flow diagram

Chapter 4

Results and discussions

4.1 Screening of *Bacillus* candidates

Four *Bacillus* candidates (3.1) were screened under analogous controlled batch conditions (3.3.3), in a fully instrumented bioreactor. Although the screening was done at one set of conditions, it should be noted that the results are comparable, since *B. subtilis* subspecies and closely related species were used. The kinetic data relating to organism growth and lipopeptide production, i.e. maximum production and yields for example, was collected and analyzed throughout each run to identify the best antifungal-producing candidate. The data collected throughout the batch culture experiments tests were compared and the most pertinent information is summarized in Table 4-1 (see Appendix A for kinetic equations).

Table 4-1: Summarized results from *Bacillus* screening experiments

Kinetic parameters	***B. amyloliquefaciens*** **(n = 2)***	***B. licheniformis*** **(n =1)***	***B. subtilis*** **(n = 1)***	***B. subtilis*** **subs *spizizenii*** **(n =2)***
μ_{max} (h^{-1})	0.43	0.30	0.45	0.39
CDW (g/L)	4.61	5.00	5.15	8.44
Antifungal parameters:				
Max concentration (mAU·min)	114.60	55.76	35.22	25.21
Max yield ($Y_{p/x}$) (mAU·min/g cells/L)	21.25	10.97	12.69	5.50
Max productivity (mAU·min/h)	3.89	1.02	1.47	1.20
Surfactin parameters:				
Max concentration (mg/L)	68	0	882.00	36.50
Max yield ($Y_{p/x}$) (g/g)	0.033	0	0.282	0.010
Max productivity (mg/L/h)	3.69	0	35.50	2.39

* (*n = 2*) Indicates multiple runs performed, where *n* refers to the number of runs

The results obtained from the comparative batch culture experiments (Table 4-1) were normalized and the relative amounts are shown in Figure 4-1. Note that the amounts were scaled relative to the parameter with the maximum value.

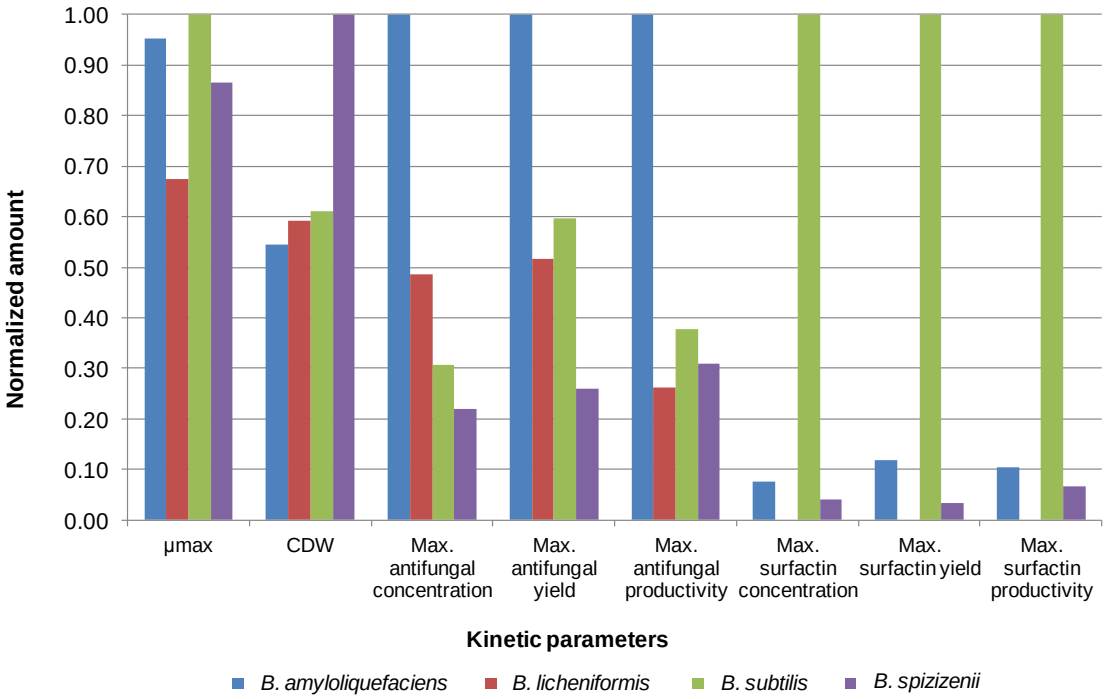

Figure 4-1: Comparison between normalized growth and production related parameters for *B. amyloliquefaciens* (n = 2), *B. licheniformis*, *B. subtilis* ATCC 21332 and *B. subtilis* subs. *spizizenii* (n = 2)

The selection of the most promising candidate was influenced most strongly by the following: first and foremost the maximum amount of antifungals produced, the product yield relative to the CDW ($Y_{p/x,\ \text{antifungals}}$) and also their maximum productivity, given that the aim is to finally use the lipopeptides produced as an antifungal agent against post-harvest diseases. *B. amyloliquefaciens* (n = 2, where *n* refers to the number of experimental runs) was shown to be the best candidate, according to these parameters, as it produced the highest concentration of antifungals (114.60 mAU·min), had the highest maximum $Y_{p/x}$ for antifungals (21.25 mAU·min/g cells/L) as well as the highest maximum productivity (3.89 mAU·min /h) as seen in Figure 4-1 and Table 4-1.

B. amyloliquefaciens also had the second highest μ_{max} of the four organisms, even though it had the lowest CDW. This suggests that the maximum CDW was reached in a shorter time compared to *B. subtilis* subs. *spizizenii* or *B. licheniformis* and that the energy was being diverted to lipopeptide production rather than biomass production. *B. spizizenii* (n = 2) had the highest CDW, with minimal surfactin and antifungal production. This suggests that the organisms preferred to utilize its energy resources for biomass rather than lipopeptide production, making it an undesirable candidate. *B. subtilis* ATCC 21332 produced 882 mg/L surfactin, which compares well with the 439 mg/L from Davis *et al*. (1999) and the 242 mg/L from Coutte *et al*. (2010), making *B. subtilis* ATCC 21332 a better candidate for

surfactin production rather than antifungal production. *B. licheniformis* produced no surfactin and only about half the amount of antifungals compared to *B. amyloliquefaciens*.

B. amyloliquefaciens was identified as the optimum organism for further experiments which included process development and optimization, as well as antifungal efficacy testing.

4.2 Process optimization for lipopeptide production

4.2.1 Effect of increased nitrate concentrations on growth and lipopeptide production

During the batch culture experiments at 4g/L NH_4NO_3 and 21% O_2, it was observed that oxygen became the limiting factor (DO = 0% at 8 hours), after which nitrate was utilized (Figure 4-2) for lipopeptide production (Figure 4-3). Note that experimental repeatability is demonstrated in section 4.4.2.

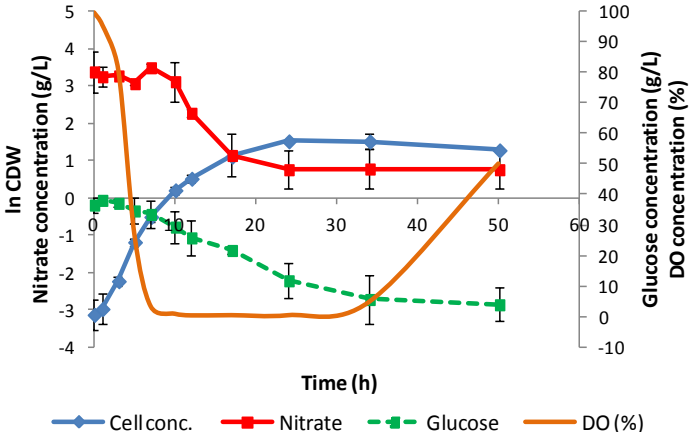

Figure 4-2: Substrate utilization and subsequent cell growth for *B. amyloliquefaciens* (n = 2) in medium containing 4 g/L NH_4NO_3. Error bars indicate standard deviation from mean value.

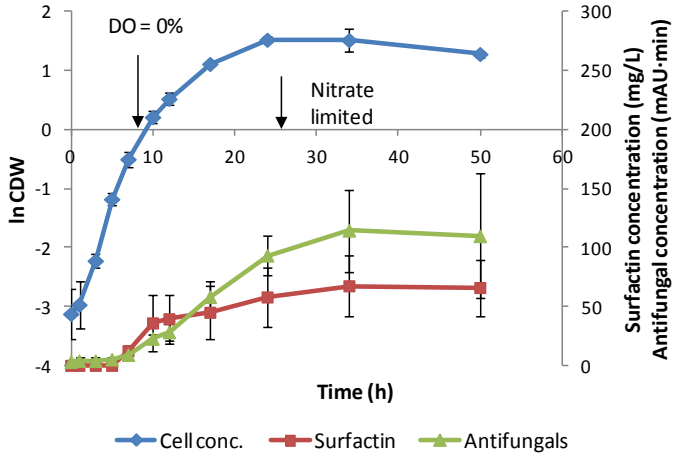

Figure 4-3: Lipopeptide production by *B. amyloliquefaciens* (n = 2) in medium containing 4 g/L NH_4NO_3. Error bars indicate standard deviation from mean value.

The production trend from Figure 4-2 and Figure 4-3 shows that lipopeptide production is initiated once nitrate utilization occurs, around 8 hours, and that production is hampered once nitrate concentrations become limiting. Nitrate was assumed limiting, as lipopeptide (both antifungal and surfactin) production plateaued at minimum nitrate concentrations, while glucose was still in excess. This was observed after about 25 hours, and prompted further investigation into the effect of increased nitrate concentrations on lipopeptide production. Of special interest was whether increased nitrate concentrations could increase antifungal production. The process performance was investigated at 8 g/L, 10 g/L and 12 g/L NH_4NO_3, and the results shown in Figures 4-4 and 4-5.

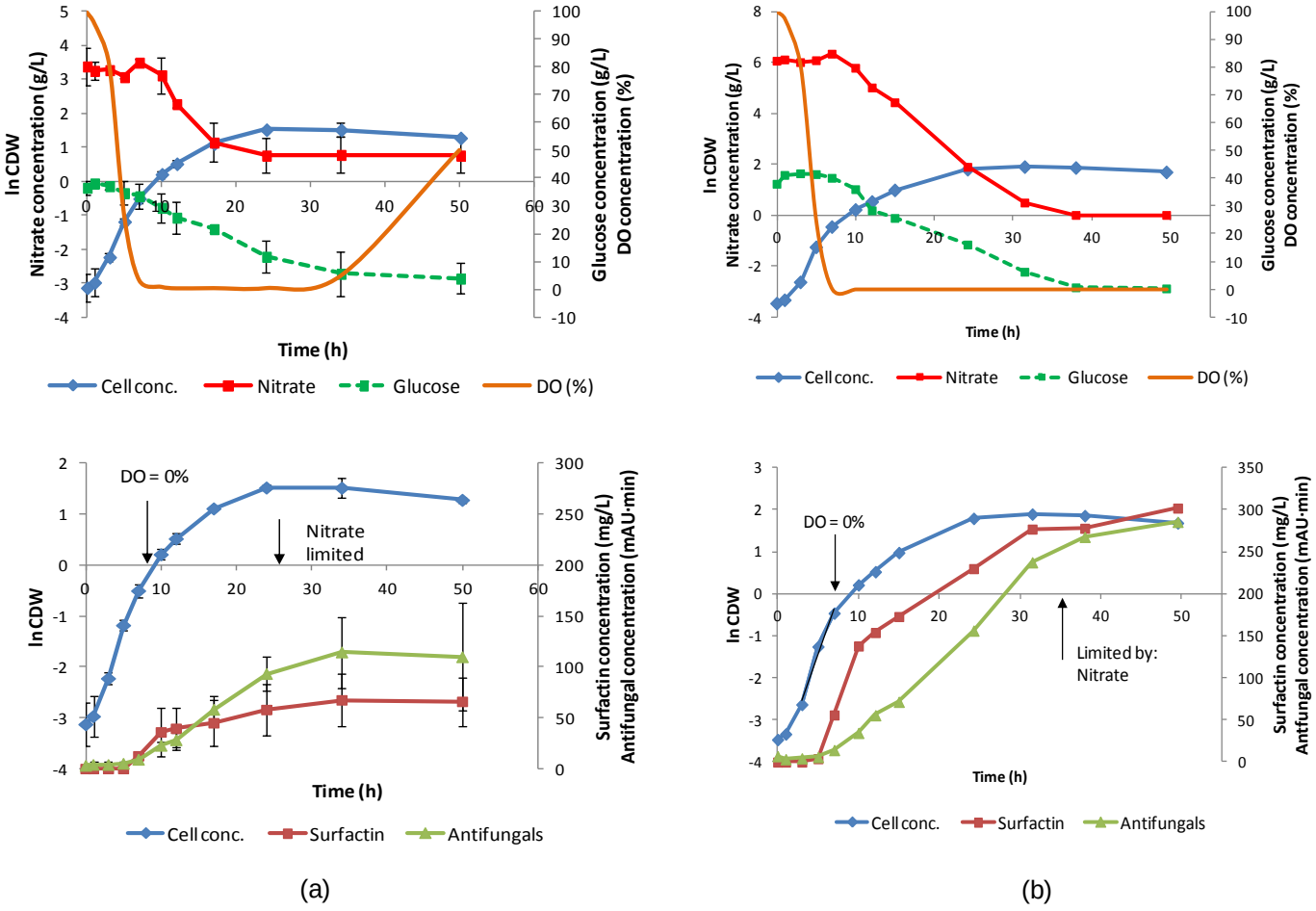

Figure 4-4: Comparison between (a) 4 g/L (n = 2) and (b) 8 g/L NH_4NO_3 growth and lipopeptide production. Error bars indicate standard deviation from mean value.

By increasing the NH_4NO_3 concentration from 4 to 8 g/L in the growth medium, the maximum CDW increased by 1.45-fold from 4.6 to 6.7 g/L (see Figure B-1, Appendix B). The maximum amount of antifungals produced during the experiment increased 2.5-fold from 114.6 to 285.7 mAU·min and the maximum surfactin produced increased 4.44-fold from 68 to

302 mg/L (Figure 4-4). The increase in nitrate concentration also increased the maximum growth rate (μ_{max}, h^{-1}) from 0.43 to 0.54 (Table 4-2). The statistical significance of the increases in antifungal and surfactin production will be discussed in section 4.2.1.1

Figure 4-4 (a) shows that when the nitrate supply is depleted around 26 hours, the lipopeptide production is hampered and plateaus. For the 4 g/L NH_4NO_3 (at 21% oxygen concentration) later referred to as the "base case", the nitrate utilization period was observed to be at its maximum between about 8 and 20 hours during the experimental run (Figure 4-4 a). When increasing the nitrate concentration, the maximum nitrate utilization period was extended to between 8 and 32 hours (Figure 4-4 b). This indicates that an increase in nitrate concentration allows for an extension of the nitrate utilization period as well as an extension of the lipopeptide production phase (around 8 to 38 hours), in turn allowing for a higher concentration of lipopeptides. It should be noted that the nitrate levels indicated in Figures 4-2 to 4-5 indicate lower values than the 4, 8 or 12 g/L NH_4NO_3 that was put into the system. This is due the fact that with nitrate ion chromatography, only the nitrate ion is measured, not the entire NH_4NO_3 molecule.

