Kinetic evaluation of lipopeptide production by *Bacillus amyloliquefaciens*

by

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Abstract

Crop losses, caused by phytopathogens and pests, are estimated to be as high as 83% worldwide. These losses along with the world's growing population put additional strain on food production and security which emphasise the need for improved crop protection strategies.

Chemically derived pesticides and fungicides are the preferred control strategy against post-harvest diseases, however their detrimental effect on the environment and human life have directed research towards alternative strategies. Biocontrol have been identified as an alternative since they are environmentally safe, biodegradable and show antagonistic behaviour against fungi, bacteria and even viruses.

Bacillus spp. have been shown to be effective as they produce the lipopeptides surfactin, iturin and fengycin. Direct application of the organism as cells and spores have been well documented and is the focus of commercially developed products. However, cell free lipopeptides have achieved greater inhibition against phytopathogens and are less sensitive to environmental factors.

The study optimised upstream production of antifungal lipopeptides, iturin and fengycin by *Bacillus amyloliquefaciens* in controlled batch cultures. The effect of nitrogen source, concentration and dissolved oxygen availability were quantified through rigorous kinetic evaluation and validated through antifungal efficacy tests. Kinetic evaluation relied on shake flasks and a fully instrumented bioreactor cultures, from where lipopeptide sample were taken for analysis by high performance liquid chromatography.

Increased nitrogen (4 to 8 g/l NH_4NO_3) in bioreactor cultures decreased μ_{max} from 0.237 h^{-1} to 0.19 h^{-1}, increased maximum antifungal concentration from 247.2 to 340.7 mAU*min, maximum specific antifungal production from 43.7 to 124.35 mAU*min/g/l, maximum antifungal productivity from 19 to 26.21 mAU*min/h and antifungal selectivity from 73.8% to 92.0%.

The use of two distinct nitrogen sources (NH_4Cl and $NaNO_3$) had an optimum ratio (NH_4-N to NO_3-N) for biomass and lipopeptide production kinetics. Antifungal production was maximised with the 0:1 or 0.5:0.5 nitrogen ratio. The optimum μ_{max} of 0.258 h^{-1} was obtained with the 0.75:0.25 ratio. The optimum for antifungal production was the 0.5:0.5 ratio, which had the second highest maximum concentration (888.3 mAU*min), highest maximum specific

production (158.15 mAU*min/g/l), highest maximum productivity (26.92 mAU*min/h) and competitively high selectivity (86.7%).

A decrease in dissolved oxygen availability, decreased antifungal lipopeptide production kinetics. Low oxygen conditions forced nitrate to be used as an alternative electron acceptor, decreasing the amount of nitrate available for lipopeptide production.

Optimum conditions cultured in the bioreactor performed better with respect to antifungal kinetics (maximum concentration, specific production and productivity) except μ_{max} and CDW compared to the optimum reported in a previous study (8 g/l NH_4NO_3). Maximum concentration increased from 285.66 to 290.17 mAU*min, specific antifungal production from 51.85 to 58.1 mAU*min/g/l and productivity from 5.67 to 22.32 mAU*min/h.

Culture supernatant, concentrated by acid precipitation, were used for antifungal efficacy tests. Fungal inhibition was observed against *Botrytis cinerea, Alternaria brassicicola, Monilinia fructigena, Penicillium expansum* and *Rhizopus stolonifer* while no inhibition was observed against *Aspergillus sclerotiorum.*

The high effectiveness of antifungal lipopeptides in combination with kinetic data from this study indicate the potential to develop a standardised antifungal product for use against phytopathogens affecting post-harvest fruit. The effect of the process parameters on homologue production and ratio should be investigated, which could allow antifungal products to be tailored to contain specific homologues effective against specific phytopathogens. The use of continuous cultures for further kinetic evaluation and optimisation should be considered as it's been shown, in this study, to be possible.

Samevatting

Daar word beraam dat gewasverliese, wat veroorsaak word deur fitopatogene en peste, so hoog kan wees as 83% wêreldwyd. Hierdie verliese, saam met die groeiende wêreldbevolking, plaas addisionele druk op voedselproduksie en –sekuriteit, wat die behoefte aan verbeterde gewas beskermingstrategieë beklemtoon.

Chemikalieë wat verkry word uit plaag- en swamdoders is die gekose beheerstrategie teen na-oes siektes, alhoewel hul nadelige effek op die omgewing en menslike welstand, navorsing gerig het op alternatiewe beheerstrategieë. Bio-beheer is geïdentifiseer as 'n alternatief aangesien dit omgewingsvriendelik is, bio-afbreekbaar is en antagonistiese gedrag toon teenoor fungi, bakterieë en selfs virusse.

Bacillus spp. het effektief getoon as biologiese beheermaatreël aangesien dit die lipopeptiede surfactin, iturin en fengycin produseer. Direkte toediening van die organisme as selle en spore, is goed gedokumenteer en is die fokus van kommersieel ontwikkelde produkte. Sel-vrye lipopeptiede het egter groter inhibisie teen fitopatogene bereik en is minder sensitief vir omgewingsfaktore.

Die studie optimaliseer die produksie van anti-fungale lipopeptiede, iturin en fengycin, deur *Bacillus amyloliquefaciens* in gekontroleerde lottestande. Die effek van stikstof bron, konsentrasie en opgeloste suurstof beskikbaarheid, is gekwantifiseer deur streng kinetiese evaluering en bevestig deur anti-fungale effektiwiteitstoetse. Kinetiese evaluering het staatgemaak op skudflesse en volledige instrumentele bio-reaktor kulture, van waar lipopeptiede monsters geneem is vir analise, deur middel van hoë-vloeistof chromatografie.

Verhoogde stikstof (4 tot 8 g/l NH_4NO_3) in bio-reaktor kulture het μ_{max} laat afneem van 0.237 h^{-1} na 0.19 h^{-1}. Die maksimum anti-fungale konsentrasie het toegeneem van 247.2 na 340.7 mAU*min, die maksimum spesifieke anti-fungale konsentrasie van 43.7 na 124.35 mAU*min/g/l, maksimum anti-fungale produktiwiteit van 19 na 26.21 mAU*min/h en anti-fungale selektiwiteit het toegeneem van 73.8% na 92.0%.

Die gebruik van twee unieke stikstof bronne (NH_4Cl en $NaNO_3$), het 'n optimum verhouding (NH_4-N tot NO_3-N) vir bio-massa en lipopeptiede produksie kinetika gehad. Anti-fungale produksie is gemaksimeer met die 0:1 of 0.5:0.5 stikstof verhouding. Die optimum μ_{max} van 0.258 h^{-1} was behaal met die 0.75:0.25 verhouding. Die optimum vir anti-fungale produksie was die 0.5:0.5 verhouding, aangesien dit die tweede hoogste maksimum konsentrasie (888.3 mAU*min), hoogste maksimum spesifieke produksie (158.15 mAU*min/g/l), hoogste

maksimum produktiwiteit (26.92 mAU*min/h) en kompeterend hoë selektiwiteit (86.7%) gehad het.

'n Afname in opgeloste suurstof beskikbaarheid het anti-fungale lipopeptiede produksie kinetika verminder. Lae suurstof toestande noodsaak die gebruik van nitraat as alternatiewe elektron akseptor, wat gevolglik die hoeveelheid beskikbaar vir lipopeptiede produksie laat afneem.

Optimum toestande gekweek in die bio-reaktor het beter presteer met betrekking tot anti-fungale kinetika (maksimum konsentrasie, spesifieke produksie en produktiwiteit) behalwe μ_{max} en CDW met vergelyking tot die optimum van 'n vorige studie (8 g/l NH_4NO_3). Maksimum konsentrasie het toegeneem van 285.66 na 290.17 mAU*min, spesifieke anti-fungale produksie van 51.85 na 58.1 mAU*min/g/l en produktiwiteit van 5.67 na 22.32 mAU*min/h.

Kultuur supernatant, gekonsentreer deur suur neerslag, is gebruik vir anti-fungale effektiwiteitstoetse. Fungale inhibisie is waargeneem teen *Botrytis cinerea, Alternaria brassicicola, Monilinia fructigena, Penicillium expansum* en *Rhizopus stolonifera,* terwyl geen inhibisie waargeneem is teen *Aspergillus sclerotiorum* nie.