The comparison between 4, 10 and 12 g/L NH_4NO_3 showed similar trends as those seen in Figure 4-4. The trends from the 12 g/L NH_4NO_3, the highest nitrate concentration, were as follows:

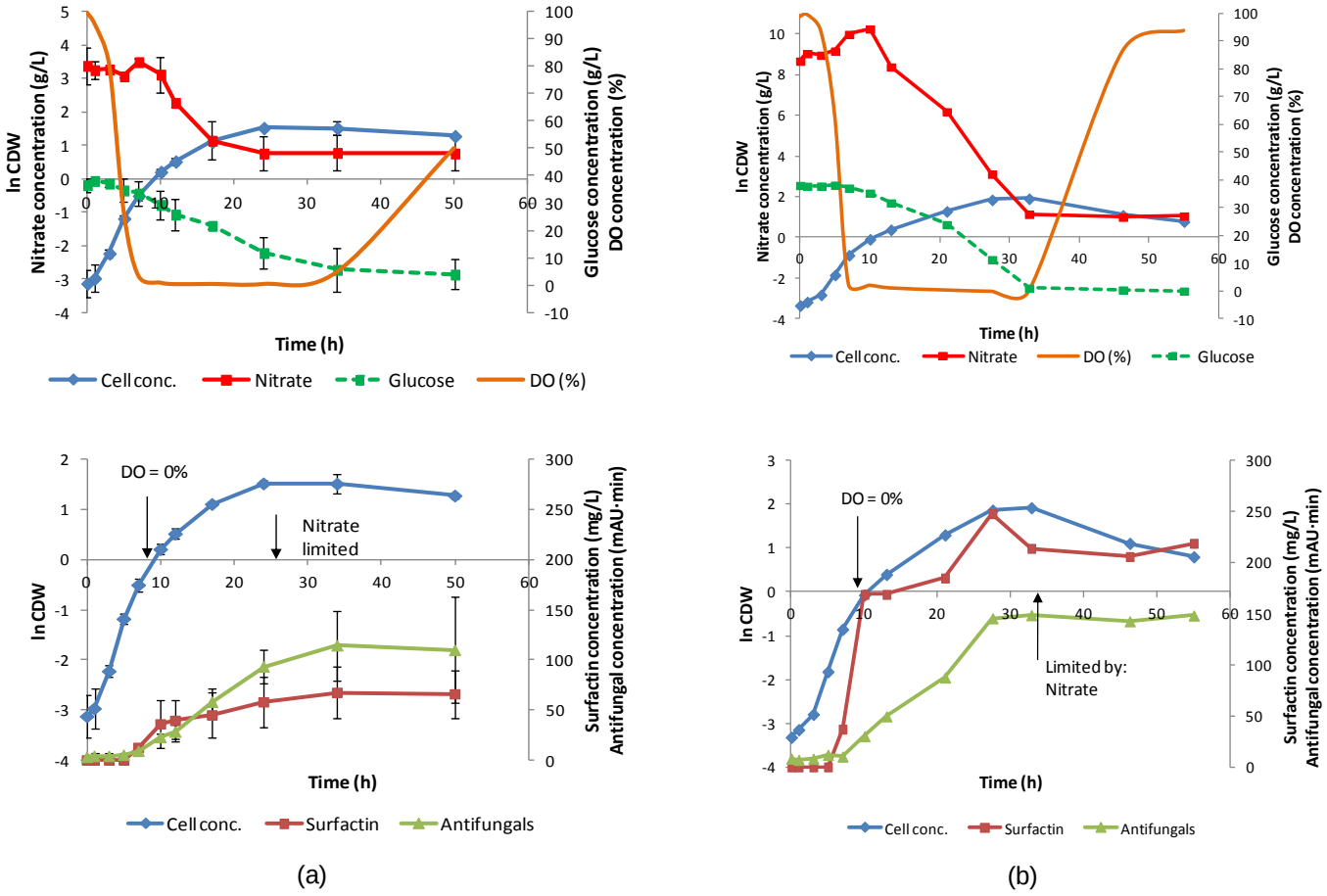

Figure 4-5: Comparison between (a) 4 g/L (n = 2) and (b) 12 g/L NH4NO3 growth and lipopeptide production. Error bars indicate standard deviation from mean value.

The increase from 4 to 12 g/L NH_4NO_3 led to a 1.48-fold increase in CDW, from 4.6 g/L to 6.8 g/L. The maximum antifungal concentration increased by 1.3-fold, from 114.6 to 148.6 mAU·min and the maximum surfactin concentration increased 3.66-fold, from 68 to 248 mg/L (Figure 4-5). Furthermore, the μ_{max} (h^{-1}) increased from 0.43 to 0.49. Even though the increase in nitrate from 4 to 12 g/L clearly improved both growth and lipopeptide production, it did not improve the CDW significantly and did not improve the lipopeptide production or μ_{max} as much as when compared with the 8 g/L run (Figure 4-4).

To determine the optimum NH_4NO_3 concentration, different results from the 4, 8, 10 and 12 g/L runs were compared. These included the lipopeptide production profiles for the antifungals and surfactin, the μ_{max}, as well as the yields and productivity.

4.2.1.1 Lipopeptide production profiles

The production profiles of the antifungals (Figure 4-6) as well as surfactin (Figure 4-7) were evaluated at different NH_4NO_3 concentrations to determine which affected either lipopeptide the most.

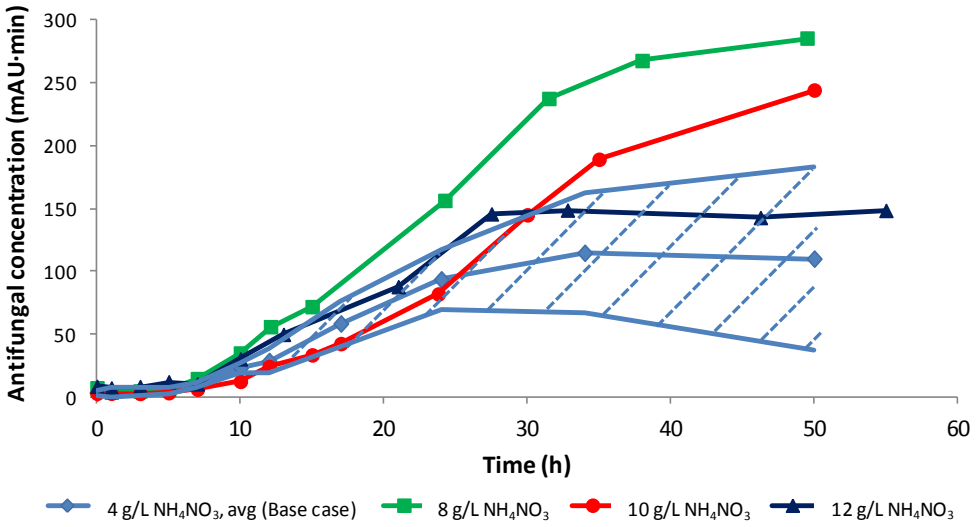

Figure 4-6: Antifungal production profiles in media containing 4 (n = 2), 8, 10 and 12 g/L NH_4NO_3, with diagonally shaded area indicating 95% confidence interval

The results from the increased nitrate experiments show that an optimum nitrate concentration exists. This is evident from the increase in antifungal production at 8 g/L, but the lower concentrations produced at both 10 and 12 g/L NH_4NO_3. Further evidence of the decrease in production at a too high nitrate concentration is the fact that the 12 g/L production profile falls within the 95% confidence interval (indicated by the diagonally shaded area) of the 4 g/L base case (Figure 4-6). This suggests that the increase in antifungal production obtained by increasing the NH_4NO_3 concentration from 4 g/L to 12 g/L is not significant. The maximum antifungal concentration produced at 4 g/L NH_4NO_3 (n = 2) was 114.6 mAU·min, while 8 g/L resulted in 285.7 mAU·min, 10 g/L resulted in 244.2 mAU·min and 12 g/L yielded 148.6 mAU·min, as mentioned previously (section 4.2.1).

The 95% confidence interval was constructed from duplicate experiments using 4 g/L NH_4NO_3 (Figures 4-6 and 4-7). Confidence intervals were not constructed for the 8, 10 and 12 g/L NH_4NO_3 experiments as these were not carried out in duplicate. Nevertheless, the 95% confidence interval for the 4 g/L experiments confirms a significant difference between the 4 g/L and 8 g/L NH_4NO_3 experiments, because an assumed similar confidence interval for 8 g/L (assuming similar variance) would exhibit no overlap of the intervals for 4 and 8 g/L.

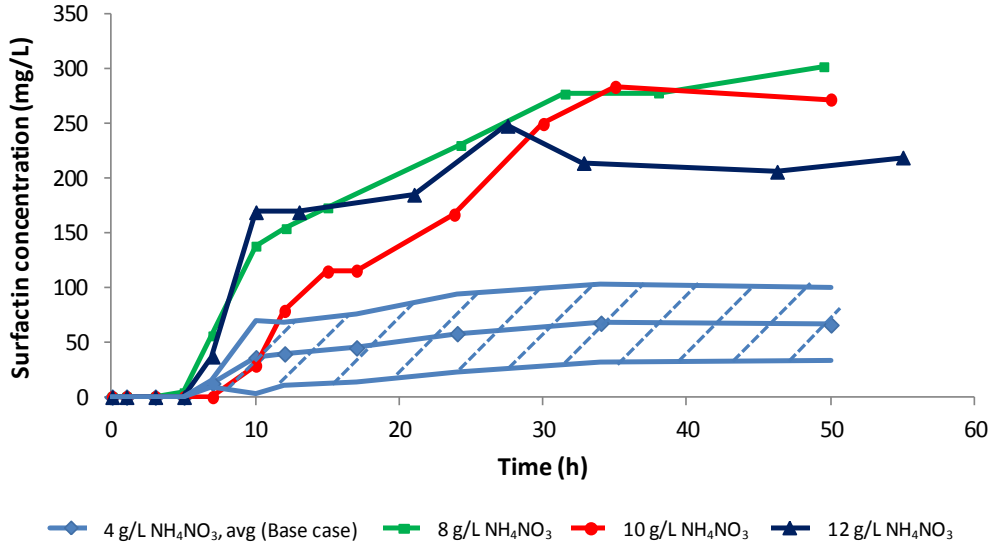

Figure 4-7: Surfactin production profiles in media containing 4 (n = 2), 8, 10 and 12 g/L NH4NO3, with diagonally shaded area indicating 95% confidence interval

The increase in NH_4NO_3 clearly increased surfactin production (Figure 4-7), although the production trends are less obvious than with the antifungals. When considering both maximum and final surfactin concentrations, once again the 8 and 10 g/L NH_4NO_3 runs outperformed the 12 g/L run. The surfactin results further differ from the antifungal results as none of the increased NH_4NO_3 profiles fall within the 95% confidence interval, suggesting that even though the 12 g/L run was less effective than the 8 or 10 g/L run in increasing the surfactin concentrations, its effect was still statistically significant (as was the case for the 8 and 12 g/L). The maximum surfactin concentration obtained from the 4 g/L (n = 2) run was 68 mg/L, while 302 mg/L, 284 mg/L and 248 mg/L were obtained at 8, 10 and12 g/L NH_4NO_3, respectively. The following plots summarize the results obtained from the increased nitrate experiments.

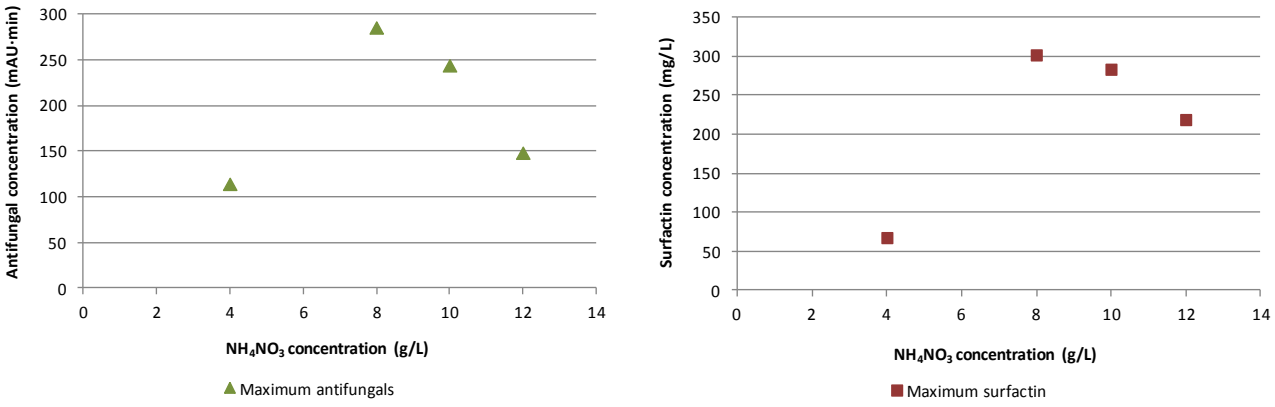

Figure 4-8: Optimum NH_4NO_3 concentration, in bioreactor, for maximum (a) antifungal and (b) surfactin production

Even though literature (Abushady *et al.*, 2005:337-344) suggests that around 4.6 g/L NH_4NO_3 is the optimum nitrate concentration for lipopeptide production, the results obtained from the bioreactor experiments suggest that the optimum concentration is higher, around 8 g/L, for both antifungal (fengycin and iturin) and surfactin production (Figure 4-8). This difference could be attributed to the fact that Abushady *et al*. (2005) performed their experiments in shake flasks.

4.2.1.2 Maximum growth rate, μ_{max}

Table 4-2 displays the growth rate results from the increased nitrate experiments.

Table 4-2 : Growth rate summary from increased nitrate experiments. R^2 denotes the accuracy of the curve fitting done (see Appendix A1) in order to determine μ_{max}

NH_4NO_3 (g/L)	μ_{max} (h^{-1})	R^2
4 (n = 2)	0.43	0.99
8	0.54	0.98
10	0.58	1.00
12	0.49	1.00

As with the lipopeptide production, an optimum NH_4NO_3 concentration seems to exist in terms of maximum growth rate. This optimum appears to be at 10 g/L NH_4NO_3 as opposed to 8 g/L as seen with the lipopeptide production. The μ_{max} values obtained from the bioreactor experiments were higher than those reported in literature for other *B. subtilis* strains. Chenikher *et al.* (2010) reported a μ_{max} of 0.35 (h^{-1}) by *B. subtilis* ATCC 6633, while Chen *et al.* (2006:1923-1931) reported a value of 0.284 (h^{-1}) using *B. subtilis* BBK006. One of the primary differences, excluding the bacterial strain, between these studies and the present study was the growth media used. Chenikher *et al.* (2010) only used 1 g/L NH_4Cl in their growth medium, while Chen *et al*. (2006:1923-1931) used 2.3 g/L $(NH_4)_2SO_4$ in their growth medium, compared to the 4 – 12 g/L NH_4NO_3 used in the present study.

4.2.1.3 Kinetic parameter comparison

The comparison of kinetic parameters facilitated evaluating the process performance at the different NH_4NO_3 concentrations by not only considering the maximum production, but also certain aspects such as the product yield ($Y_{p/x}$) or productivity. Due to the fact that the cells exited the stationary phase and started entering the death phase after about 32 hours in

the majority of the experiments, except for the 10 g/L which remained in the stationary phase until termination of the experiment, it was decided to compare the kinetic parameters at the point before the death phase was reached. This was done to ensure that kinetic parameters dependent on the CDW would not be overestimated or incorrectly interpreted as a result of the reducing CDW. The results from this comparison are summarized in Figure 4-9.

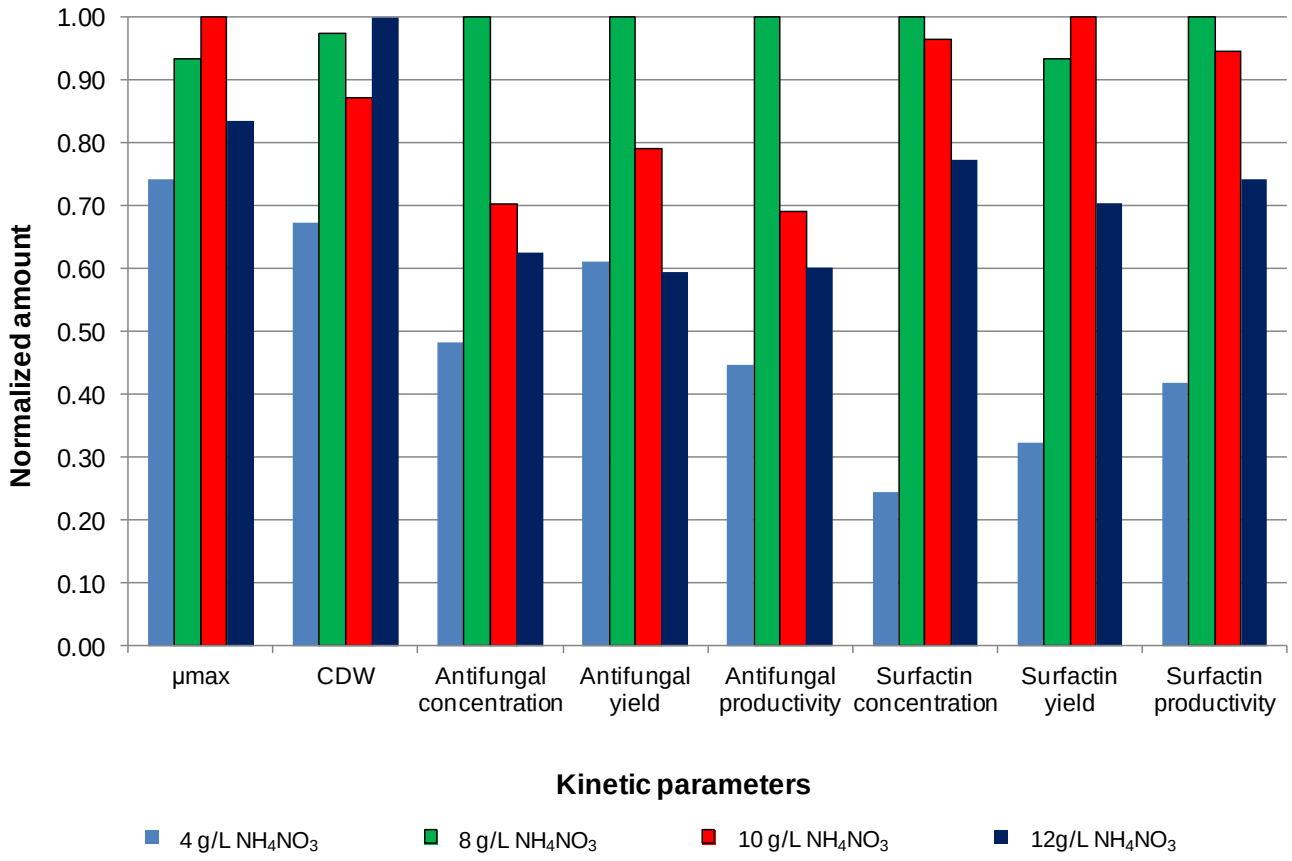

Figure 4-9: Comparison between normalized growth and lipopeptide production related parameters at 32 hours for different NH_4NO_3 treatments

As previously discussed (see section 4.2.1.2), the highest μ_{max} was achieved at 10 g/L NH_4NO_3. A noticeable increase in CDW was observed when increasing the NH_4NO_3 from 4 g/L, as mentioned previously (section 4.2.1), shown again in Figure 4-9 where a minimum of 50% increase in CDW was noted for the 8, 10 and 12 g/L runs.