Die hoogste effektiwiteit van anti-fungal lipopeptiede in kombinasie met kinetiese data uit hierdie studie toon die potensiaal om 'n gestandaardiseerde anti-fungale produk te ontwikkel vir gebruik teen fitopatogene wat na-oes vrugte affekteer. Die effek van die parameters op homoloog produksie en verhoudings moet ondersoek word. Dit sal tot gevolg hê dat anti-fungale produkte ontwerp kan word om spesifieke homoloë te bevat wat effektief is teen spesifieke fitopatogene. Die gebruik van aaneenlopende kulture vir verdere kinetiese evaluering en optimalisering moet oorweeg word aangesien dit, soos aangedui in hierdie studie, moontlik is.

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Introduction

In the absence of crop protection, crop losses are estimated to be as high as 48-83% worldwide. However, with current control methods in place these losses are between 27% and 42%, which are the result of various types of pests including pathogens and invertebrates (Glare *et al*., 2012). This emphasises the importance of effective control strategies in order to limit losses and ensure sufficient production.

Current control strategies rely on chemically synthesised pesticides and fungicides to prevent post-harvest disease and crop spoilage. These strategies have been the preferred method due to their effectiveness against a wide range of pathogens (Glare *et al*., 2012). However, public concern regarding their detrimental effects on the environment and human life has led to stricter regulations and a need for a safe and environmentally benign alternative (Yánez-Mendizábal *et al*., 2011; Glare *et al*., 2012).

A promising alternative is the use of specific microorganisms and their metabolites as biocontrol agents due to their antagonistic nature against fungi and other microorganisms (Ongena and Jacques, 2008). Biocontrol holds a variety of advantages over their chemically synthesised counterparts, such as less strict government regulations, being environmentally benign, biodegradable and exhibiting low toxicity towards organisms not present in the target spectrum (Yánez-Mendizábal *et al*., 2011).

The use of living organisms as either cells or spores has been documented extensively and has led to the development of commercial products, with varying degrees of success (Glare *et al*., 2012). Serenade® is one such product that achieved commercial success due to its wide range of targeted fungal species (Marrone, 2002). The effectiveness of these products, and the cells/spores they are derived from, is dependent on suitable environmental conditions. Furthermore, consistency in production is variable due to the biological nature of the cells/spores that make up these products (Pretorius *et al*., 2015).

The use of cell free metabolites, as opposed to cells/spores for biocontrol, has received considerably less research and is not well documented. The lipopeptides, surfactin, iturin and fengycin have been identified as some of the most effective and promising metabolites for both bacterial and fungal control. *In vitro* tests have revealed that the use of lipopeptides achieved greater fungal inhibition than the use of cell/spores (Yánez-Mendizábal *et al*., 2011).The effectiveness of lipopeptides is independent of environmental conditions, and process control can be implemented to ensure consistent products. Furthermore, lipopeptide

or any metabolic based product would be exempt from quarantine restrictions applicable to living organisms (Pretorius *et al*., 2015).

The current study focuses on the production of antifungal lipopeptides by *Bacillus amyloliquefaciens,* a widely reported organism for biocontrol against phytopathogens. The effect of production parameters that potentially influence lipopeptide production, such as nitrogen concentration, nitrogen source and dissolved oxygen availability were investigated through rigorous kinetic analysis of the biomass growth and lipopeptide production in shake flasks and controlled batch bioreactor cultures. The optimum conditions from these kinetic studies were cultured under controlled conditions in the bioreactor. The resulting cell free supernatant from the optimum culture was tested for its efficacy against six phytopathogens that affect post-harvest fruit, namely *Botrytis cinerea, Alternaria brassicicola, Aspergillus sclerotiorum, Monilinia fructigena, Penicillium expansum* and *Rhizopus stolonifer*.

This thesis follows with background information with regards to the control of phytopathogens, the *Bacillus* genus, biosurfactants, production conditions and strategies in the literature review (Chapter 1), the hypotheses and objectives (Chapter 2), material and methods employed (Chapter 3), and representation and discussion of experimental results (Chapter 4). Lastly, the conclusions, importance and impact of the study, and recommendation to improve the study will be discussed (Chapter 5).

Chapter 1: Literature review

1.1. Control of phytopathogens

1.1.1. Control strategies

Crop protection strategies can be applied to either pre-harvest or post-harvest crops, depending on the type of control and are classified as physical, chemical or biological controls (Saranraj et al. 2012).

Physical strategies are aimed at creating unfavourable conditions for phytopathogens by temperature manipulation (cold-chains), drying and even irradiation (Saranraj *et al*., 2012). Chemical pesticides and fungicides have been the most effective and preferred method for dealing with crop spoilage, however their detrimental effect on the environment, increased concerns about food safety and the occurrence of resistant organisms have led to stricter regulations which have resulted in the removal of many pesticides/fungicides, such as dichlorodiphenyltrichloroethane (DDT). This created a need for an environmentally benign and safe alternative (Glare *et al*., 2012). Biological control, or biocontrol, offers such an alternative.

Biocontrol is not able to completely replace chemically derived pesticides and fungicides as a control strategy, but it can be used in combination with chemicals or other control strategies in the form of integrated pest management (IPM). The rational is that the combined strategies overcome any shortcomings of individual strategies. Various tactics are considered to make up an IPM system, including the use of low risk chemicals, crops bred with resistance against pest, plant extracts and biocontrol (Chandler et al. 2011).Biocontrol includes the use of biological agents such as bacteria, fungi and viruses, as well as bioactive compounds (metabolites) produced by these agents to suppress phytopathogens. Through increased attention and research into the viability of biological control, a number of potential biocontrol agents have been identified, of which the *Bacillus* genus holds many promising species (Xu *et al*., 2013).

1.1.2. Biocontrol strategies

Bacteria have a number of mechanisms at their disposal for phytopathogen suppression, which include: antibiosis by bioactive compounds (lipopeptides), growth promotion, competition and induction of systemically acquired resistance (SAR) (Xu *et al*., 2013). Through these methods the bacteria can protect the host plant from phytopathogens, as is the case

with *B. amyloliquefaciens* which suppresses *Fusarium oxysporum* in cucumber rhizosphere (Xu *et al*., 2013).

It is these abilities of natural occurring bacteria that have led to research where organisms (cells/spores and metabolic products) as biocontrol agents are used. Research focussing on the use of living organisms as control strategy, through the direct application of cells and spores, has been well documented and has even led to the development of commercial products.

Serenade®, containing spores of *B. subtilis* QST-713 (Marrone, 2002), is a biopesticide that has achieved commercial success due to its ability to target a range of fungi. Commercially less successful examples are Chontrol®, derived from *Chondrosterum purpureum,* and Sarritor®, derived from *Sclerotinia minor,* regardless of their ability to target multiple species (Glare *et al*., 2012).

The use of the metabolic products, biosurfactants, as control strategies have been researched significantly less. However, some research has been conducted with respect to metabolic product use. Yánez-Mendizábal *et al*., (2011) showed that the use of cell free supernatant (biosurfactants) displayed better performance than living organisms (cells and spores). *In vitro* studies conducted with *B. subtilis* CPA-8 on known fungal pathogens (*Botrytis cinerea, Penicillium digitatum and Monilinia fructicola*) showed 89-100% inhibition with cell free supernatant compared to 40-73% inhibition with samples containing cells and spores.

The use of biocontrol agents holds a variety of advantages over their chemically derived counterparts, and research has led to improvements, including their activity spectrum and implementation options. However, these natural alternatives are still lacking, specifically with regards to financial viability (Ongena & Jacques 2008).

1.1.2.1. Advantages of biocontrol

The major advantages of natural occurring organisms and their metabolic products are that they are biodegradable, have low toxicity towards organisms not in the target spectrum, and are environmentally friendly (Yánez-Mendizábal *et al*., 2011). Their lack of chemicals exempts them from strict regulations and governments favour their use. In some cases, biopesticides can deliver additional benefits like increased soil nutrient uptake and plant growth benefits (Glare *et al*., 2012). Furthermore, biopesticides can simply be applied to crops through a farmer's spray equipment. There is no difference in the method of application from currently used chemicals nor a need for different equipment (Chandler et al. 2011).

The use of metabolic products have some advantages over the use of living organisms, namely in the sense that cells and spores require a suitable environment to remain active or

effective, while biosurfactants (lipopeptides) are less sensitive to environmental changes like temperature and pH (Pretorius *et al*., 2015). Products containing cells and spores may be subjected to quarantine restrictions due to the presence of living organisms, a restriction that does not apply to the metabolic products. Furthermore, biosurfactants achieve improved phytopathogen inhibition as opposed to cells and spores (Yánez-Mendizábal *et al*., 2011).

1.1.2.2. Limitations of biocontrol

As with most new and innovative research, there exists a variety of limitations that prevent mainstream acceptance and use. This is no different for biocontrol, both metabolic and organism based. Biopesticides are expensive to produce, with regards to both time and money. In 2008, it was reported that approximately $5 million and 3 years are required to develop a biopesticide (Marrone, 2008).

Many biopesticides are too specific as they tend to only target a single pest or genus, such as the biopesticide Bioshield®. There are however biopesticides that target a larger spectrum. Furthermore, due to their biodegradability, these substances rarely remain active for prolonged periods of time and need to be applied regularly (Ongena & Jacques 2008)

Continuous research and development is required in order to improve the limitations associated with biopesticides and ensure effective, large scale production. In some cases this may prove problematic as many companies keep production methods and formulation of biopesticides confidential (Glare *et al*., 2012).

1.1.3. Target phytopathogens

Post-harvest diseases are caused by a range of microorganisms. Post-harvest diseases on fruits are generally caused by fungi while post-harvest diseases on vegetables are caused by bacteria (Sholberg & Conway 2004). These organisms are currently controlled by means of chemically synthesised fungicides and pesticides, however biological strategies making use of cells, spores and cell fee supernatants have been shown to be effective against some of the most widespread culprits (Yánez-Mendizábal et al. 2011). Six known phytopathogens, which specifically affect postharvest fruits (*Botrytis cinerea, Alternaria brassicicola, Aspergillus sclerotiorum, Monilinia fructigena, Penicillium expansum* and *Rhizopus stolonifer),* were used for efficacy tests to assess the effectiveness of antifungal lipopeptides.

Botrytis cinerea targets pome fruits (apples), stone fruits and grapes, causing grey mould. *Monilinia fructigena* targets stone fruits (cherries, peaches, plums and nectarines), causing brown rot. *Penicillium expansum* caused blue mould on pome fruits (apples) and stone fruits. The fungus also produces the mycotoxin patulin, which is carcinogenic (Yánez-Mendizábal *et al*., 2011).

Alternaria brassicicola targets *brassica* species, which include cabbage, broccoli, cauliflower, mustard and canola, causing black spot disease/dark leaf spot (Nordberg *et al*., 2014). *Rhizopus stolonifer* is known as black bread mould, but can also target soft fruits, vegetables and other common food sources (Nishijima *et al*., 1990). *Aspergillus sclerotiorum* is found on a range of raw foods, some of which include beans, peas, nuts and rice. The fungus is responsible for the production of ochratoxins, which has been found to be genotoxic, teratogenic and carcinogenic in a variety of mammalian species and thus a potential safety concern for humans (Ploetz, 2003).

1.1.4. Alternative application

Biocontrol, specifically the use of cell free metabolites, can potentially be applied to more than just post-harvest fruit. The project focussed on the effect of cell free lipopeptides on phytopathogens, thus lipopeptides can be applied to any scenario where these phytopathogens are present, including pre-harvest and possible seed treatment.

The use of biocontrol during pre-harvest would be advantageous in the sense that post-harvest applied biocontrol is not able to control latent infections, that can occur during various stages of development or during the harvesting process. These latent infections have been observed in a variety of fruit including stone fruit, citrus and grapes (Ippolito & Nigro 2000).

A variety of post-harvest diseases have been successfully controlled through pre-harvest application, including rot caused by *Botrytis*, *Rhizopus and Aspergillus.* The use of cell free metabolites for pre-harvest control would not differ all that much from post-harvest however, if living cells and spores were to be used, the chosen antagonist would need to fulfil certain requirements. A pre-harvest antagonist would need to be resistant to environmental conditions such as low nutrients, climate change and UV radiation. Furthermore, the antagonist would also need to be able to attach to the host surface and colonise on the fruit (Ippolito & Nigro 2000).

Seed treatment would not rely on biocontrol on its own, but a combination of both chemical and biological control in the manner of IPM. In this manner, the chemical treatment assists in early season protection while the biological aspect ensures protection during the later season (Chen 2014).

Successful seed treatment strategies are dependent on a variety of criteria including, uniform coverage, adhesion, seed safety, operator safety and environmental safety. The use of biological seed treatment is being studied extensively and is expected to be a fast growing sector (Chen 2014).

1.2. The *Bacillus* genus

1.2.1. Overview

The genus *Bacillus* is a large and diverse group of gram positive bacterial species belonging to the *Bacillaceae* family. These species exhibit diverse physiological and morphological features while retaining some shared characteristics (Todar, 2008). These shared characteristics include: universal occurrence, exceptional colonisation capacity, production of antibiotic substances, versatility in protecting plants from phytopathogens (Xu *et al*., 2013) and several *Bacillus* spp. are generally recognized as safe (GRAS) organisms including *B. subtilis and B. amyloliquefaciens. Furthermore* they also have the ability to sporulate, which guarantees survival as their endospores have high resistance against heat, irradiation (ultraviolet), organic solvents and desiccation (Yánez-Mendizábal *et al*., 2011; Romero *et al*., 2007; Ongena and Jacques, 2008).

The genus associates itself with a complex surface structure, comprising a capsule, a surface layer made up of protein or glycoprotein subunits (S-layer), an arrangement of peptidoglycan sheeting and proteins present on the outside of the cell membrane (Todar, 2008). Furthermore, the species exhibits a uniform flagellum distribution, known as peritrichous flagella which is used for motility (Muradian, 2014; Todar, 2008).

Bacillus species are chemotrophs (Todar, 2008), capable of both aerobic and anaerobic respiration (facultative anaerobes). Anaerobic respiration is achieved by making use of nitrate as an electron acceptor, as opposed to oxygen which is used during aerobic respiration (refer to section 1.4.1.2). Fermentation also occurs in the absence of oxygen, however it is not considered a form of respiration as the electron transport chain is not used (Nakano and Zuber, 1998).

Most *Bacillus* species are mesophiles, i.e. organisms that thrive under moderate temperatures. However, the genus also holds extremophiles, organisms that are not only tolerant of extreme environmental conditions, but thrive under such conditions.

There are many different types of extremophiles, all of which have evolved and adapted in order to survive. Extremophiles can inhabit a range of extreme conditions and it is these environmental conditions that define them. Extremophiles can thrive under acidic conditions (acidophilic), basic conditions (alkaliphilic), high temperatures (thermophilic), extremely high temperatures (hyperthermophilic), at low temperatures (psychrophilic) and many more (Niederberger, 2015).Microorganisms make use of a wide variety of metabolic pathways for substrate conversion and production of metabolites, amino acids and enzymes. A broad overview is given in Figure 1 below.

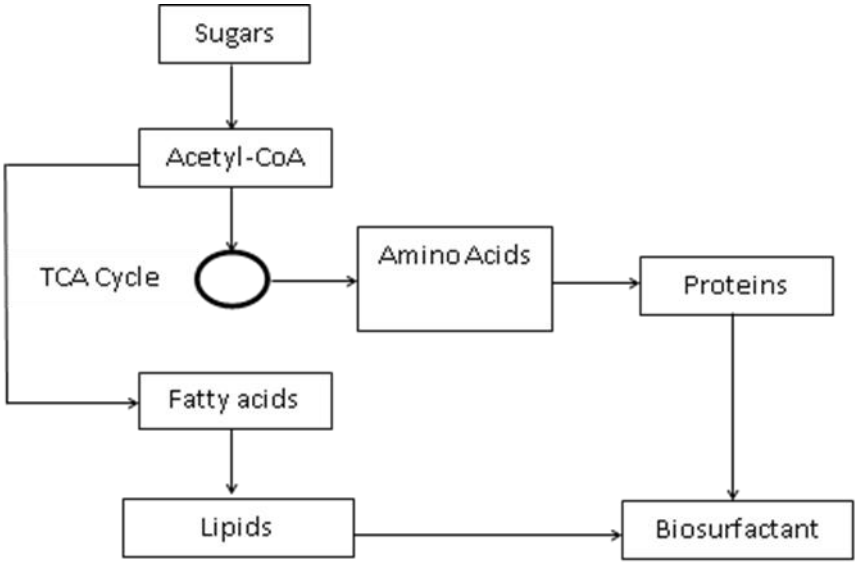

Figure 1: Broad overview of the metabolic pathways employed by *Bacillus* species for biosurfactant production

The outline in Figure 1 shows that sugars are converted to acetyl-coA, which is in turn used in the tricarboxylic acid cycle (TCA cycle) to produce intermediates for amino acid production. Alternatively, acetyl-coA can be used to produce fatty acids via the fatty acid biosynthesis pathway. Both pathways undergo further conversion to obtain the building blocks of biosurfactants, namely proteins and lipids (Clarke, 2013).