Figure 4-9 suggests that optimum surfactin production occurs at 10 g/L NH_4NO_3, but as 8 g/L has already been proven to be the optimum (Figure 4-8) this result will not be taken into account. The reason for the discrepancy is most probably due to the fact that the surfactin concentration profiles are very close to each other for the 8 and 10 g/L cases between 30 and 50 hours (Figure 4-7). The maximum antifungals produced at 32 hours do however

reflect the results from the antifungal profile as a whole (Figure 4-6) and can be taken into account.

The highest surfactin yield ($Y_{p/x,surfactin}$) was obtained at 10 g/L NH_4NO_3 due to the fact that at that point in time, the highest surfactin concentration was coupled with the lowest CDW. The $Y_{p/x,surfactin}$ obtained at this point for 10 g/L was 0.045 g/g. This corresponds well with the 0.021 – 0.075 g/g, depending on the conditions, that Davis *et al.* (1999) obtained for their surfactin production with *B. subtilis* ATCC 21332. The highest antifungal yield ($Y_{p/x,antifungals}$) was obtained at 8 g/L NH_4NO_3, corresponding to the treatment where the maximum antifungals were obtained. The yield at 8 g/L NH_4NO_3 was 31.04 mAU·min/g cell/L (section 3.4.4.2).

The 8 g/L NH_4NO_3 treatment out-performed the other treatments with regard to productivity for both surfactin and antifungals. This corresponded to the results obtained in section 4.2.1.1. The antifungal productivity at 32 hours for the 8 g/L treatment was 6.59 mAU·min/h. The surfactin productivity at 32 hours more accurately reflects the surfactin production profile as a whole (Figure 4-7), showing that the highest productivity, 8.79 mg/L/h, was observed under the 8 g/L conditions.

Depending on the desired kinetic parameter, the data from Figure 4-9 can be used as a guide to optimize the production process. The data further suggest that the 8 g/L NH_4NO_3 treatment is highly recommendable, due to the fact that it generally out-performed the other treatments. Another reason to consider the 8 g/L treatment would be the economic implications of adding more NH_4NO_3 and whether these additions would actually be justified with regard to production.

4.2.1.4 Lipopeptide initiation phase

Due to the widespread controversy in literature (section 1.2.3) regarding the phase with which lipopeptide production is associated, i.e. whether they are primary or secondary metabolites, the association between rate of cell growth and lipopeptide production was also investigated. The results from two runs, 4 g/L NH_4NO_3 and 8 g/L NH_4NO_3, are shown in Figure 4-10.

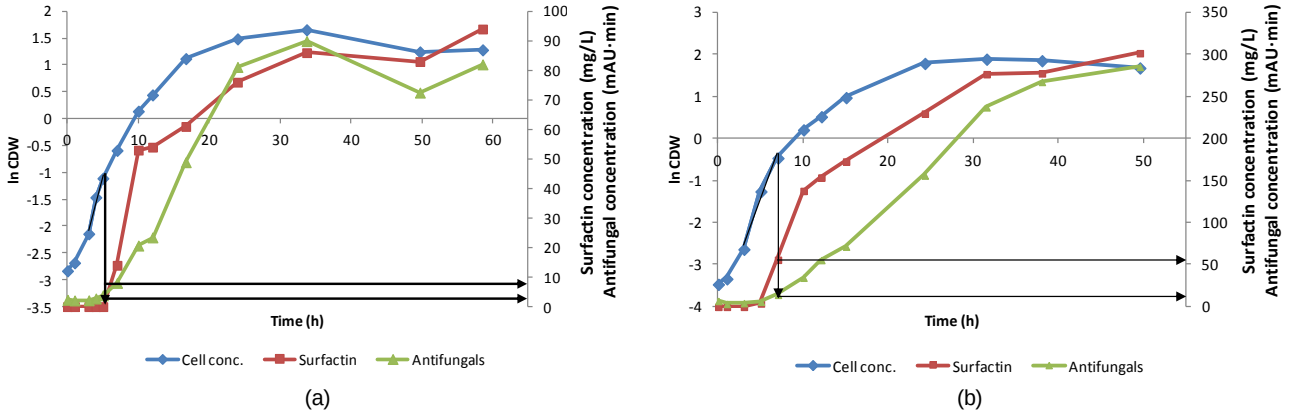

Figure 4-10: Growth and production trends in (a) 4 g/L and (b) 8 g/L NH_4NO_3 treatments indicating lipopeptides from *B. amyloliquefaciens* are secondary metabolites

Lipopeptide production, both surfactin and the antifungals, only commences toward the end of the exponential or start of the stationary phase (Figure 4-10). As previously shown (Figure 4-2), lipopeptide production only commences once the oxygen supply becomes limited. Both facts support the claim that the lipopeptides produced by *B. amyloliquefaciens* DSM 23117 are secondary metabolites (see section 1.2.3). This conclusion is in agreement with that of Arguelles-Arias *et al.* (2009) who stated that the lipopeptides produced by *B. amyloliquefaciens* GA1 were secondary metabolites. Lipopeptides being secondary metabolites agrees with the claim that they are used as a defence mechanism by the organism, and are not directly involved with the growth, development or reproduction of the organism.

4.2.2 Effect of increased oxygen supply concentrations on growth and lipopeptide production

The experiments in which the NH_4NO_3 concentrations were increased from 4 g/L to 8, 10 and 12 g/L were evaluated and showed that lipopeptide production was dependent on DO utilization. The effect of increased oxygen supply concentration was investigated to determine what its effect was on growth and lipopeptide production.

The effect of using 30% enriched air (30% O_2) on growth and lipopeptide production is shown in Figure 4-11 where the effect of 21% O_2 and 30% O_2 is compared. Note that for both 21% and 30% O_2 supply, the initial NH_4NO_3 concentration in the medium was 4 g/L.

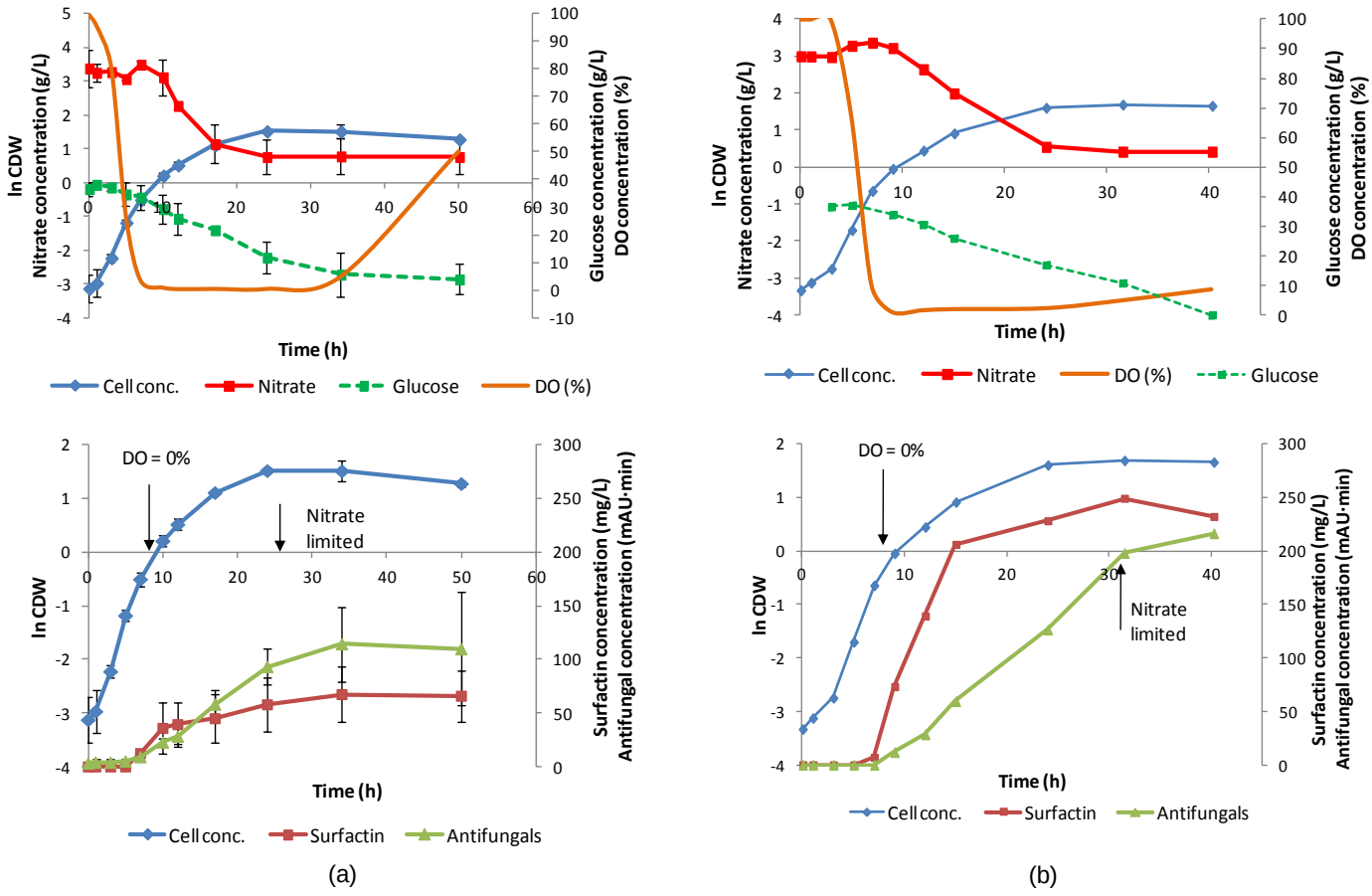

Figure 4-11: Comparison of growth and lipopeptide production at (a) 21% (n = 2) and (b) 30% O_2 supply concentration. Error bars indicate standard deviation from mean value.

Similar trends to those seen in the increased nitrate experiments (Figure 4-4 and Figure 4-5) manifested in the results from the enriched oxygen run. An unexpected occurrence was that with the 30% O_2, the DO supply became depleted around the same time as with the 21% O_2 runs (around 8 to 9 hours into the run). This depletion once again initiated nitrate utilization, however the main difference between the base case run (21% O_2) and the 30% O_2 run is the fact that the nitrate utilization period was extended (to between 8 – 24 hours), as seen with the increased nitrate runs (Figure 4-4 and Figure 4-5). This suggests that the increase in oxygen supply concentration allows for an increased nitrate availability indicated by the extended nitrate utilization period (Figure 4-11 b).

The maximum CDW increased 1.17-fold from 4.6 to 5.4 g/L when increasing the oxygen in the supply and the μ_{max} increased from 0.43 to 0.53 h^{-1}. In addition, the maximum lipopeptide production also increased. This in part is a consequence of the increase in CDW, but also a likely result of an increase in the availability of nitrate under these conditions. Maximum antifungal production increased 1.9-fold, to 216.3 mAU·min and maximum surfactin production increased 3.7-fold, to 249 mg/L. These results suggested that with an

increased oxygen supply, more oxygen is available to act as the electron acceptor, allowing the nitrate to be utilized for lipopeptide production.

These results, i.e. increase in biomass and lipopeptide production, suggest that the process performs better under increased oxygen availability conditions. To evaluate the true performance of the process under enriched oxygen conditions, however, certain parameters, including maximum lipopeptide production, yields and productivity, must be considered and compared to the results obtained from the base case runs (4 g/L NH_4NO_3, 21% O_2). The lipopeptide production profiles are compared in section 4.2.2.1 and the kinetic parameters are compared in section 4.2.2.2.

4.2.2.1 Lipopeptide production profiles

The antifungal production profiles (Figure 4-12) and surfactin production (Figure 4-13) profiles for the 30% enriched air treatment are compared to the results obtained from the base case runs to ascertain whether the enriched treatment had a significant effect on lipopeptide production.

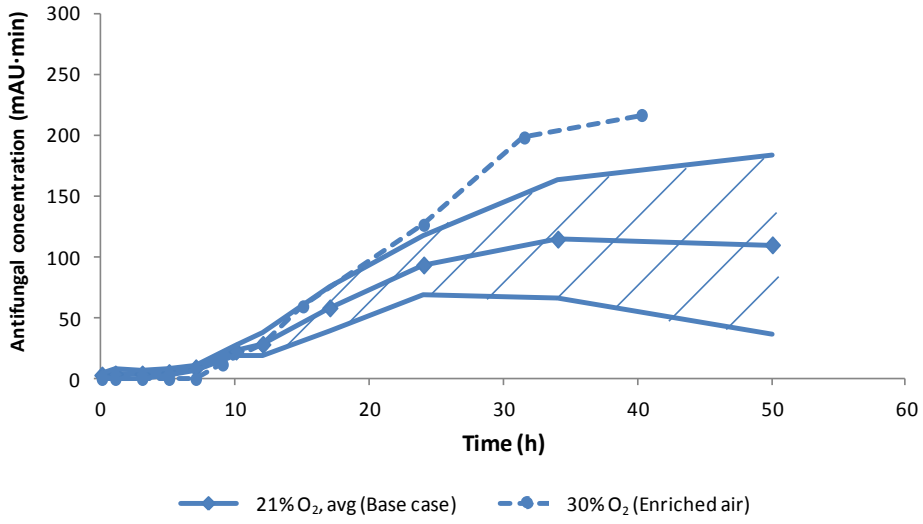

Figure 4-12: Antifungal production profile in system with 21% and 30% O_2 supply concentration, with diagonally shaded area indicating 95% confidence interval

The antifungal production profiles (Figure 4-12) suggest that, within a 95% confidence interval, the increase in antifungal production for the 30% enriched air treatment is significant. However, it is interesting to note that the 216.3 mAU·min obtained from the 30% enriched air treatment at 4 g/L NH_4NO_3 is 32% and 13% lower than the 285.5 or

244.3 mAU·min from the 8 g/L and 10 g/L NH_4NO_3 experiments respectively. Considering that both 8 and 10 g/L nitrate experiments out-performed the 30% enriched air run in terms of lipopeptide production and that the nitrate treatment is less expensive than the enriched air treatment, increased nitrate treatments would seem to be the preferable and more economic treatment option.

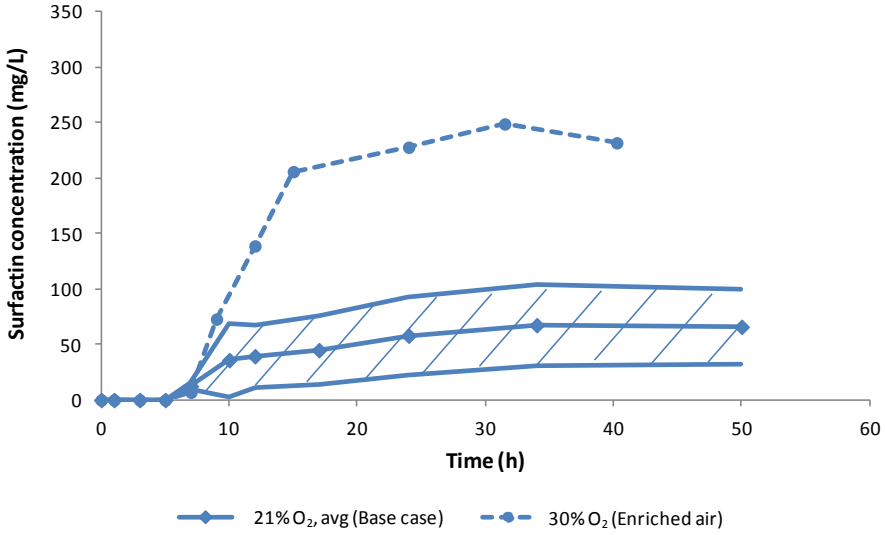

Figure 4-13: Surfactin production profile in system with 21% and 30% O_2 supply concentration, with diagonally shaded area indicating 95% confidence interval

Similar results were observed with surfactin production (Figure 4-13). As with the antifungal production, the effect on surfactin production was significant within a 95% confidence interval. Here again, the maximum surfactin concentration of 249 mg/L was lower than the 302 or the 284 mg/L produced from the 8 and 10 g/L NH_4NO_3 treatments respectively.

4.2.2.2 Kinetic parameter comparison

The normalized kinetic parameters are shown in Figure 4-14.