1.2.2. *Bacillus* screening

Bacillus spp. are the best-known lipopeptide producers, of which different species have been evaluated as lipopeptide producers, most notably, *B. subtilis* (Besson & Michel 1992; Deleu et al. 2008), *B. amyloliquefaciens* (Arguelles-arias et al. 2009; Cawoy et al. 2015) *and B. licheniformis* (Javaheri et al. 1985; Patel et al. 2004).

Pretorius *et al*., (2015) identified four of the most promising lipopeptide producing *Bacillus* species. These species were screened by experimental means in order to obtain the optimum lipopeptide producer, specifically the antifungal lipopeptides iturin and fengycin. The species considered were *B. subtilis* ATCC 21332, *B. subtilis subsp. spizizenii* DSM 347, *B. amyloliquefaciens* DSM 23117 and *B. licheniformis* DSM 13 (Pretorius *et al*., 2015).

Screening experiments were used to quantify growth and production kinetics through controlled batch bioreactor cultures, which were conducted at the same conditions for each of the four species. The optimum candidate was obtained by comparing the kinetic parameters of the different species. The comparison is outlined in Table 1 below.

Table 1: Comparison of kinetic parameters between screened *Bacillus* species in controlled batch bioreactor cultures (Pretorius *et al*., 2015)

Kinetic Parameter	*B. amyloliquefaciens*	*B. licheniformis*	*B. subtilis*	*B. spizizenii*
Growth				
μ_{max} (h^{-1})	0.43	0.30	0.45	0.39
CDW (g/l)	4.61	5	5.15	8.44
Antifungal				
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				
Max concentration (mg/l)	68	0	882	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

The kinetic parameters in Table 1 show that *B. subtilis* (0.45 h^{-1}) had the fastest growth rate and *B. licheniformis* (0.30 h^{-1}) the slowest. Maximum CDW was greatest with *B. spizizenii* (8.44 g/l) and lowest with *B. amyloliquefaciens* (4.61 g/l). *B. amyloliquefaciens* outperformed all other *Bacillus* spp. with regards to antifungal parameters (max concentration, specific production and productivity), while *B. subtilis* did the same with regards to surfactin parameters. The optimum antifungal producer is thus *B. amyloliquefaciens* and the justification for its use in the present study.

1.2.3. *Bacillus amyloliquefaciens*

B. amyloliquefaciens is a gram positive bacteria, of which there are two subspecies; *B. amyloliquefaciens subsp. amyloliquefaciens* and *B. amyloliquefaciens subsp. plantarum* (Costin, 2012). It has been found, through ssRNA analysis (Todar, 2008), that *B. amyloliquefaciens* and *B. subtilis* are closely related, sharing many homologous genes. Furthermore, these two species appear visually identical (Muradian, 2014).

1.3. Biosurfactants

1.3.1. Overview, classification and structure

A variety of microorganisms are capable of producing biosurfactants as metabolites (refer to Table 2) - whether primary or secondary has not been conclusively established - which are either secreted extracellularly or attached to cells (Okoliegbe and Agarry, 2012). Furthermore, these substances can potentially be used in a range of commercial industries such as agriculture, pharmaceutical, cosmetic and microbiology (Salihu *et al*., 2009).

Synthetic surfactants can be, and are currently used in these industries. However, biosurfactants are superior due to their low toxicity, biodegradability, ecological acceptance and the fact that they can be produced from a range of low cost materials like molasses, vegetable oil and some industrial waste (Gudiña *et al*., 2013; Okoliegbe and Agarry, 2012; Salihu *et al*., 2009).

Biosurfactants are structurally diverse amphiphillic molecules, which means they consist of both polar (hydrophilic) and non-polar (hydrophobic) moieties (Rodrigues and Teixeira, 2010; Salihu *et al*., 2009). Generally saturated or unsaturated fatty acids make up the hydrophobic moiety, while the hydrophilic moiety consists of carbohydrates, cyclic peptides, phosphates or amino acids in either a simple or a complex structure (Okoliegbe and Agarry, 2012; Siñeriz *et al*., 2001).

Biosurfactants were initially classified based on molecular weights into either low or high molecular weight biosurfactants. The former are capable of decreasing surface and interfacial tension while the latter act as emulsifiers (Okoliegbe and Agarry, 2012; Salihu *et al*., 2009). Currently, chemical structures are the preferred classification method and biosurfactants are classified as glycolipids, lipopeptides, polymeric surfactants, phospholipids, fatty acids or neutral lipids (Rodrigues and Teixeira, 2010; Prommachan, 2002).

Table 2: Typical biosurfactants and their best known producers (Prommachan, 2002)

Biosurfactants	Microorganism
Glycolipids	
Rhamnolipids	*Pseudomonas* sp. *Pseudomonas aeruginosa*
Trehalolipods	*Rhodococcus erythropolis* *Nocardia erythropolis* *Mycobacterium* sp.
Sophorolipids	*Torulopsis apicola* *Torulosis bombicola* *Torulosis petrophilium*
Lipopeptides	
Surfactin	*Bacillus subtilis* *B. amyloliquefaciens*
Fengycin	*Bacillus subtilis* *B. amyloliquefaciens*
Iturin A	*Bacillus subtilis* *B. amyloliquefaciens*
Subtilisin	*Bacillus subtilis*
Peptide-lipid	*Bacillus licheniformis*
Gramicidins	*Bacillus brevis*
Polymeric surfactants	
Emulsan	*Acinetobacter calcoaceticus*
Liposan	*Candida lipolytica*
Mannan-lipid protein	*Candida tropicalis*
Phospholipids, fatty acids and neutral lipids	
Phospholipids	*Thiobacillus thiooxidans*
Fatty acids	*Candida lepus*
Neutral lipids	*Nocardia erythropolis*

*This table does not reflect all biosurfactants, or all their producing microorganisms, only the most common.

1.3.1.1. Glycolipids

Glycolipids are mono or polysaccharide carbohydrates, including glucose, rhamnose, galactose and mannose. These carbohydrates are joined to long chains of aliphatic or hydroxyaliphatic acids by means of either an ester or ether functional group (Okoliegbe and Agarry, 2012). The three best known glycolipids are; rhamnolipids, trehalolipods and sophorolipids (Rodrigues and Teixeira, 2010; Okoliegbe and Agarry, 2012).

1.3.1.2. Lipopeptides

Lipopeptides are complex structures consisting of a protein ring, made up of amino acids connected by peptide bonds, which are attached to a fatty acid chain (Pathak, 2011). Lipopeptides produced by *Bacillus* are non-ribosomally synthesised through the multi-enzyme complex non-ribosomal peptide synthetases (NRPSs) (Ongena and Jacques, 2008).

The best known lipopeptides are surfactin, iturin and fengycin, all of which are produced by a variety of mesophilic species in the *Bacillus* genus including, but not limited to, *B. subtilis, B. amyloliquefaciens, B. licheniformis* and *B. subtilis subsp. Spizizenii*(Pathak, 2011; Ongena and Jacques, 2008).

Extremophile *Bacillus* species are also capable of producing certain lipopeptides, these compounds are highly resistant and tolerant to temperature, pH and pressure (Makkar and Cameotra, 1997a) and are thus perfectly suited for harsh conditions such as oil recovery from drilling operations (Makkar and Cameotra, 1998).

Makkar and Cameotra, 1998 studied lipopeptide production of a thermophilic *B. subtilis* (MTCC 1427) strain under thermophilic conditions (45°C). The biosurfactant, which was similar to surfactin confirmed through tin-layer chromatography (TLC) and infrared (IR) analysis, remained stable at 100°C, and between a pH range of 3 and 11.

Surfactin is produced by the NRPS surfactin synthetase, which is made up of three enzymatic subunits. These subunits are structured into seven modules, involving 24 catalytic domains. A minimum set of three domains is present in each module, which allows specific amino acids to be assimilated into the peptide chain. The three domains are: adenylation (A), thiolation (T) and condensation (C) (Pathak, 2011).

Surfactin typically consists of a cyclic lactone ring made up of 7 amino acids connected to a β-hydroxyl fatty acid chain, 13 to 15 carbons in length (Ongena and Jacques, 2008). Seven homologues in the surfactin family have been reported (Pathak and Keharia, 2014). These homologues differ at the 2^{nd}, 4^{th} and 7^{th} amino acids of the peptide ring and in the length of the fatty acid chain (Pathak, 2011; Prommachan, 2002).

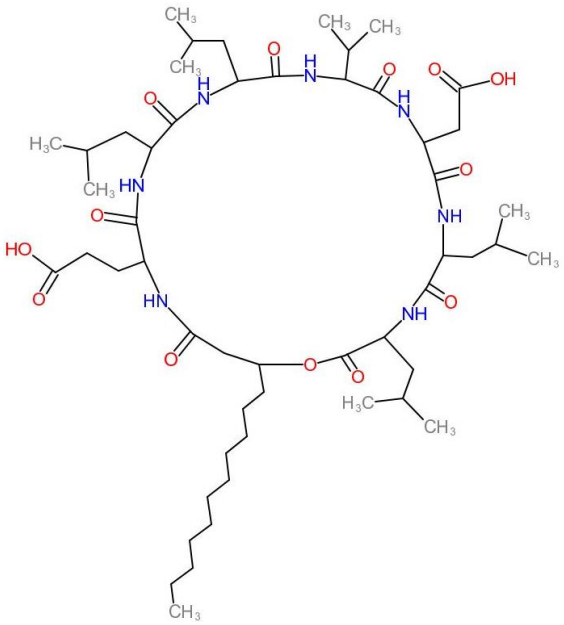

Figure 2: Chemical structure of typical surfactin redrawn from Ongena and Jacques (2008) with Chem4word add in

Iturin's structure (Figure 3) is similar to that of surfactin, consisting of a heptapetide (7 amino acids) ring attached to a β-amino fatty acid chain ranging from 14 to 17 carbons. Pretorius (2014) identified between 40 and 75 iturin homologues. These homologues differ at the 1st, 4th, 5th, 6th and 7th amino acid and in the length of the carbon chain (Pathak, 2011; Prommachan, 2002).

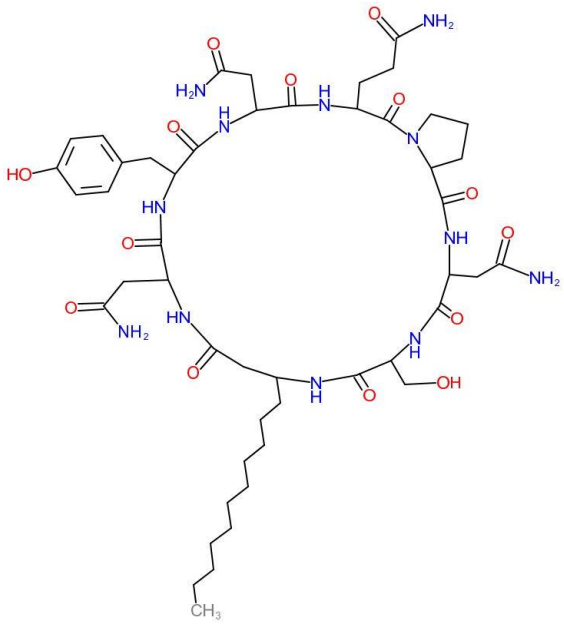

Figure 3: Chemical structure of typical iturin redrawn from Ongena and Jacques (2008) with Chem4word add in

The structure of fengycin differs from that of surfactin and iturin. It consists of a decapeptide chain of which 8 amino acids are connected via lactone bonds to form an internal lactone ring,

which is attached to a β-hydroxyl fatty acid chain, varying between 14 and 18 carbons. Pretorius (2014) identified between 40 and 70 fengycin homologues. These homologues differ at the 6th amino acid and in the carbon chain length (Pathak, 2011; Prommachan, 2002; Ongena and Jacques, 2008).

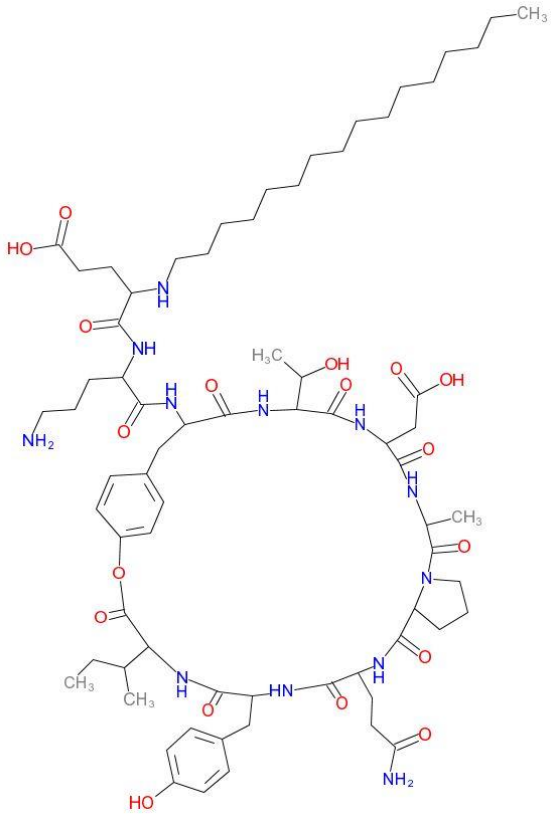

Figure 4: Chemical structure of typical fengycin redrawn from Ongena and Jacques (2008) with Chem4word add in

1.3.1.3. Polymeric surfactants and phospholipids

Polymeric surfactants are polysaccharide-protein complexes consisting of a heterosaccharides backbone with covalently bonded fatty acids (Okoliegbe and Agarry, 2012; Shoeb *et al*., 2013). The most common producer is *Acinetobacter calcoaceticus* (Rahman and Gakpe, 2008; Shoeb *et al*., 2013). The most studied types are emulsan, liposan and mannoprotein (Shoeb *et al*., 2013).

Phospholipids are an important compound that makes up microbial membranes. They play a role in the formation of the protective lipid bilayer. Phospholipids are commonly produced by *Thiobacillus thioxidans* (Rahman and Gakpe, 2008).

1.3.2. Cellular mechanism of lipopeptide action

Biosurfactants, specifically lipopeptides and their producers are of interest as biocontrol agents due to their ability to suppress and kill phytopathogens. The interaction and mechanism by which these compounds achieve this is discussed in this section.

1.3.2.1. Surfactin

Surfactin is one of the strongest known biosurfactants, exhibiting excellent foaming and emulsifying properties, as well as antibacterial activity against a variety of both gram-negative and gram-positive bacteria (Ongena and Jacques, 2008). Furthermore, surfactin is a strong antiviral agents against enveloped viruses, some of which include herpes simplex, retrovirus and vesicular stomatitis (Pathak, 2011).

Surfactin has the ability to interfere with cell membranes by interacting and attaching to lipids in the lipid bilayer which is achieved by its three dimensional and amphiphillic structure. Polar moieties reach into the hydrophilic part of the membrane while nonpolar moieties reach into the hydrophobic part of the membrane (Ongena and Jacques, 2008).

Ongena and Jacques (2008) found that the extent and effect of interference is concentration dependent, defined by the surfactant to lipid mole ratio (X_s) present in the membrane. At a low ratio (X_s <0.04), surfactin attaches to the outer layer of the membrane, causing minimal disruption to the organism. At an intermediate ratio ($0.05 < X_s < 0.11$) the attached surfactin molecules cause permeabilisation, while a high ratio ($0.1 < X_s < 0.22$) results in the formation of permanent pores. Surfactin, at the critical micelle concentration (CMC) (X_s >0.22), completely disrupts and solubilises the lipid bilayer. The last two ratios result in the release of cytoplasmic components and cell death.