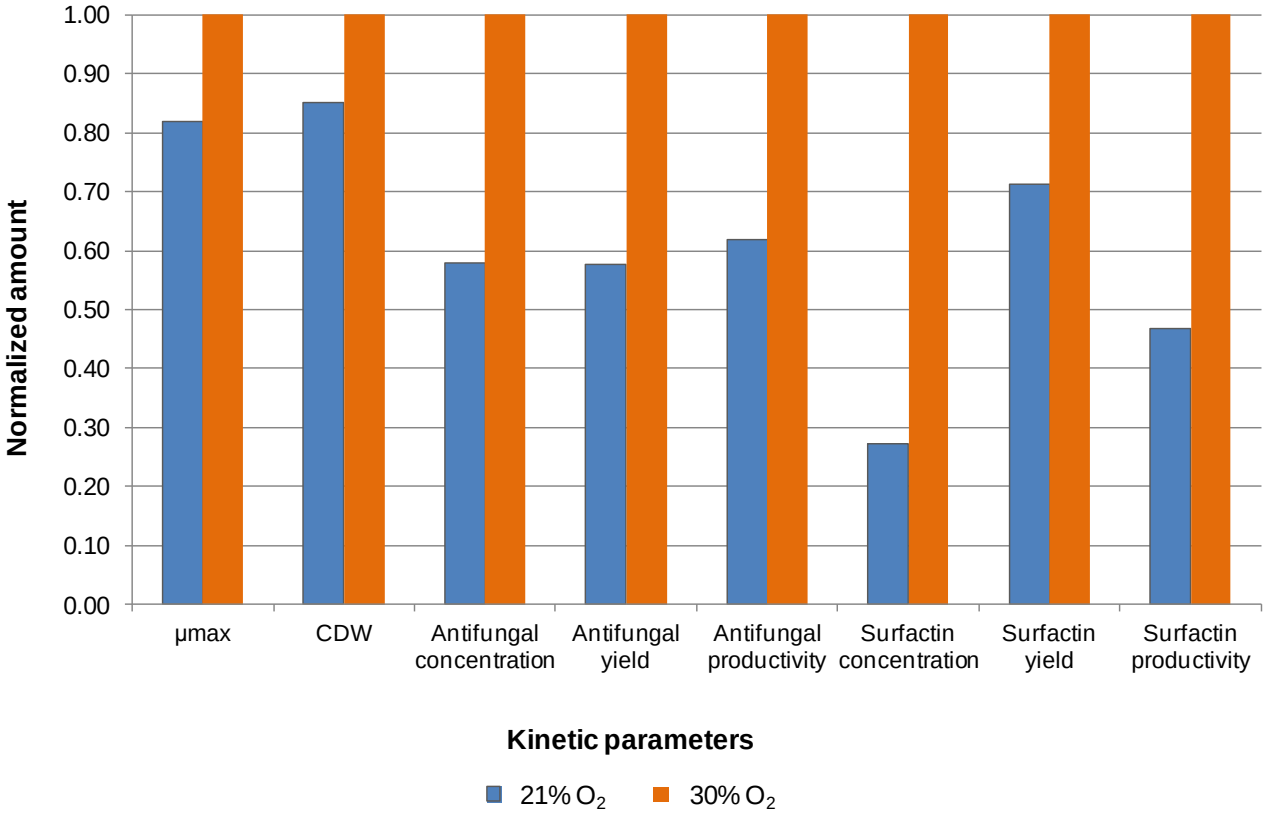

Figure 4-14: Comparison between normalized growth and lipopeptide production related parameters at 32 hours for different oxygen supply treatments

Kinetic parameters obtained from the 30% O_2 out-performed the 21% O_2 parameters, both obtained at 4 g/L NH_4NO_3. The surfactin yield ($Y_{p/x,surfactin}$) for the 30% O_2 run was 0.046 g/g which similar to the 0.045 g/g obtained from the 10 g/L NH_4NO_3 experiment. Antifungal yield ($Y_{p/x,antifungal}$) for the 30% O_2 run was 36.85 mAU·min/g cells/L, similar to the 31.04 mAU·min/g cells/L obtained from the 8 g/L NH_4NO_3 run. The 30% O_2 surfactin productivity at 32 hours was 7.91 mg/L/h, similar to the 8.79 mg/L/h from the 8 g/L NH_4NO_3 experiment. This was also the case for the antifungal productivity, where the enriched air treatment yielded 6.28 as opposed to the 6.59 mAU·min /h from the 8 g/L NH_4NO_3 treatment.

Increased oxygen availability in the 30% O_2 experiment (demonstrated by DO = 0% at the same time in the 21% and 30% O_2 experiments) enabled more cells to utilise O_2. Furthermore, the increase in oxygen availability resulted in an increase in lipopeptide production, both antifungals and surfactin, (Figure 4-14). As noted in section 4.2.2, this increase in lipopeptide production could, in part, be a result of the increase in CDW. This, however, is not the sole cause behind the increase in lipopeptide production, as is clear from the increased lipopeptide yield obtained under enriched oxygen conditions. This increase in yield (both antifungal and surfactin) shows that, not only were more cells available to produce

the lipopeptides, but that these cells had a higher production capacity and were functioning better under the oxygen rich conditions. It can be postulated that the controversies regarding O_2-limitation seen in literature, exist because lipopeptide production coincides with O_2-limitation in batch cultures, as seen in Figures 4-2 and 4-3.

To evaluate the effect of the optimization and determine which run was better suited for antifungal production, the results from sections 4.2.1.3 and 4.2.2.2 are shown in the following table (Table 4-3).

Table 4-3: Kinetic parameter summary at 32 hours, corresponding with Figure 4-9 and 4-14

NH_4NO_3	Oxygen	Antifungal concentration (mAU·min)	Antifungal yield (mAU·min/g cells/L)	Antifungal productivity (mAU·min/h)	Surfactin concentration (mg/L)	Surfactin yield (g/g)	Surfactin productivity (mg/L/h)	CDW (g/L)	μ_{max} (h^{-1})
4 g/L	21%	114.55	21.25	3.38	68.00	0.015	1.99	4.61	0.43
8 g/L	21%	237.15	34.76	7.54	277.00	0.042	8.79	6.66	0.54
10 g/L	21%	166.93	27.52	5.22	267.00	0.045	8.32	5.97	0.58
12 g/L	21%	148.61	20.66	4.53	214.00	0.031	6.52	6.83	0.49
4 g/L	30%	197.88	36.85	6.28	249.00	0.046	7.90	5.41	0.53

Focussing on the antifungal-related parameters, the 8 g/L NH_4NO_3 (21% O_2) run resulted in the highest antifungal concentration (237.15 mAU·min) and the highest antifungal productivity (7.54 mAU·min/h), while the 30% enriched air run (4 g/L NH_4NO_3) resulted in the highest antifungal yield (36.85 mAU·min/g cells/L). These conditions should be considered for further optimization purposes.In addition to the changes in lipopeptide production observed with changes in nitrate and oxygen concentrations, it was observed that the ratio in which different homologues were produced, were also affected. These results will be shown and discussed in the following section (4.2.3).

4.2.3 Homologue production under different process conditions

The primary homologue analysis was done from the HPLC results obtained from the different samples. In order to distinguish between some of the specific homologues within the lipopeptide groups, i.e. surfactin, fengycin or iturin, some samples were also analyzed *via* LC-MS.

4.2.3.1 Homologue production profiles

Homologue profiles from the base case (4 g/L NH_4NO_3, 21% O_2), 8 g/L, 10 g/L and 12 g/L NH_4NO_3 experiments as well as the 30% enriched air experiment were analyzed. These results will be reported in this section. Figures 4-15 to 4-19 show the homologue profiles produced under the increased nitrate conditions as well as the 30% enriched air conditions.

Note that the retention times are labled as “t 15.6” for example which would indicate a retention time of 15.6 minutes (Figure 4-15 to Figure 4-19).

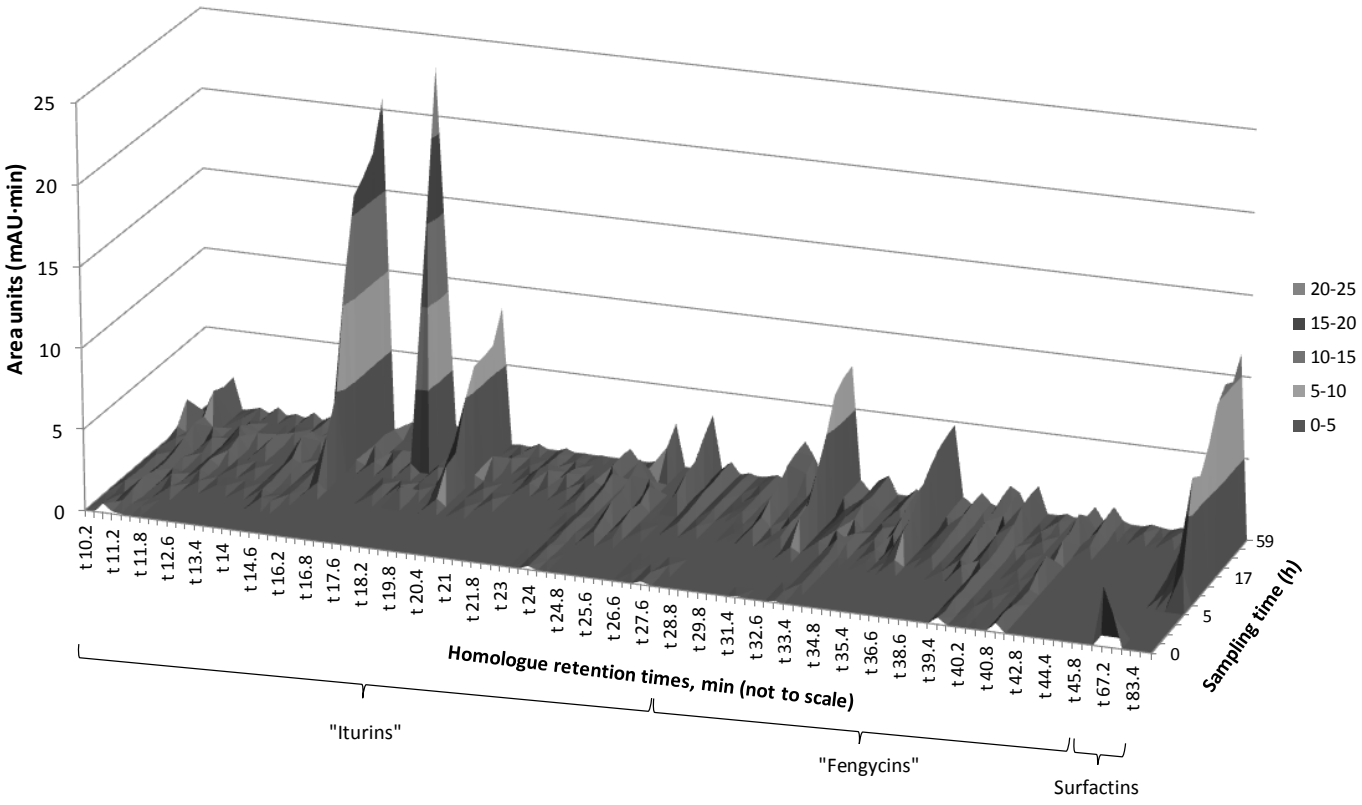

Figure 4-15: Base case (4 g/L NH_4NO_3, 21% O_2) homologue profile based on HPLC results

Iturin and surfactin standards were purchased for quantification, but unfortunately no standard is available for fengycin. Therefore the quantification that could be done for the homologues present in the available standards, was not possible for the fengycin group. Instead of quantification in mg/L, the amounts for each peak was expressed in HPLC area units, mAU·min. *B. amyloliquefaciens* seemed to produce more iturin homologues than were present in the iturin standard, complicating the distinction between the two antifungal

lipopeptide groups. However, from the HPLC spectra (using a match factor of 900 out of a possible 1000 with Chromeleon software) and the peak retention times (which correlate with the elution acetonitrile percentage), it was possible to deduce the boundaries between the homologue groups.Consequently the iturin and fengycin groups were labled as “Iturin” and “fengycin” in the homologue profiles (Figure 4-15). The elution order was determined by using the available standards as well as literature, which indicated that iturins eluted first, follwed by the fengycins, and then the surfactins (Romero *et al*., 2007:430-440). Better classification would be possible with LC-MS (see section 3.4.5).

For the base case (4 g/L NH_4NO_3, 21% O_2) run 105 peaks were identified. The most prominent peaks were observed in the “Iturin” range. Some peaks corresponding to those seen in the iturin standard (Figure 3-6), in terms of retention times, were observed in the base case run (Figure 4-15). However, additional peaks were observed that were not present in the iturin standard, possibly indicating production of partially formed lipopeptides, intermediate products, previously unidentified iturins or iturins that are not included in the standard that was used. To determine this, additional analyses and possible classification would be required. It should be noted that the number of peaks identified were more than reported by Romero *et al*. (2007), but the column used in the current study was 10 cm longer than the 15 cm column used by Romero *et al.* (2007), This allows for better separation and identification of compounds, explaining the increased number of peaks.

Of the 105 peaks from the base case run, only 5 peaks were in the surfactin range. Of these 5 peaks, only 3 corresponded to the surfactin standard (Figure 3-4), the other 2 peaks were in the surfactin range, but were not exact matches to the surfactin standard. The peaks observed in both Figure 4-15 and the surfactin standard were Surfactin 3, Surfactin 4 and Surfactin 5&6.

Of the other possible 100 peaks, 60 fell into the “Iturin” category, while the remaining 40 fell into the “Fengycin” category. The prominent iturin peaks were observed at retention times of 15.6, 17.8 and 19.8 minutes, while the most prominent fengycin peaks were observed at 32.4 and 35.8 minutes.

The 8 g/L nitrate run (Figure 4-16) showed more peaks compared to the base case (Figure 4-15), along with higher maximum concentrations – a 4-fold increase for some peaks. Of the possible 133 peaks, 5 were in the surfactin range and all 5 corresponded with the peaks in the surfactin standard. 63 of the peaks fell into the “Iturin” group and the remaining 65 fell into the “Fengycin” group. As with the base case, peaks corresponding to the iturin

standard were present, but again, additional peaks not present in the standard were observed. The most prominent iturin peak once again eluted at a retention time of 15.6 minutes, with a maximum concentration of 80.5 mAU·min, 4-times more than the same peak in the base case. Other major iturin peaks eluted at 19.2 and 19.8 minutes, with maximum peak values of 26.7 and 34.5 mAU·min respectively. The major fengycin peaks were observed at retention times of 31.8, 34.8, 35.4 and 40.8 minutes respectively.