1.3.2.2. Iturin

Iturin has shown exceptional *in vitro* antifungal activity against a wide range of fungi including *Penicillium chrysogenum, Microbotrytum violaceum* and *Candida albicans* (Ongena and Jacques, 2008; Romero *et al*., 2007). However, it exhibits poor antibacterial activity, limited to *Micrococcus* spp. (Pathak, 2011).

Iturin's cellular interaction differs from that of surfactin as it is not able to disrupt and solubilise membranes. Instead it diffuses through the membrane by means of osmotic perturbation, where it forms ion conducting pores by interacting with lipids (Ongena and Jacques, 2008). This also allows iturin to interact with nuclear and cytoplasmic organelle membranes. These membrane interactions and pore formation facilitate the release of cytoplasmic components, which leads to cell death (Pathak, 2011; Rodrigues and Teixeira, 2010).

1.3.2.3. Fengycin

Fengycin, similarly to iturin, shows strong antifungal activity, specifically against filamentous fungi, including *Botrytis cinerea, Mycosphaerell pinodes* and *Fusarium moniliforme* (Pathak, 2011; Ongena and Jacques, 2008). The antifungal efficiency of fengycin is further increased in the presence of the other lipopeptides surfactin and iturin. However, fengycin is quite ineffective against bacteria (Pathak, 2011).

Fengycin interacts with fungi by inducing a variety of morphological changes, including bulging, curling and/or hyphae rupturing. The latter potentially explains fengycin's efficiency against filamentous fungi as hyphae are the organism's main mode of vegetative growth. Furthermore, fengycin can form complexes with sterols which is an important organic molecule vital to the structure and function of cell membranes (Pathak, 2011).

The exact mechanism by which fengycin achieves this is not fully understood yet. A dipalmitoylphosphatidylcholine monolayer has been used as a model membrane in order to study the molecular interactions of fengycin. The results suggest that, at a low fengycin to lipid mole ratio (X_f) ($0.1 < X_f < 0.5$), concentrations changes in membrane permeability is achieved by pore formation, while at high concentrations ($X_f > 0.66$) membranes are solubilised (Deleu *et al*., 2005; Ongena and Jacques, 2008).

1.4. Production conditions

The ability of microorganisms to grow and produce metabolites is influenced by the environment in which they are found. The environmental conditions, both nutrient and physiological in nature, need to be recreated and optimised in the laboratory to ensure efficient growth and production. The nutrients include a carbon and nitrogen source as well as salts and minerals, while the physiological conditions refer to oxygen availability, pH, temperature and agitation.

1.4.1. Nutrients

1.4.1.1. Carbon

Carbon is one of the most important nutrient sources for biosurfactant production, of which there are three common sources, namely hydrocarbons, carbohydrates and vegetable oils (Prommachan, 2002; Salihu *et al*., 2009).

Kim et al. (1997) investigated the effect of three carbon sources on biosurfactant production by *B. subtilis* C9. Growth on glucose, n-hexadecane and soybean oil were compared based on surface tension reduction and emulsification activity, which is a property of biosurfactants like surfactin. Thus, the greater the decrease in surface tension, the greater the production of

biosurfactants The surface tension of the culture was reduced from 72.8 dyne/cm to 28.2 dyne/cm with glucose, to 45.3 dyne/cm with soybean oil, and to 50.9 dyne/cm with n-hexadecane as the carbon source. Consequently, the most biosurfactant was produced when using glucose as the carbon source. Furthermore, the use of glucose medium was the only one that highly emulsified all three carbon sources.

Makkar and Cameotra (1998) conducted similar studies with carbohydrates (glucose, sucrose and starch) and hydrocarbons (hexadecane and dodecane) with *B. subtilis* MTCC 1427. The surface tension of the culture was reduced from 68 dyne/cm to 28 dyne/cm with glucose and sucrose, to 29 dynes/cm with starch, to 50 dynes/cm with hexadecane, and to 47 dynes/cm with dodecane.

In these two studies, it seems that the use of carbohydrates, like glucose and sucrose, are the preferred carbon source for *B. subtilis* which is closely related to *B. amyloliquefaciens* by many shared homologous genes (refer to section 1.2.3). These shared genes substantiate the assumption that *B. amyloliquefaciens* also prefers carbohydrates as the carbon source. However, further research is required before it can be confirmed.

Biosurfactant concentration increased with increased glucose concentrations up to 40 g/l, after which increased glucose concentrations yielded no improvements. The increase in concentration beyond 40 g/l led to acidification due to the accumulation of glucose, in the absence of pH control. This caused lipopeptide production to decrease. The optimum glucose concentration for production was thus found to be 40 g/l (Kim *et al*., 1997; Shaligram and Singhal, 2010). Optimal glucose levels were not investigated by Pretorius *et al.* (2015).

Makkar and Cameotra (1997b) investigated the possibility of using non-conventional sources of carbon which are readily available, inexpensive and considered waste material in many cases. Molasses was used as the carbon source with two *B. subtilis* strains, MTCC 2423 and MTCC 1427. Both strains were able to utilise molasses, however production was significantly less efficient than with conventional sources (glucose). After 96 hours strain 2423 produced approximately 1000 mg/l biosurfactant, while strain 1427 produced only about 250 mg/l. Non-conventional carbon sources are not a viable option as of yet due to their low efficiencies, however, they show promise, especially from an economical point of view.

1.4.1.2. Nitrogen

Nitrogen is another important nutrient source, since it is required for the synthesis of proteins and enzymes which are essential to microbial growth (Okoliegbe and Agarry, 2012). Organic and inorganic nitrogen sources can be used (Salihu *et al*., 2009).

Nitrogen is typically added to the medium according to the C:N stoichiometric ratio, which differs significantly between species and cultivation method. Both 13:1 and 27:1 are ratios that have been used for *B. subtilis* (Rangarajan and Clarke, 2016).

The effect of different concentrations NH_4NO_3 (4, 8, 10 and 12 g/l) on growth and lipopeptide production by *B. amyloliquefaciens* was investigated by Pretorius *et al.* (2015) in batch bioreactor cultures. The growth and production profiles achieved with these concentrations were compared. Antifungal (fengycin plus iturin) production achieved the greatest increase with 8 g/l (285.7 mAU*min), compared to the 4 g/l (114.6 mAU*min), while the 10 g/l (244.2 mAU*min) and 12 g/l (148.6 mAU*min) achieved subsequently lower production but still higher than 4 g/l NH_4NO_3. Surfactin production also increased above the 4 g/l NH_4NO_3 concentrations, the 8 g/l and 10 g/l being identified as the optimum with 12 g/l achieving slightly lower production. The maximum specific growth rate increased with increasing NH_4NO_3 concentrations until reaching a maximum of 0.58 h^{-1} at 10 g/l NH_4NO_3.

The source of nitrogen is usually some sort of nitrate or ammonium salt, or one containing both sources (Rangarajan and Clarke, 2016). However, other sources including urea, peptone, beef and yeast extract have been investigated and/or used (Makkar and Cameotra, 1997a).

Makkar and Cameotra (1997) investigated the effect of different nitrogen sources using *B. subtilis* MTCC 2423. Ten sources were considered, including ammonium nitrate, sodium nitrate, potassium nitrate, ammonium sulphate and urea. Biosurfactant production improved to 0.170 g/l with ammonium sulphate, to 0.317 g/l with ammonium nitrate, to 0.724 g/l with sodium nitrate, to 0.731 g/l with potassium nitrate and to 1.012 g/l with urea.

Kim et al. (1997) achieved a biosurfactant concentration of 7 g/l with *B. subtilis* C9 when NH_4HCO_3 was used as the nitrogen source. The optimum NH_4HCO_3 concentration for biosurfactant production was found to be 13.5 g/l.