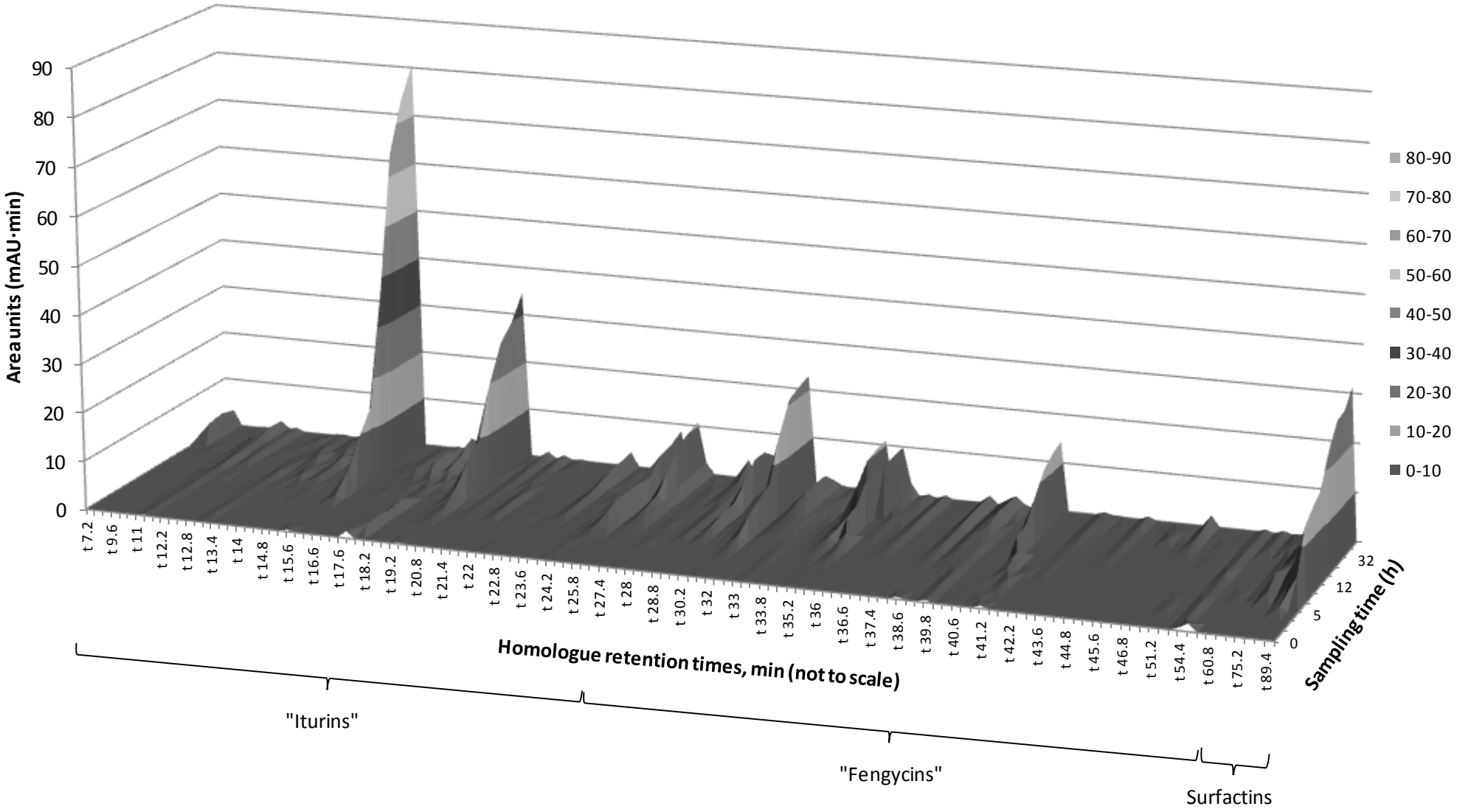

Figure 4-16: 8 g/L NH_4NO_3 homologue profile based on HPLC results

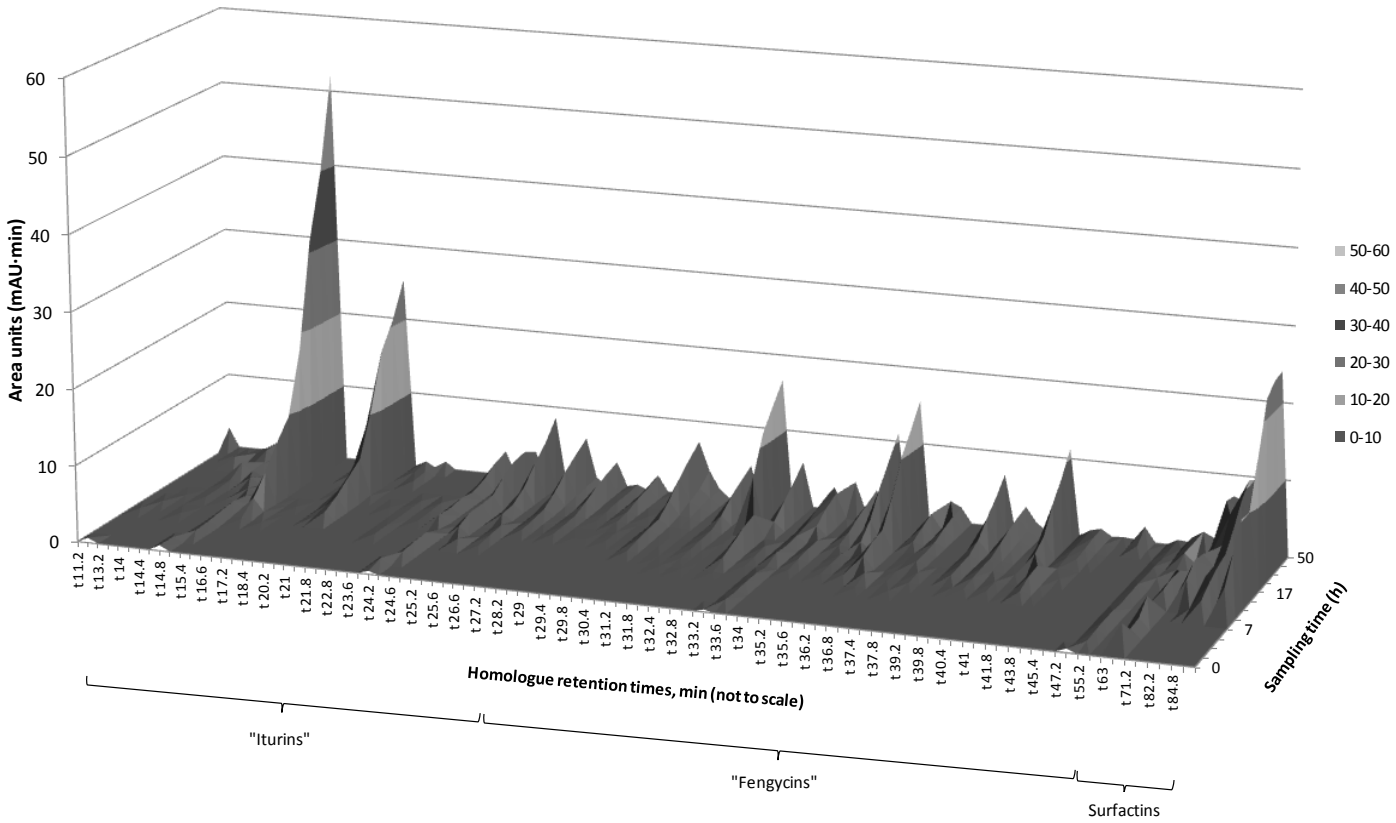

Figure 4-17: 10 g/L NH_4NO_3 homologue profile based on HPLC results

Although the 10 g/L NH_4NO_3 experiment yielded a similar number of peaks as the 4 g/L NH_4NO_3 experiment, around 102, the general trend was that the maximum peak values were higher than those seen in the 4 g/L NH_4NO_3 experiment. Of the possible 102 peaks, 6 were in the surfactin range and 5 of the 6 peaks corresponded to the peaks from the surfactin standard. The sixth peak that was observed was only observed in one of the samples taken, and had a relatively low concentration, suggesting that it could have been an intermediate product. 40 peaks fell into the “Iturin” group and the remaining 56 peaks were located in the “Fengycin” range (Figure 4-17). The most prominent iturin peaks were observed at 16, 19.6 and 20.2 minutes with maximum peak values of 52.1, 18.9 and 25.3 area units respectively. The most prominent fengycin peaks were observed at 32.6, 35.6, 36.2 and 41 minutes with maximum values of 16.6, 11.1, 15.6 and 11.3 area units respectively.

The 12 g/L nitrate run (Figure 4-18) showed more potential peaks than the base case, the 8 g/L or the 10 g/L run. Of the possible 150 peaks, 75 were from the “Iturin” group, 70 from the “Fengycin” group and 5 from the surfactin group. All 5 of the surfactin standard peaks were present during sampling. As with the 4 and 8 g/L runs, the most prominent iturin peak eluted at 15.6 minutes and it had a maximum value of 43.7 area units, 2.1 times higher

than the maximum value for the base case, but 1.8 times lower than that of the 8 g/L run homologue.

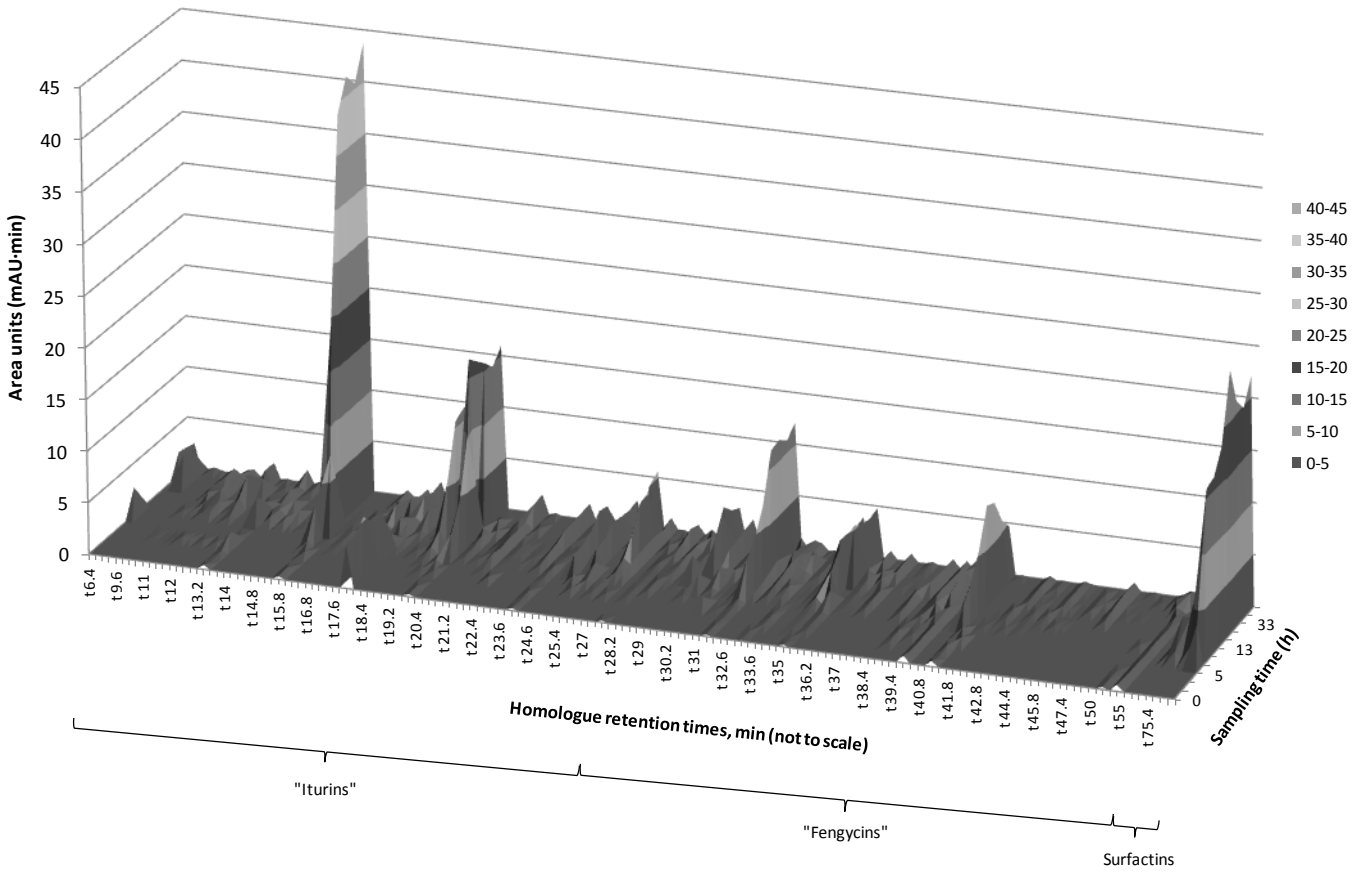

Figure 4-18: 12 g/L NH_4NO_3 homologue profile based on HPLC results

Other prominent iturin peaks were noted at 19.2, 19.8 and 20 minutes, while the prominent fengycin peaks were observed at 32 and 41 minutes. The following table summarizes the number of peaks and their distribution between the three lipopeptide groups for the four NH_4NO_3 concentrations:

Table 4-4: Peak distribution for different NH_4NO_3 concentrations

	Total number of peaks	Number of iturin peaks	Number of fengycin peaks	Ratio fengycin:iturin peak	Number of surfactin peaks
Base case (4 g/L)	105	60	40	0.67:1	5
8 g/L	133	63	65	1.03:1	5
10 g/L	102	40	56	1.4:1	6
12 g/L	150	75	70	0.93:1	5

Table 4-4 shows that by altering the nitrate concentration, the ratio of homologues produced can be manipulated. Increasing the NH_4NO_3 concentration from 4 g/L to 8 g/L resulted in a noticeable increase in the number of homologues produced, the number of iturin and fengycin peaks as well as the ratio in which these homologues are produced. A larger increase in the nitrate concentration, from 4 g/L to 10 g/L, did not yield as many homologues as the 8 g/L treatment, but the ratio of fengycin:iturin peaks, more than doubled from that seen in the base case treatment. The 12 g/L NH_4NO_3 treatment yielded the most peaks of the four nitrate treatments as well as the highest number of iturin and fengycin peaks. Note that the 8 and 10 g/L NH_4NO_3 treatments still resulted in the highest overall concentrations (see section 4.2.1).

The effect of enriched air on homologue production was also investigated. This yielded the following results:

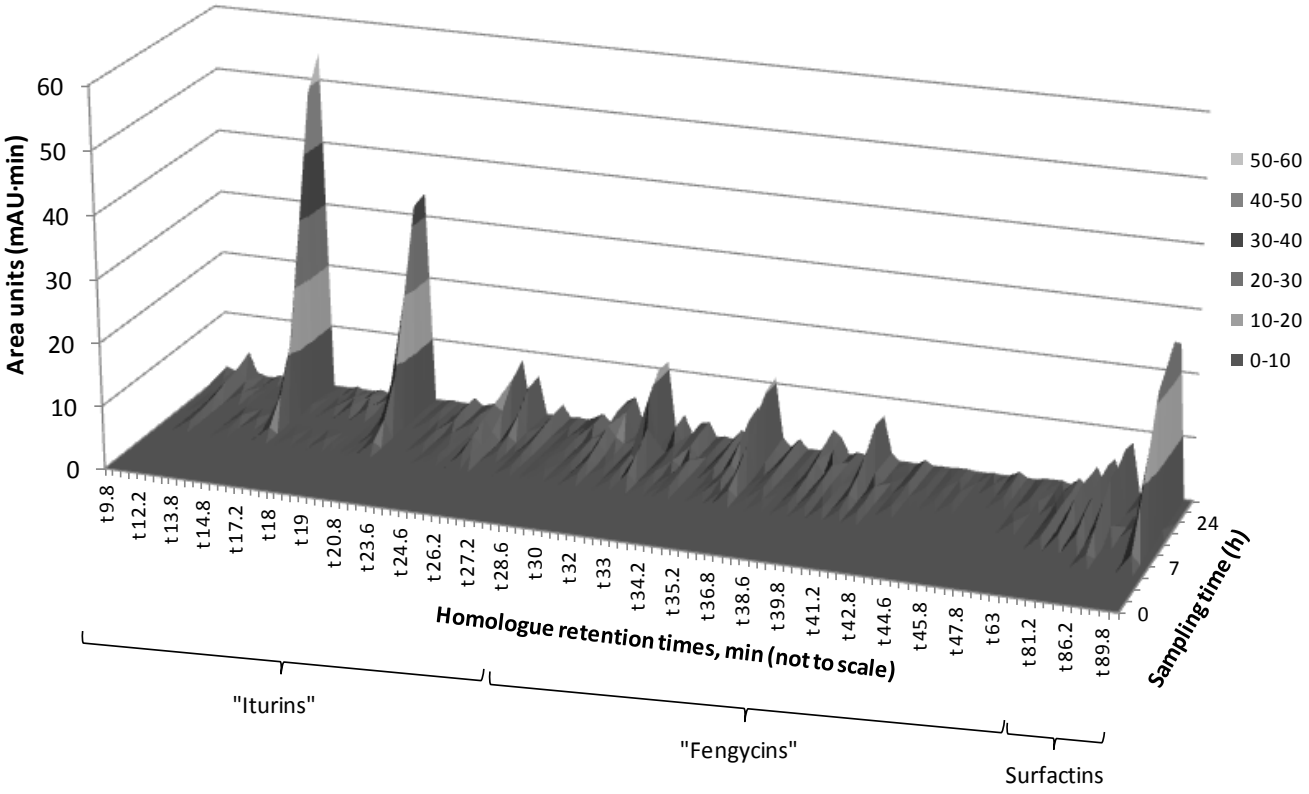

Figure 4-19: 30% Enriched air homologue profile based on HPLC results

For the enriched treatment 113 possible peaks were noted, 48 of which fell into the “Iturin” group, 53 in the “Fengycin” group and 12 in the surfactin range (Figure 4-19). The 5

surfactin standard peaks were present among the 12 in the range and the remaining 7 peaks that were observed could once again possibly indicate the production of partially formed lipopeptides, intermediate products, previously unidentified surfactins or surfactins that are not included in the standard that was used.

The most prominent iturin peak once again eluted at 15.6 minutes and had a maximum value of 54.24 area units, 2.6 times higher than seen in the base case. Other notable peaks eluted at 19.2, 19.8 and 25.2 minutes. In terms of fengycin, the most prominent peaks were noted at 32, 35.6 and 40.8 minutes.

Batch culture results at varying NH_4NO_3 (8, 10 and 12 g/L) concentrations and increased oxygen concentrations (30% O_2) have shown that the ratio in which lipopeptide homologues are produced can be manipulated. This property can be exploited when considering the development and design of a biocontrol agent specific to certain pathogens. In the case of filamentous fungi in general, a NH_4NO_3 concentration of around 8 g/L would be optimal, as this is the concentration at which antifungal production is at its maximum (section 4.2.1), a high number of fengycin homologues are produced (65 peaks) and the fengycin:iturin homologue ratio is also the second highest observed value (Table 4-4). Once studies have identified a specific pathogen, or even a range of pathogens, efficacy tests can be performed to identify which homologue is most effective against the pathogen, as fengycin and iturin have different efficacy against different fungi (see sections 1.2.2.2.2 and 1.2.2.2.3). The production process can then be altered to favour the production of the desired homologue, allowing for a tailor-made agent with increased effectiveness.

The data also shows that the organisms are sensitive to changes in their environment, i.e. the alteration of the growth medium composition or oxygen availability. This further supports the reasoning behind developing a standardized product which is less sensitive to its surroundings.