Nitrate can be utilised in two ways, through assimilation and/or anaerobic respiration, also known as dissimilation (Glaser *et al*., 1995; Painter, 1970). In the case of assimilation, nitrate is converted to nitrite by nitrate reductase, which is further converted to ammonia by nitrite reductase. Ammonia is converted into nitrogenous cell constituents by glutamine and glutamate synthase (Glaser *et al*., 1995; Painter, 1970). Dissimilation is the use of nitrate to oxidise carbon by acting as an alternative electron acceptor during anaerobic respiration (Glaser *et al.*, 1995; Painter, 1970). Strict anaerobes are capable of using nitrate reductase, but can't use nitrate as an electron acceptor for anaerobic growth. Other bacteria such as *E. coli* can grow anaerobically by nitrate respiration, but are unable to convert nitrate to ammonia

by nitrate reductase. Facultative anaerobes such as *Bacillus* spp. are capable of both nitrate assimilation and nitrate respiration. Neither nitrate assimilation nor nitrate respiration would be possible without the enzymes nitrate reductase and nitrite reductase (Glaser *et al*., 1995).

Lipopeptide production has been shown to improve under oxygen depleted nitrate limiting conditions (Davis *et al*., 1999; Rangarajan *et al*., 2015; Rangarajan and Clarke, 2016). The effect of carbon and nitrate (ammonium and nitrate) limiting conditions under aerobic and anaerobic conditions are discussed in section 1.5.4.

1.4.1.3. Mineral salts

Carbon and nitrogen aren't the only required substrates for organism growth and metabolic production. Various other substrates, including mineral salts are also required and influence growth and production to various degrees.

Cooper et al. (1981) investigated the effect of mineral salts on the growth and surfactin production of *B. subtilis* ATCC 21332. A range of salts had no effect on production or growth, some of which were $MgSO_4$, $CaCl_2$, Na_2HPO_4 and KH_2PO_4 while others ($ZnSO_4$, $CuSO_4$, $NiSO_4$, $CoSO_4$ and $Al_2(SO_4)_3$ suppressed and even inhibited growth. The addition of iron ($FeSO_4$) and manganese ($MnSO_4$) increased surfactin production. Many of these heavy metals are associated with toxic properties, which was the most likely cause for growth inhibition (Rangarajan and Clarke, 2016).

Wei and Chu (1998) showed that the addition of 1.7 mM $FeSO_4$, during the exponential phase of a culture, initially containing only 2 mM $FeSO_4$, led to an almost tenfold increase in surfactin production to around 3500 mg/l. The addition also caused acidification and required pH control to prevent the pH from dropping to unfavourable levels. Wei and Chu (2002) investigated the effect of manganese ($MnSO_4$) and found that the addition on 0.01 mM Mn^{2+} increased surfactin production from 330 to 2600 mg/l. These results were confirmed by Shaligram and Singhal (2010) who conducted similar studies on the same *Bacillus* strain.

Mn^{2+} is an important addition to nutrient medium as it promotes the synthesis of the enzyme nitrate reductase which is required for nitrate assimilation (refer to section 1.4.1.2). $Fe2^{+}$ stimulates growth and is required in the synthesis of enzymes that are involved in lipopeptide production (Rangarajan and Clarke, 2016).

Wei *et a*l. (2007) further investigated the effect of metal ions, of which the most important are Mg^{2+}, K^{+}, Mn^{2+} and Fe^{2+}. Mn^{2+} is important as it plays a role in nitrogen utilisation, K^{+} uptake as well as other biochemical functions, all of which contribute to growth and/or biosurfactant production. The optimum concentration of the individual metal ions was determined while keeping the concentration of the other salts constant. Medium containing the individually

optimised trace elements achieved a maximum surfactin production of only 1300 g/l, suggesting significant interaction between the metal ions. Statistical analysis (Taguchi method) was used to investigate the interaction between metal ions and determine the optimum concentration, which was found to be 2.4 mM Mg2+, 10 nm K+, 0.01 mM, 0.008 mM Fe2+ and 7 mM Ca2+. The optimum led to a maximum surfactin concentration of 3340 mg/l.

1.4.2. Physiological parameters

1.4.2.1. Temperature and pH

The effect of temperature and pH on both organism growth and biosurfactant production has been studied by various researchers, making use of different conditions, species/strains and production strategies.

The optimum temperatures for surfactin production by *B. subtilis* RB14 was found to be 37°C (Shaligram and Singhal, 2010). However, temperatures between 30°C and 37°C have been recommended for *Bacillus*, with most experiments conducted at 30°C (Rangarajan & Clarke 2016b; Akpa et al. 2001).

Makkar & Cameotra (1997) investigated the growth and production of *B. subtilis* MTCC 2423 with medium pH ranging from 4.5 to 10.5. The results showed biosurfactant production increased with pH 4.5 to 7, at which the maximum was observed. The optimum pH ranging between 6 and 7 was confirmed by other studies (Cooper and Goldenberg, 1987; Shaligram and Singhal, 2010).

1.4.2.2. Aeration and agitation

Aeration and agitation allows oxygen to be transferred into the aqueous culture medium. This influences the amount of dissolved oxygen available, which is an important factor in biosurfactant production (Okoliegbe and Agarry, 2012; Shaligram and Singhal, 2010).

Yeh *et al.* (2006) investigated oxygen transfer at different aeration and agitation rates. They observed a strong relation between the oxygen consumption, pH of the medium and surfactin production. Increased surfactin production was observed as aeration increased from 0.5 to 1.5 volume per volume per minute (vvm) and agitation from 200 to 300 rpm. Increasing these parameters further (above 350 rpm and 2 vvm) led to rapid foaming, overflow and decreased production. A maximum of 6450 mg/l surfactin was achieved with an aeration rate of 1.5 vvm and agitation rate of 300 rpm.

Rangarajan *et al*. (2015) investigated the effect of oxygen availability on surfactin and fengycin production. This was achieved in shake flasks by using different volume levels, the more volume in a flask, the less oxygen available. It was found that a decrease in oxygen availability,

increased the total concentration of lipopeptides. However, the decrease also shifted the selectivity towards fengycin instead of surfactin.

Pretorius *et al*. (2015) investigated the effect of oxygen availability on lipopeptide production, by *B. amyloliquefaciens* DSM 23117*,* in controlled bioreactor cultures, by supplying both 21% and 30% oxygen. The increased oxygen concentration improved growth, surfactin and antifungal related kinetic parameters, while the selectivity decreased (antifungal/surfactin ratio). In the case of antifungals, the maximum concentration increased from 114.6 to 197.9 mAU*min, the specific antifungal concentration from 21.25 to 36.85 mAU*min/g cells/l, and the productivity from 3.379 to 6.282 mAU*min/h. In the case of surfactin, the maximum concentration increased from 68 to 249 mg/l, the specific surfactin concentration from 0.015 to 0.046 g surfactin/g cells, and the productivity from 1.995 to 7.905 mg/l/h). The maximum CDW increased from 4.607 to 5.406 g/l and maximum specific growth rate form 0.430 h^{-1} to 0.525 h^{-1}

1.5. Production strategy

Microorganisms have been, and continue to be studied extensively. With each discovery, new research opportunities present themselves. These studies rely on successful culturing of organisms, of which there are a few methods available. Process operational strategy include, batch, fed-batch or continuous, also known as a chemostat.

1.5.1. Batch culture

Batch cultures provide an unsteady environment where growth and production parameters vary over time. These parameters refer to nutrient and product concentrations as well as growth rates and even growth phases. Batch culture operation and setup is relatively easy, making it ideal for lab scale experiments.

The familiar growth cycle (lag, exponential, stationary and death phases) of microorganisms are the result of the organism's interaction with its environment. The transition between phases is caused by changes in the environment, the depletion of nutrients and the increase of toxic by-products (Hoskisson and Hobbs, 2005). The growth cycle is almost exclusively observed during batch cultures.

Batch cultures are however limited in their ability to define physiological changes and trends in growth, nutrient limitations and subsequent responses as these are often masked by secondary growth effects (Hoskisson and Hobbs, 2005). Continuous cultures are suited for these studies as secondary growth effects are prevented by continuously providing nutrients.

Batch cultures can be conducted in both shake flasks and bioreactors. However, shake flasks offer less control of operating conditions and typically becomes oxygen-limited faster than bioreactor cultures under the same conditions.