4.2.3.2 Identification of homologues

Lipopeptide classification was also done *via* LC-MS analysis. The data presented in this section was obtained from the analysis of a sample from the 10 g/L NH_4NO_3 experiment at 35 hours as there were no indications that it would differ substantially from other concentrations. Unfortunately, due to the slight difference in the eluent used in the LC-MS methodology (formic acid for LC-MS versus trifluoroacetic acid for HPLC), the chromatogram

profiles for the LC-MS and the HPLC analyses differ slightly, i.e. the retention times shifted slightly as seen in Figure 4-20 and the magnitude of the peaks also differed to those obtained *via* HPLC analysis. Figure 4-20 shows a prominent peak around 28 minutes, which was not the case in the HPLC analysis (Figure 4-17). This, however, did not influence the compound classification, it only made it less likely to draw an exact correlation between the HPLC and LC-MS results.

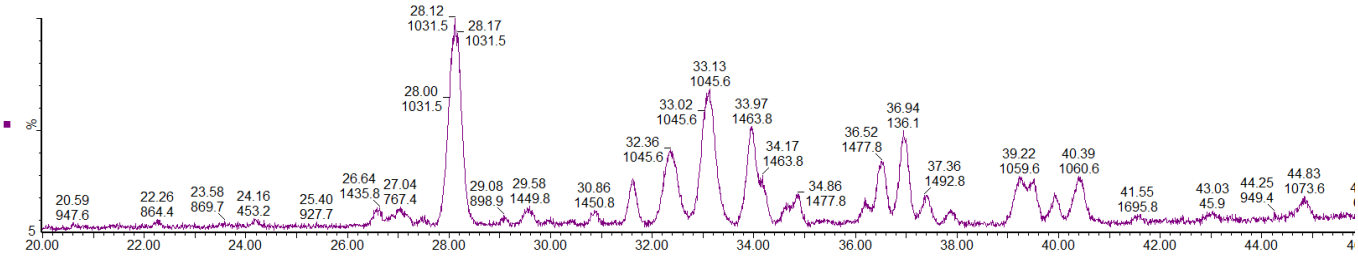

Figure 4-20: LC-MS chromatogram for 10 g/L NH_4NO_3 treatment at 35 hours

The MS analysis of the different peaks defined the surfactin, iturin and fengycin groups based on their resulting *m/z* (mass-to-charge ratio) values. This ratio correlates the mass of a specific ion to the number of elementary charges that the ion carries. This, with the retention time profile, specifically identifies the relevant compounds. Figures 4-21 to 4-23 will show examples of the MS data used to identify the lipopeptide homologues.

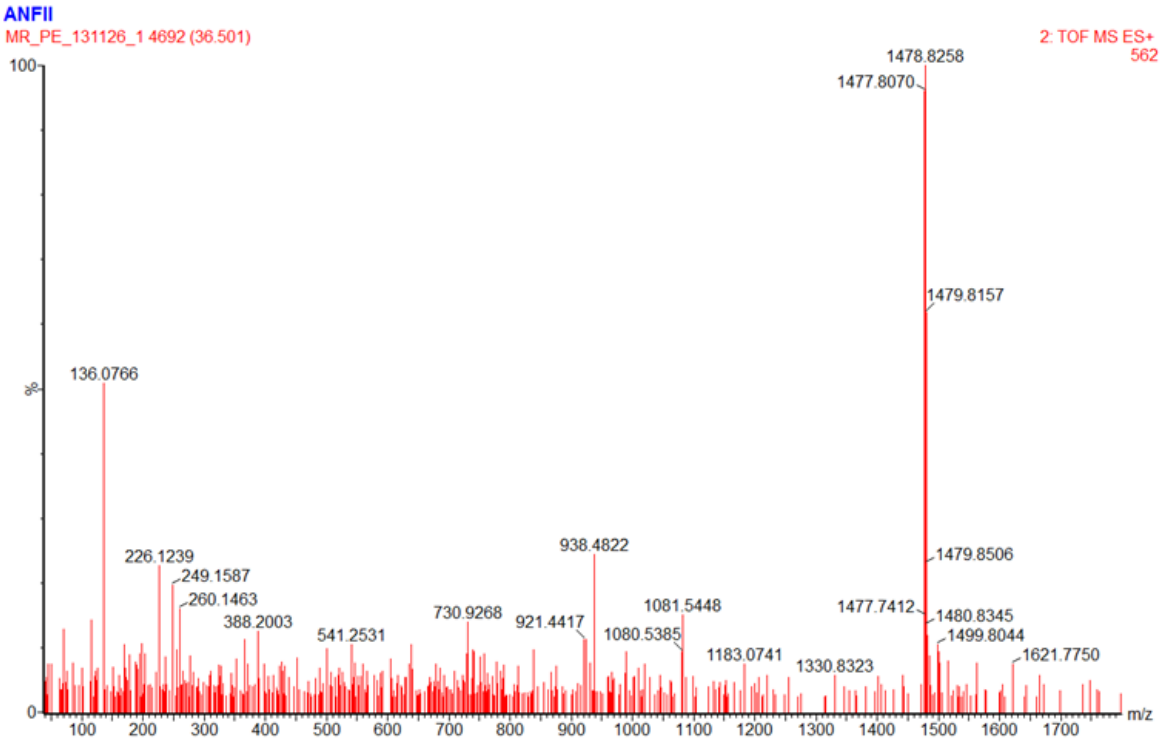

Figure 4-21: Mass spectra corresponding to peak *m/z* 1478.8, retention time 36.5 minutes

Figure 4-21 shows the mass spectrum for the protonated molecule $[M+H]^+$ at *m/z* 1478.8. Literature (Caldeira *et al.*, 2011:1738; Pecci *et al.*, 2010:772) has shown that this compound is fengycin, and depending on the retention time, could be either C_{17}-fengycin A or C_{15}-fengycin B. Other fengycin peaks were found at *m/z* 1434.8 thought to be C_{14}-fengycin A as well as the following protonated molecules: at 1449.8 thought to be C_{15}-fengycin A, at 1463.8 thought to be a fengycin A, at 1491.8 thought to be C_{16}-fengycin B and at 1505.8 thought to be C_{17}-fengycin B. Peaks observed at intervals of 14, such as 1491.8 and 1505.8 can be attributed to a variation in the number of methylene groups (-CH_2-) in their side chains (Pecci *et al.*, 2010:772).

When comparing the mass spectra of iturins and surfactins, the chromatography retention times as well as the possibility of adduct ion formation should be taken into account. Some peaks in an electrospray mass spectrum represent sodium or potassium adducts, i.e. $[M+Na]^+$ or $[M+K]^+$. Figure 4-22 shows the spectra of a sodiated, $[M+Na]^+$, molecule at *m/z* 1067.5 and the protonated molecule at *m/z* 1045.5.

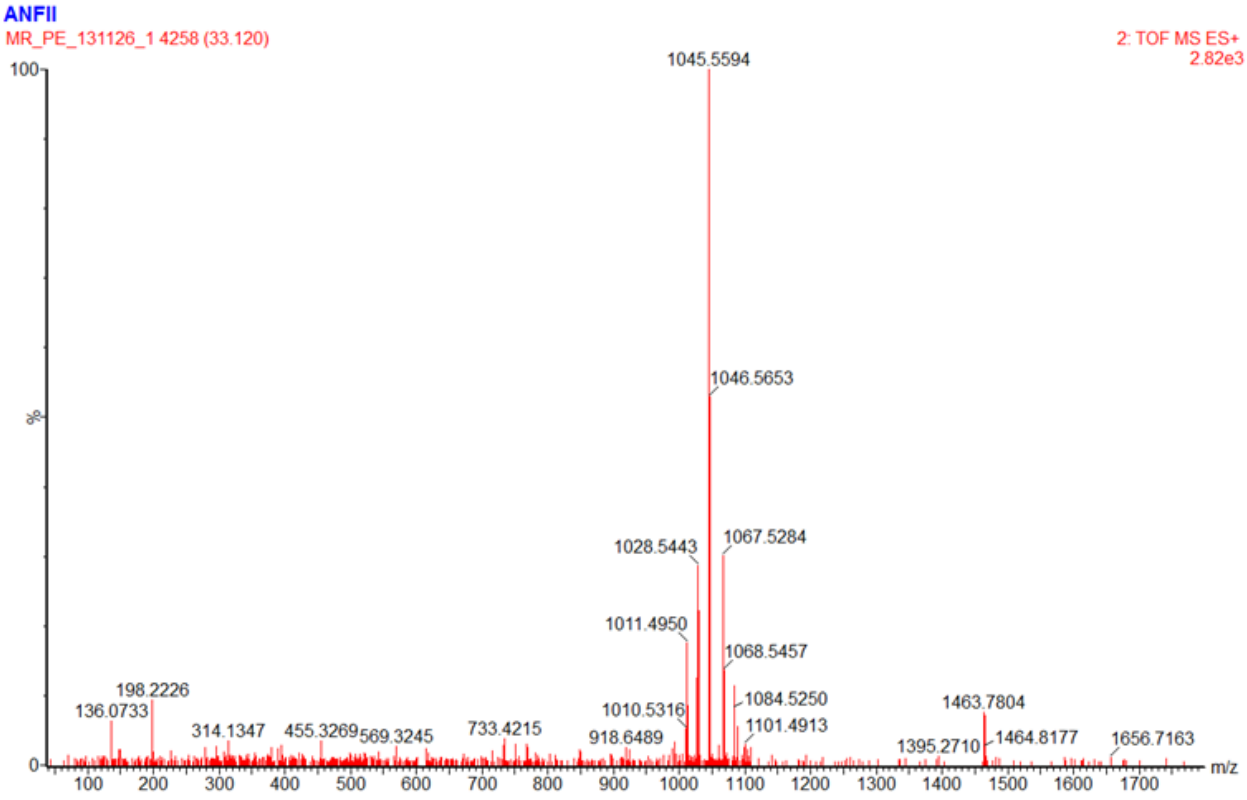

Figure 4-22: Mass spectra corresponding to peak m/z 1045.5, retention time 33.1 minutes

From the MS value (*m/z* 1045.5) (Arguelles-Arias *et al.*, 2009; Hiradate *et al.*, 2002:693), as well as the relatively low retention time, it can concluded that this compound is an iturin and most probably iturin A2. Iturin peaks were also identified at *m/z* 1059.6 as well as 1073.6. Again sodiated molecules were observed.

As previously mentioned, the differentiation between surfactin and iturin spectra could be difficult at first glance, however the retention times can be used to distinguish between the two groups as surfactins elute later than iturins (Figures 4-15 to 4-19).

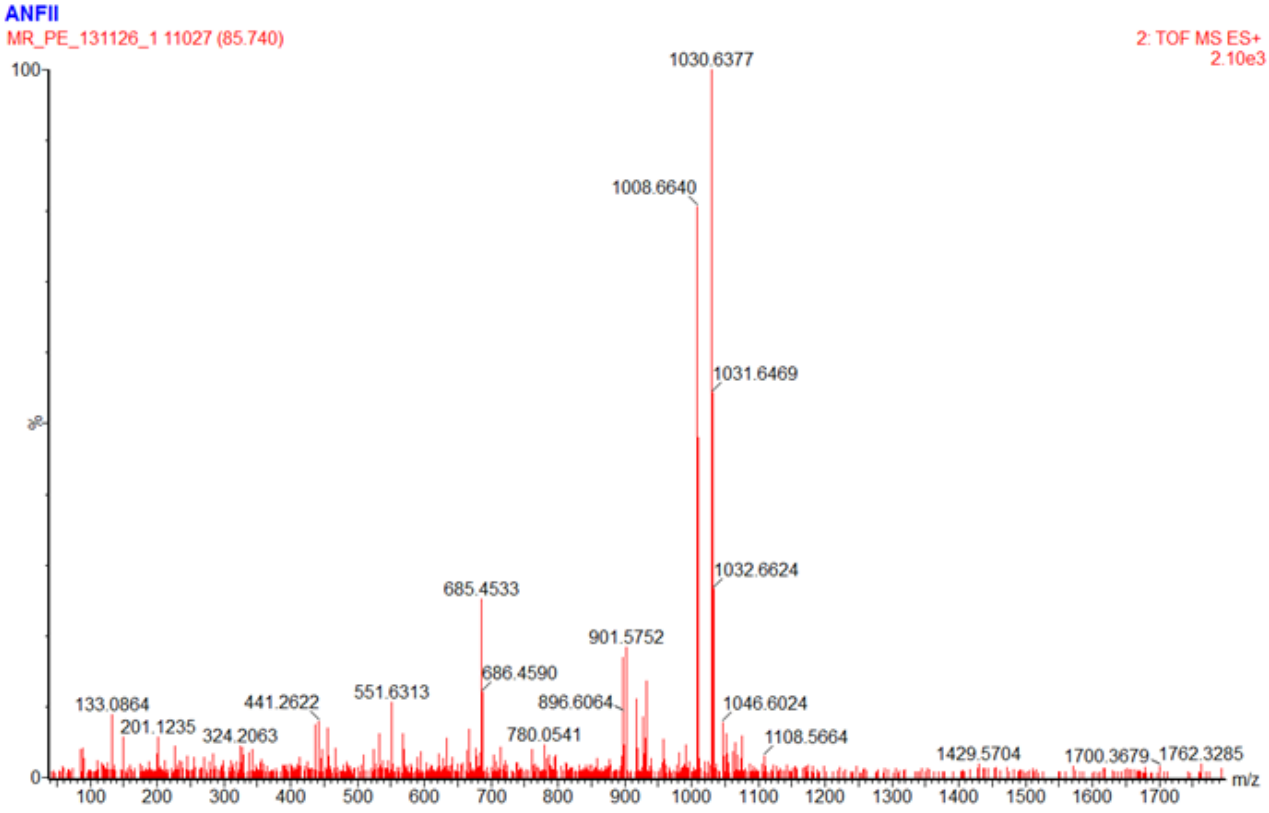

Figure 4-23: Mass spectra corresponding to peak m/z 1030.6, retention time 85.7 minutes

Figure 4-23 shows the sodium adduct $[M+Na]^+$ at a *m/z* of 1030.6 while the *m/z* of 1008.6 represents the protonated adduct $[M+H]^+$. Here the resulting molecular mass of the compound would be 1007.6, which corresponds well with the results obtained by Pecci *et al*. (2010). Literature (Arguelles-Arias *et al.*, 2009) has identified this molecule as C_{13}-surfactin. Other surfactin peaks were identified at *m/z* 1022.7 as well as the sodium adducts at *m/z* 1044.7 thought to be C_{14}-surfactin and at 1058.7 thought to be C_{15}-surfactin.

The results from the LC-MS analysis show that this technique, when combined with HPLC analyses, is a powerful tool in the identification and classification of lipopeptides. Even

without a fengycin standard it was possible to determine which peaks belong to which lipopeptide group. Even though this eliminates the need for a fengycin standard as a means of classification, it does not allow for quantification in terms of grams or milligrams.

4.2.4 Lipopeptide stability

The stability of the lipopeptides after prolonged periods of freezing was tested. This was done by freezing cell-free samples in a commercial freezer at -18°C. The concentrations of the samples were determined before and after freezing *via* HPLC. Samples were taken from all the runs. The results are shown in Table 4-5.

Table 4-5: Summarized results from lipopeptide freezing and stability testing

Run	Sample time (h)	Concentration before freezing		Time frozen (months)	Concentration after freezing			
		Antifungals (mAU·min)	Surfactin (mg/L)		Antifungals (mAU·min)	Surfactin (mg/L)	% Antifungal reduction	% Surfactin reduction
4 g/L NH_4NO_3; 21% O_2	24	113.01	248	10	95.15	202	16	19
8 g/L NH_4NO_3; 21% O_2	32	237.51	277	5	163.14	217	31	22
12 g/L NH_4NO_3; 21% O_2	33	148.61	214	5	41.93	92	72	57
4 g/L NH_4NO_3; 30% O_2	32	197.88	249	4	176.57	207	11	17

Considering that the 4 g/L NH_4NO_3, 21% O_2 sample was frozen for the longest time period (10 months) compared to the 8 and 12 g/L samples, it showed the lowest percentage reduction in both antifungal and surfactin concentration.

The data suggest that with an increase in nitrate, an increase in concentration reduction can be expected for both surfactin and the antifungals. This can be seen from the increase in % reduction for the antifungals from 31 to 72 % and from 22 to 57 % for surfactin, respectively, when nitrate was increased from 8 to 12 g/L.

Minimal reduction was recorded with an increase in oxygen supply (4 g/L NH_4NO_3, 30% O_2), compared to increases in nitrate. The 17% reduction in surfactin was close to the 22% reduction observed for the 8 g/L NH_4NO_3 run, even though the nitrate experiments were frozen for 1 month longer than the 30% enriched air run.

It should be noted that these were only initial observations and for a better understanding of the results, further experiments need to be performed, under exactly the same conditions and the results need to be quantified. The initial observations suggest that the homologue ratios may influence the stability of the lipopeptides.

The results obtained from the freezer experiments suggest that long-term storage in a freezer might not be ideal for these specific lipopeptides and that an alternative freezing method, like freeze-drying, should be considered if long-term storage is required.

4.3 Antifungal efficacy of lipopeptides produced

To test the antifungal efficacy of the lipopeptides produced from the different runs, culture supernatant from the base case (4 g/L NH_4NO_3, 21% O_2), 8 g/L NH_4NO_3, 12 g/L NH_4NO_3 and 30% enriched air at about 32 hours was used in antifungal assays against *Botrytis cinerea*, *Botryotinia fuckeliana* and *Penicilium digitatum*.

4.3.1 Efficacy against *Botrytis cinerea*

No clearance zones were observed after 21 days of growth. Nevertheless, white “fungal walls” were observed on the plates corresponding to wells containing the supernatant samples, while no “fungal walls” were observed near the control wells as seen in Figure 4-24. A fungal wall refers to the dense white lines that separate the fungal colony in the centre of the plate from the wells containing the supernatant, seen in Figure 4-24.

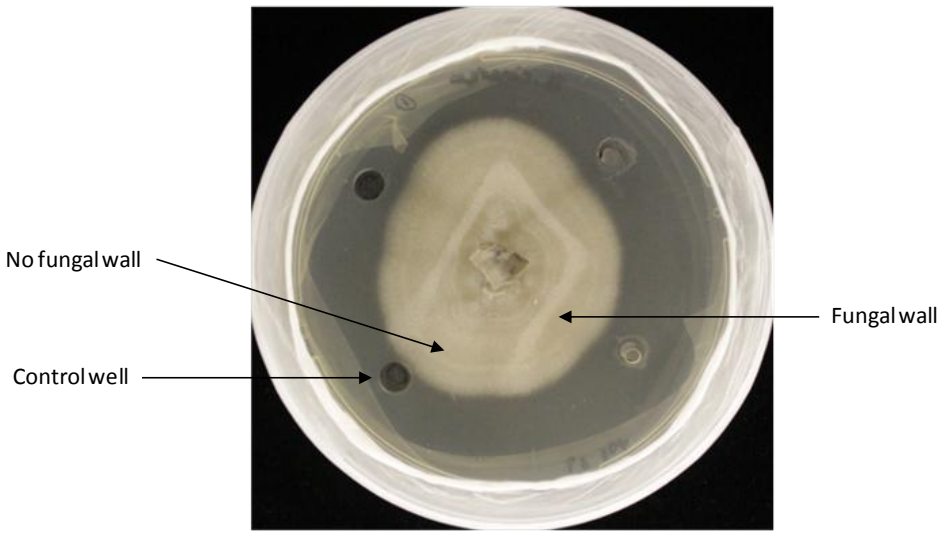

Figure 4-24: Fungal growth interference of *B. cinerea* on PDA plate

A microscopic view (Figure 4-25) around the fungal wall revealed that fungal growth was more dense on the inside (Figure 4-25 a) of the wall, towards the middle of the plate, than on the outside (Figure 4-25 b) of the plate towards the wells. This suggested that the lipopeptides hindered the growth of the *B. cinerea* and that the fungal wall could be a form of defence mechanism employed by the fungus. Hang *et al.* (2005) also found evidence of lipopeptides interfering with the growth abilities of *B. cinerea*, as demonstrated by the abnormal mycelia growth observed in their studies. No fruiting bodies or spores were observed in the current study, indicating vegetative mycelia growth.

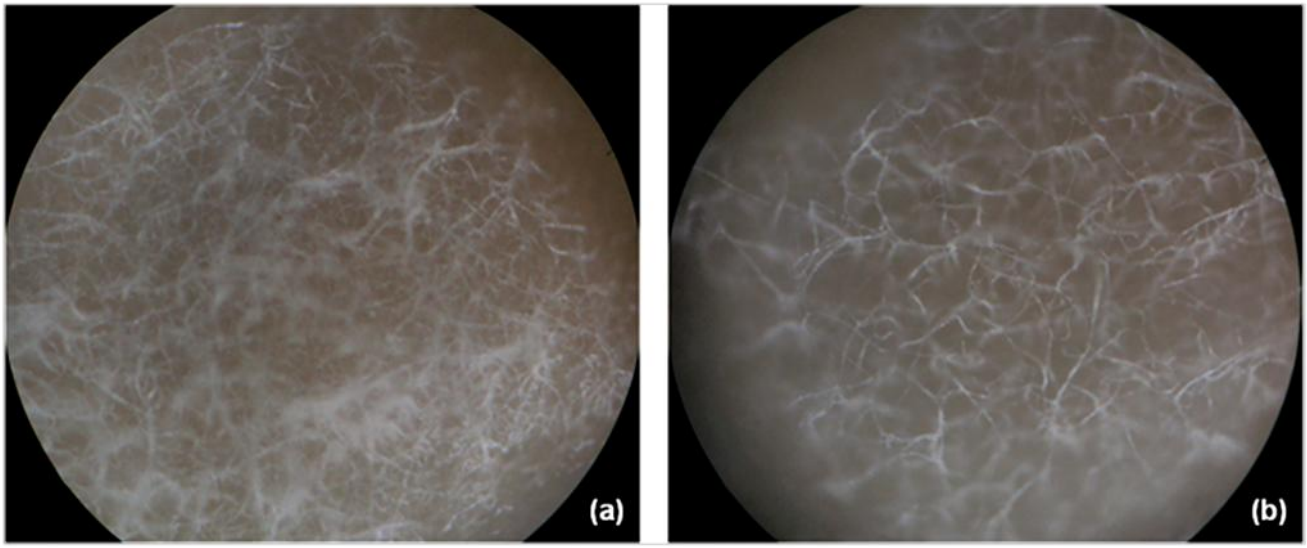

Figure 4-25: Microscopic view of fungal growth (*B. cinerea*); (a) inside the fungal wall, (b) outside the fungal wall

The distances from the wells to the fungal walls are summarized in Table 4-6 and Figure 4-26.

Table 4-6: *B. cinerea* fungal growth interference summary

	Base case	8 g/L NH_4NO_3	12 g/L NH_4NO_3	30% Enriched air
Antifungal concentration (mAU·min)	89.9	163.1	41.9	176.6
Distance to fungal wall (mm) (Mean ± standard deviation)	11.83 ± 1.13	13.17 ± 2.50	11.08 ± 1.47	13.45 ± 0.98

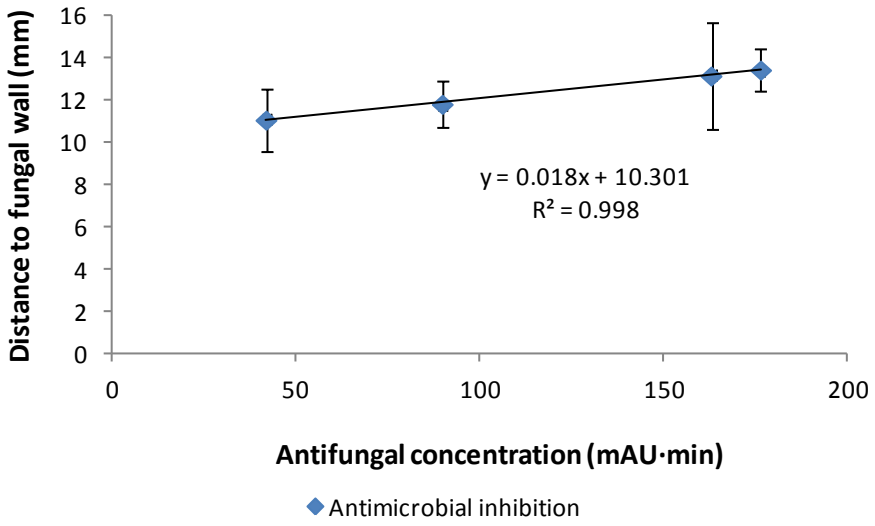

Figure 4-26: Correlation between lipopeptide concentration and fungal growth interference for *B. cinerea.* Error bars indicate standard deviation from mean value

Even as independent samples (each point represents an independent experiment), the data shows that there is a linear correlation between lipopeptide concentration and fungal growth interference (Figure 4-26).

A possible reason for the reduced effectiveness against this specific fungus could be that the rate at which the supernatant diffused through the agar was higher than the rate at which the fungus could grow, in effect reducing the lipopeptide effectiveness by the time it reached the fungal colonies. The lipopeptides still had an effect on the fungus, i.e. by interfering with the growth and stimulating the development of a fungal wall (Figure 4-24), but the concentration reaching the fungal colony itself might have been too low to inhibit growth completely. For this, minimal inhibition concentration (MIC) studies would need to be performed to determine the minimal lipopeptide concentration required to ensure complete fungal inhibition. Subcultures were made to rule out the effect of the culture's age and colonial growth was in accordance to literature (Samson *et al.*, 2002).

4.3.2 Efficacy against *Botryotinia fuckeliana*

After 3 days fungal inhibition became evident (Figure 4-27). The plates were incubated for another day until the fungal colonies had spread over the entire plate and the clearance halos were measured.

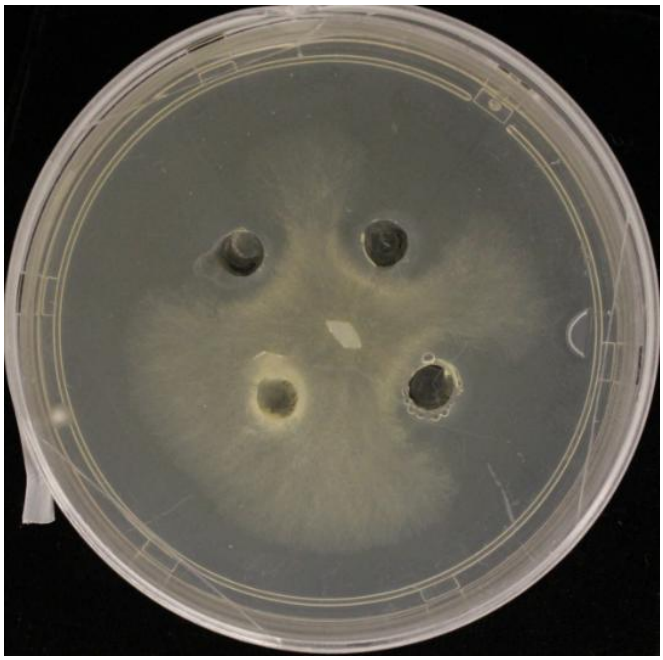

Figure 4-27: *B. fuckeliana* growth inhibition after 3 days with 4 g/L NH_4NO_3

The lipopeptide effect on *B. fuckeliana* was more obvious than that seen on *B. cinerea*, as seen by the clearance halos in Figure 4-27. As previously mentioned, this could be attributed to the growth rate of the fungi, as the *B. fuckeliana* grew much faster than the *B. cinerea* culture (section 4.3.1). The growth inhibition results for the increased nitrate experiments are given in Figure 4-28 and the 30% enriched air results are given in Figure 4-29.

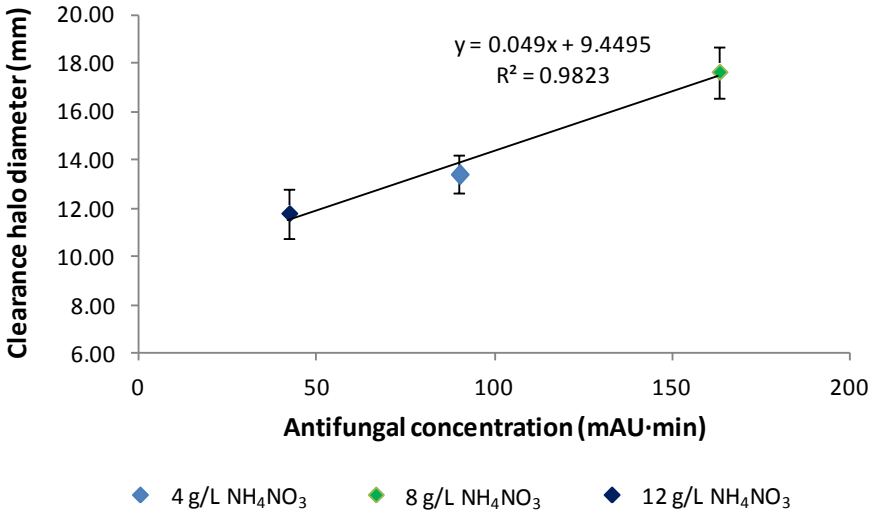

Figure 4-28: Correlation between lipopeptide concentration and fungal growth inhibition for *B. fuckeliana* (NH_4NO_3 results). Error bars indicate standard deviation from mean value

The results shown in Figure 4-28 suggest that there is a linear correlation between lipopeptide concentration and clearance halos measured. The halo diameters compare well with the 11 – 26 mm seen in literature for *Botrytis* spp. (Donmez *et al.*, 2011:758; Hang *et al.*, 2005:59). The 30% enriched air treatment yielded the results seen in Figure 4-29.

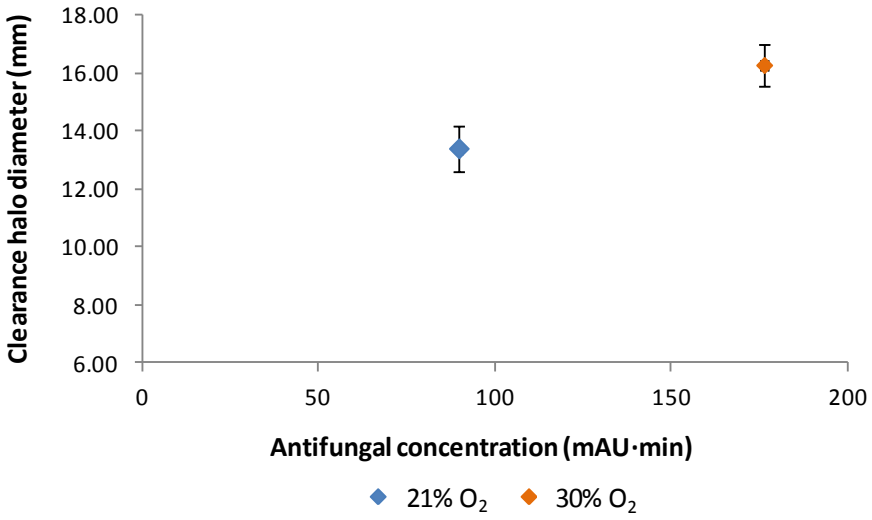

Figure 4-29: Correlation between lipopeptide concentration and fungal growth inhibition for *B. fuckeliana* (30% enriched air results). Error bars indicate standard deviation from mean value

Figures 4-28 and 4-29 show that the *B. amyloliquefaciens* lipopeptides, even in crude extract form, are effective against *B. fuckeliana*. Downstream processing, such as purification and concentration processes, could enable more effective use of the products and could allow growth inhibition in the case of the *B. cinerea* strain used, as the control agent would be concentrated and more potent.

4.3.3 Efficacy against *Penicillium digitatum*

After 21 days no clearance halos or growth hindrance was evident on the *P. digitatum* plates. Microscopic investigation confirmed that the lipopeptides had no effect on the fungus, i.e. effect on the mycelia. This could be attributed to the slow growth of the fungal colonies, as seen with the *B. cinerea* strain. Alternatively, it could indicate that the homologues required for fungal inhibition in the case of *P. digitatum* had not been produced or had not been produced in adequate concentrations in the lipopeptide samples used for these experiments.

4.4 Analytical accuracy and statistical repeatability

4.4.1 Analytical error

Samples from one of the 4 g/L NH_4NO_3 experiments were tested in triplicate to determine the repeatability and validity of the analytical methods employed. For the glucose and lipopeptide analyses, samples were taken at 24 hours for one of the 4 g/L NH_4NO_3 experiments, to ensure that the levels would be mid-range, whereas the nitrate samples were taken during the first 8 hours to ensure quantifiable amounts were present. The following results were obtained:

Table 4-7 : Analytical error summary

	% Error
Glucose	5.4
Nitrate	2
Antifungal	8.3
Surfactin	4.3

The low error results (Table 4-7) show that the analytical methods employed were accurate and repeatable and that the results obtained were credible.

4.4.2 Experimental repeatability

Assessment of the experimental repeatability was divided into two parts, one dealing with the repeatability of the parameters pertaining to growth and process consistency, e.g. initial glucose or nitrate concentration (Figure 4-30), and the other part dealing with production parameters, e.g. maximum concentration or productivity (Figure 4-31). Note that for consistency, these comparisons were done between two experimental runs, under the base case conditions (4 g/L NH_4NO_3, 21% O_2), at 32 hours. The repeatability of the experiments was calculated using Equation 4-1:

$$Repeatability\ (\%) = \left(1 - \frac{standard\ deviation}{average}\right) \times 100\% \qquad \text{Equation 4-1}$$

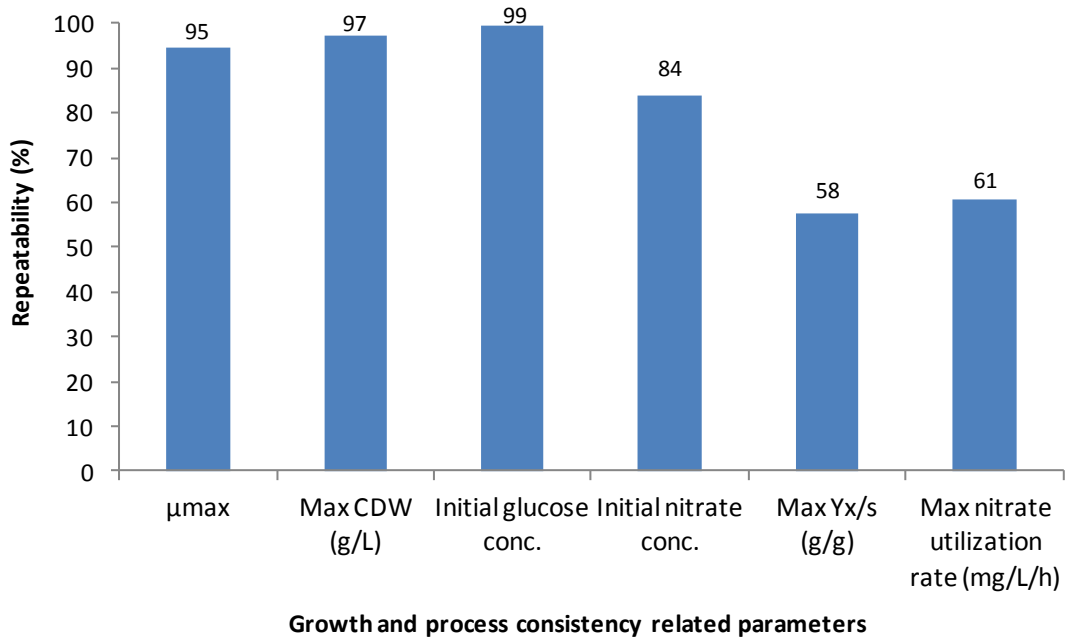

Figure 4-30: Repeatability of growth and consistency related parameters (n = 2)

The high repeatability (%) for μ_{max} and maximum CDW show that the growth and growth rate of the organism are predictable. High initial glucose and nitrate repeatability values were also obtained, showing that the process setup was consistent.

The lower values obtained for the cell yield from glucose ($Y_{x/s}$) can be attributed to this parameter being dependent on the biomass as well as the amount of substrate utilized at that specific point. Due to the relatively small amounts, 0.2 g/g for example, a small difference in substrate utilization at a specific point, can have a noticeable effect on the final $Y_{x/s}$ value, reducing the repeatability of $Y_{x/s}$.

Even though the nitrate utilization followed a similar trend in both experiments, i.e. nitrate utilization commencing and terminating at similar time periods, the maximum utilization rate differed slightly, with one run having a utilization rate of 398 mg/L/h and the other a rate of 226 mg/L/h, hence the variation in repeatability.

Lipopeptide production related parameters were compared and the results shown in Figure 4-31.

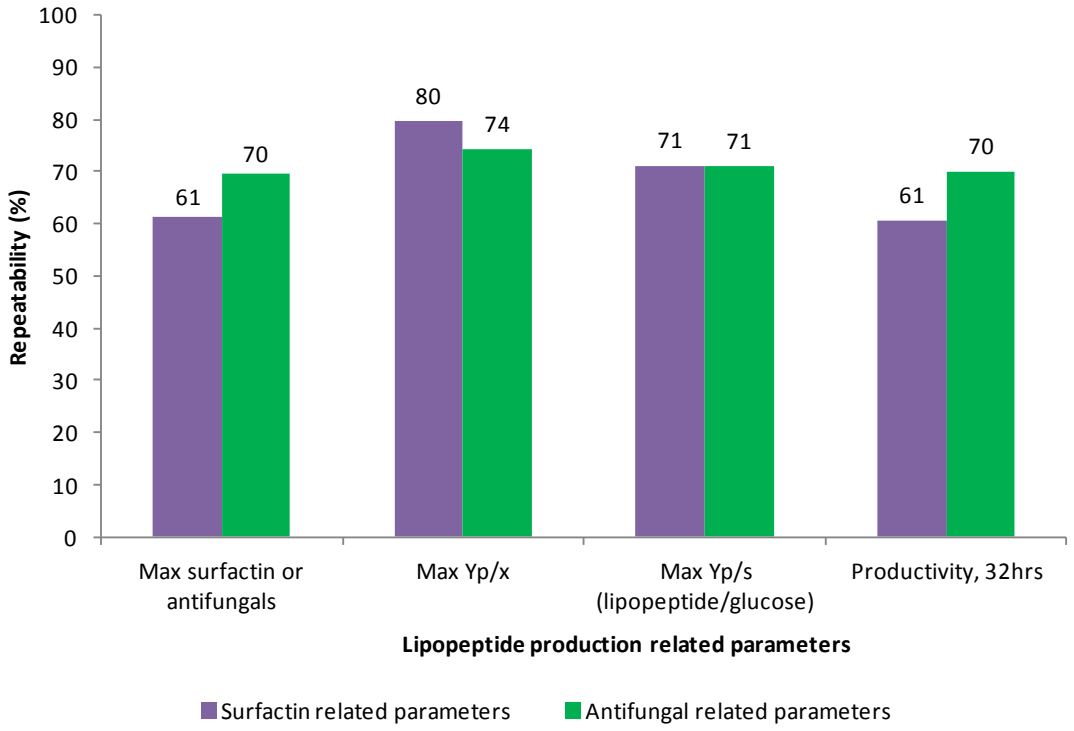

Figure 4-31: Repeatability of lipopeptide production related parameters (n = 2)

The antifungal repeatability of 70% is acceptable, especially when considering that this is a biological system. The surfactin production showed a lower repeatability than maximum antifungal production. The lower surfactin repeatability is attributed to one run having a maximum concentration of 50 mg/L and the other 86 mg/L. Potential causes for reduced reproducibility were investigated. These included sporulation and the effect of antifoam on the lipopeptide production or lipopeptide analyses. Sporulation as cause was eliminated through microscopy and no clear correlation was evident from the antifoam data.

The repeatability of maximum $Y_{p/x}$ for both surfactin and the antifungals were high, showing that lipopeptide production per cell was consistent for the experiments. The average $Y_{p/x,surfactin}$ was 0.015 g/g and the average $Y_{p/x,antifungals}$ was 21.25 mAU·min/g cells/L. The repeatability of maximum $Y_{p/s}$ for surfactin and the antifungals were 71%, indicating an acceptable level of repeatability regarding lipopeptide production from glucose.

The results for the productivity mirror those of the maximum production. This is due to the fact that the productivity is dependent on the lipopeptide production as well as the time period in which the lipopeptides are produced.

Considering that this was a biological system, Figure 4-30 and Figure 4-31 have shown that the results obtained from the experiments were reliable.

Chapter 5

Conclusions

With research focussing on alternatives to synthetic pesticides, *Bacillus* spp. lipopeptides as biocontrol agents have shown great potential. This study focussed on the production of antifungal lipopeptides by *Bacillus* spp., and whether these could be implemented as a biocontrol strategy against phytopathogens diseases such as *Botrytis cinerea* and *Penicillium digitatum*, which cause post-harvest diseases. Following the results and discussion in Chapter 4, the following conclusions were drawn based on the hypotheses stated in Chapter 2:

1) *B. amyloliquefaciens* is the strongest potential antifungal producing candidate.

For potential antifungal applications, *Bacillus amyloliquefaciens* DSM 23117 was found to be the best candidate of the four that were tested, under the specified conditions. This conclusion was based on the fact that it out-performed the other organisms in terms of maximum antifungals produced, maximum antifungal yield per cells ($Y_{p/x,antifungal}$) as well as having the highest antifungal productivity value.

2) An optimum nitrate concentration exists for antifungal lipopeptide production.

An optimum NH_4NO_3 concentration exists above which CDW is no longer increased significantly, μ_{max} decreases and lipopeptide (both surfactin and antifungals) production also decreases.

The optimum NH_4NO_3 concentration for μ_{max} was found to be 10 g/L and the optimum NH_4NO_3 concentration for lipopeptide production was found to be 8 g/L.

3) The lipopeptides produced by the selected *Bacillus* spp. are secondary metabolites.

The lipopeptides, surfactin and the antifungals, produced by *B. amyloliquefaciens* are secondary metabolites, regardless of the treatment employed, i.e. increased nitrate concentrations or enriched air conditions. This, coupled with the information obtained from literature, suggests that a batch configuration or continuous, provided that it is

operated at a low dilution rate, would be ideal for the production of these compounds, as they are nutrient-limited and with lipopeptide production occurring toward the start of the stationary phase.

4) An increase in oxygen supply concentration will increase growth as well as antifungal lipopeptide production.

Increasing the oxygen supply to 30% O_2 stimulated cell growth and lipopeptide production. Comparatively, the 30% O_2 experiment out-performed the 21% O_2 in terms of all the pertinent kinetic parameters. Antifungal (fengycin + iturin) production was increased 1.73-fold and surfactin production was increased 3.66-fold when increasing the O_2 supply concentration.

Using 30% enriched air also extended the nitrate utilization period, which suggests that with the increased oxygen supply concentration more oxygen is available to act as the electron acceptor, allowing the nitrate to be used for lipopeptide production.

5) Homologue ratios can be manipulated by altering process conditions.

Increased nitrate generally increased the total number of homologues produced as well as the number of iturin and fengycin homologues produced.

An optimum NH_4NO_3 concentration exists in terms of the ratio of fengycin:iturin homologues produced, with the highest ratio occurring at 10 g/L NH_4NO_3. The 8 g/L NH_4NO_3 should rather be considered for a biocontrol application, as it yielded more fengycin homologues (65 peaks) and a higher maximum antifungal production.

Process conditions, such as oxygen supply concentration or nitrate concentration can be used to manipulate the ratio in which lipopeptide homologues are produced. This allows for the possibility of a tailor-made biocontrol agent with increased efficacy against selected pathogens.

In order to identify and classify the lipopeptides produced by *Bacillus* spp. LC-MS can be utilized, although it does not replace the need for a fengycin standard for quantification purposes.

6) Lipopeptides remain stable over extended periods of time.

After being frozen for a more than 4 months, the concentrations of the lipopeptides in samples were reduced. This is attributed to the high moisture content, as literature stated that freeze-drying the samples would ensure stability as well as the ability to be kept at below zero conditions for prolonged periods of time.

7) *Bacillus* spp. lipopeptides are effective against phytopathogens such as *Botrytis cinerea* and *Penicillium digitatum*.

In vitro antifungal tests showed that the lipopeptides produced by *B. amyloliquefaciens* were very effective against *B. fuckeliana* and also interfered with fungal growth, by stimulating the development of fungal walls, in *B. cinerea*. No inhibitive effect was observed on *P. digitatum*, suggesting that lipopeptide concentrations were too low or that homologues responsible for the inhibition of *P. digitatum* were not present.

By using the lipopeptides produced by *Bacillus amyloliquefaciens* instead of applying the organism directly as a biocontrol agent, the problem of potential quarantine restrictions has been eliminated. The lipopeptides also allow for the development and production of a standardized, tailor-made product. The lipopeptides produced by *B. amyloliquefaciens* are very effective against *Botryotinia fuckeliana* and also display a fungal growth interference effect on *Botrytis cinerea.* This, along with the data obtained from this study indicates the potential for further development and future implementation of these lipopeptides as applicable biocontrol agents.

Recommendations

The following recommendations have been made based on the results and conclusions presented in Chapters 4 and 5. The recommendations that will be presented fall into one of three possible categories, namely Process development and optimization, Analytical considerations and downstream processing as well as Storage and application methods.

Process development and optimization

- It was found that nitrogen, in this case in the form of nitrate, was vital in ensuring lipopeptide production. It is important that a sufficient nitrogen source is available under aerated conditions, such as those found in a controlled, aerated bioreactor.
- The production process should be scaled up to a 7.5 L bench top reactor, as this would allow for the comparison between different oxygen transfer areas.
- Utilization of a fed-batch system rather than a batch system must be investigated to determine which process is better suited for the production of the desired antifungal lipopeptides.
- Further investigation into the manipulation of the homologue ratios produced under different conditions is recommended. This should include the addition of different amino acids, as these have been shown to alter the ratio in which different homologues are produced and certain homologues have been shown to be more effective as antifungal agents than others. This could allow for a tailor-made post-harvest biocontrol agent.
- An in-depth analysis of the molecular interaction between the antifoam and the lipopeptides is recommended to determine whether an alternate foam-control method should be considered or whether the foaming capability of the broth itself should be utilized with a device such as a foam-trap.

Analytical considerations and downstream processing

- The addition of solids, such as activated carbon, has shown a lot of potential in systems with living organisms. The addition of these solids should be investigated, especially if the process is to be altered slightly to include a foam-trap for example.
- The iturin homologues produced by *B. amyloliquefaciens* DSM 23117 need to be investigated and analyzed, as this strain has shown the potential to produce a wider range of iturins than those found in the commercial iturin standard.
- Possible concentration and purification methods should be investigated when considering potential commercial implementation of this biocontrol strategy.

Storage and application method

- A quantitative, comparative investigation, performed under exactly the same conditions for the same periods of time, is recommended to determine the effect on lipopeptide stability. This will also elucidate the affect that specific homologues have on the stability
- Normal freezing over an extended period has been shown to degrade the lipopeptides and freeze-drying has been recommended, as this method ensures product stability. Freeze-drying will also allow for a longer storage period than normal freezing.
- The application process, i.e. a single application versus multiple applications, must be evaluated as this will determine which application method will be best suited against the selected phytopathogens. A study on the effectiveness of the application method will help to refine the product for potential commercial implementation. Minimal inhibition concentration (MIC) studies are also recommended.

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Appendices

Appendix A: Equations

A1 Maximum growth rate, μ_{max} (graphical method)

$$ln\, x = ln\, x_0 + \mu t$$

Where μ_{max} can be obtained from plotting *ln x* vs. *t*, with μ_{max} represented by the maximum slope of the plot.

A2 Yield, product per cells, $Y_{p/x}$

$$\boldsymbol{Y_{p/x} = \frac{p_i - p_0}{x_i - x_0}}$$

Here the subscript "*0"* indicates conditions at inoculation or at time = 0 and "*i*" refers to the conditions in the reactor at any sampling point thereafter.

A3 Yield, product per glucose, $Y_{p/s}$

$$\boldsymbol{Y_{p/s} = \frac{p_i - p_0}{s_0 - s_i}}$$

A4 Yield, cells per glucose, $Y_{x/s}$

$$\boldsymbol{Y_{x/s} = \frac{x_i - x_0}{s_0 - s_i}}$$

A5 Productivity

$$\boldsymbol{Productivity = \frac{p_i - p_0}{t_i - t_0}}$$

Appendix B: *B. amyloliquefaciens* CDW results at different nitrate concentrations

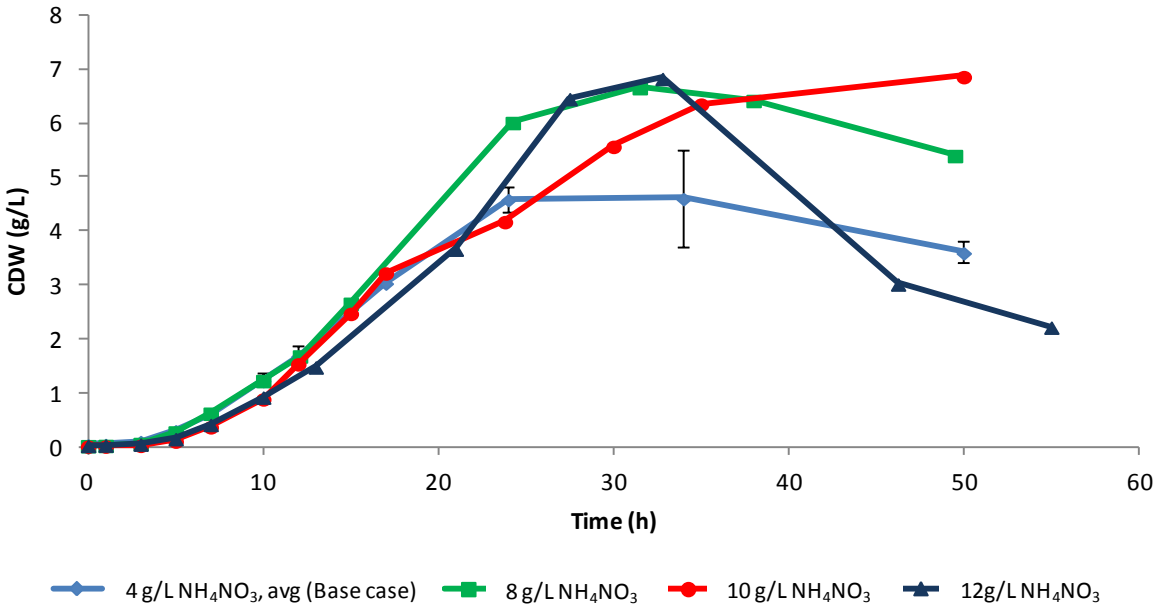

Figure B-1: *B. amyloliquefaciens* CDW results at 4 (n=2), 8, 10 and 12 g/L NH_4NO_3 respectively. Error bars indicate standard deviation from mean value