1.5.2. Fed-batch culture

Fed-batch cultures are superior to simple batch processing as changes in nutrient concentrations during batch cultures have a detrimental effect on yield and production (Lee *et al*., 1999). Fed-batch can make use of an exponential feeding rate for nutrient supply, in order to keep the growth rate (μ) constant (Chenikher *et al*., 2010). Fed-batch can however be quite complex since specific feeding strategies are required to prevent either over or underfeeding, both of which are detrimental. Regardless of the added complexity, fed-batch cultures have been used extensively for metabolite production, both primary and secondary (Lee *et al*., 1999).

Chenikher *et al.* (2010) and Guez *et al.* (2007) both conducted fed-batch studies for lipopeptide production by *B. subtilis* BBG100. The production of lipopeptides were maximised when the bioreactor was operated to achieve a specific growth rate of 0.05 h^{-1} and 0.07h^{-1}.

1.5.3. Continuous culture

A chemostat is a system that employs nutrient addition and removal at a constant rate in order to maintain a constant volume of homogeneous distribution, achieved by constant mixing. This ensures that bacteria grow at a constant rate and can reach a steady state since their environment does not change over time. The specific growth rate (μ) can be manipulated by defining the dilution rate (D) since μ = D, under constant volume operation (Hoskisson and Hobbs, 2005; Rao *et al.*, 2009).

The greatest advantage of the chemostat is its ability to study the effect of a single parameter while all others are kept constant. This ensures that secondary growth and bacterial stress effects that may hide physiological changes, are eliminated (Hoskisson and Hobbs, 2005).

Chen *et al.* (2006) investigated the effect of various dilution rates on growth and surfactin production of *B. subtilis* BBK 006. They predicted that the maximum dilution rate should not exceed 0.4 h^{-1}, as washout could occur. Furthermore, they concluded that low dilution rates maximised surfactin production, the optimum range being between 0.1 and 0.2 h^{-1}.

Chen *et a*l. (2006) also compared continuous and batch cultures, operated within the same medium and conditions. They observed higher cell and surfactin concentrations with batch cultures, 520 mg/l as opposed to 290 mg/l, and 136 mg/l as opposed to 12.2 mg/l. The same

trend was observed by Lin *et al.* (1994) 5.3 mg/l biosurfactant in continuous cultures as opposed to 33 mg/l in batch with *B. licheniformis* JF-2.

1.5.4. Lipopeptide production under oxygen and nutrient limiting conditions

The use of nutrients by the bacterium eventually leads to nutrient limited growth, which is when one or more key nutrient(s) has been exhausted. The type of nutrient limiting growth is important for optimisation of lipopeptide production. Davis *et al*., 1999 investigated the effect of carbon and nitrogen limiting growth during aerobic and anaerobic growth on surfactin production by *B. subtilis* ATCC 21332.

During aerobic respiration ammonium is used before nitrate since oxygen is available as an electron acceptor. Only once ammonium was depleted did nitrate utilisation start. In the absence of oxygen, nitrate was used regardless of ammonium's presence. Nitrate and subsequently nitrite were used as electron acceptors alternative to oxygen (Davis *et al*., 1999; Rangarajan *et al*., 2015).

In both aerobic and anaerobic respiration, surfactin production was observed to increase during nitrate utilisation. The only difference was that during anaerobic respiration, surfactin production could continue after nitrate had been depleted since nitrite would be used as an electron acceptor (Davis *et al*., 1999; Rangarajan *et al*., 2015). This was however only possible if nitrate became depleted in anaerobic conditions. Anaerobic growth would cease once glucose became depleted, regardless of the nitrate concentration (Davis *et al*., 1999).

Surfactin production was optimum in conditions where nitrate was utilised until depleted and then became growth limited. Similarly, Rangarajan *et al*. (2015) observed that fengycin production, just like surfactin, was promoted during nitrate limiting conditions. In the case of Davis *et al*. (1999), a maximum surfactin concentration of 439 mg/l was achieved during anaerobic nitrate limited growth, a factor 10 times higher than the other configurations Whether the same trend and optimum is true for iturin is unclear as no information pertaining to iturin production was given by Davis *et al*. (1999) or Rangarajan *et al*. (2015).

Chapter 2: Hypotheses and objectives

2.1. Hypotheses

The project identified and quantified parameters influencing antifungal lipopeptide production, namely nitrogen concentration, source and dissolved oxygen availability. These parameters were evaluated through rigorous kinetic analysis in shake flask and bioreactor cultures to optimise upstream production by *B. amyloliquefaciens* for use as biocontrol against fungal phytopathogens. To date, the systematic quantification kinetics of lipopeptide production and lipopeptide selectivity have not been well documented. Accordingly, the hypotheses are outlined below.

Hypothesis 1: The nitrogen concentration in the medium significantly influences the process kinetics in terms of growth and antifungal lipopeptide (iturin and fengycin) production, and the selectivity for antifungal lipopeptides relative to the co-produced antibacterial lipopeptides (surfactin).

> Nitrogen plays an important role in the cellular metabolism of *Bacillus* spp. secondary only to the carbon source. Firstly, it is involved in the synthesis of proteins and enzymes, which are essential for microbial growth, and in turn in the synthesis of antifungal lipopeptides. Furthermore, nitrogen supplemented in the form of nitrate acts as an alternative electron acceptor in the absence of oxygen. There are significant research works which investigated the influence of carbon source and its concentration on the *Bacillus* spp. growth and lipopeptide production. However, the effect of nitrogen concentration on the microbial growth and lipopeptide production has not been systematically investigated. Also, the fact that lipopeptides are produced during the nitrogen utilisation period prompts the investigation of optimal nitrogen concentration that facilitates optimal lipopeptide production.

Hypothesis 2: The nitrogen source in the medium significantly influences the process kinetics in terms of growth and antifungal lipopeptide production, and the selectivity for antifungal lipopeptides relative to the co-produced antibacterial lipopeptides.

> Nitrogen is typically added to the medium as a nitrate or ammonium salt, or one containing both. Nitrogen facilitates a dual purpose. Ammonium is primarily used for microbial growth while nitrate is involved in antifungal lipopeptide production. In the presence of both ammonium and nitrate, a diauxic consumption profile has been observed (ammonium utilisation followed by nitrate utilisation). However, the effect of

varying concentrations of nitrogen supplied by ammonium (NH_4-N), and nitrogen supplied by nitrate (NO_3-N) in the medium on antifungal production, is still unexplored.

Hypothesis 3: The oxygen availability significantly influences the process kinetics in terms of growth and antifungal lipopeptide production, and the selectivity for antifungal lipopeptides relative to the co-produced antibacterial lipopeptides.

Facultative organisms like *Bacillus* spp. can grow both in the presence and in the absence of oxygen. Also, it has been well established that *Bacillus* cells utilise NO_3^- as the final electron acceptor during oxygen depleted conditions, thus sustaining the cellular metabolism towards growth and product formation. In particular, oxygen depleted conditions have been shown to improve lipopeptide production. However, at the same time, contradictory results also exist, showing both an increase and decrease in antifungal selectivity during oxygen limited growth conditions.

Hypothesis 4: *B. amyloliquefaciens* produces a culture supernatant containing antifungal lipopeptides effective against phytopathogens prevalent in postharvest fruit.

Bacillus spp. as cells/spores have been widely tested for their antifungal efficacies. However, there are very few reports on testing efficacy of crude lipopeptide products derived from these bacteria as cell free supernatant.

2.2. Objectives

The gap identified through comprehensive literature surveys and the hypotheses arrived at based on the key questions, led to the design of the following objectives.

1. To evaluate the effect of increased nitrogen concentration on biomass concentration, growth rate, lipopeptide production (specifically antifungals) and other kinetic parameters ($Y_{x/s}$, $Y_{p/s}$, $Y_{p/x}$, productivity and selectivity).
2. To evaluate the effect of nitrogen source (ammonium and nitrate) on biomass concentration, growth rate, lipopeptide production (specifically antifungals), and other kinetic parameters ($Y_{x/s}$, $Y_{p/s}$, $Y_{p/x}$, productivity and selectivity).
3. To evaluate the effect of oxygen availability on biomass concentration, growth rate, lipopeptide production (specifically antifungals), and other kinetic parameters ($Y_{x/s}$, $Y_{p/s}$, $Y_{p/x}$, productivity and selectivity).
4. To evaluate the efficacy of antifungal lipopeptides against *Botrytis cinerea, Alternaria brassicicola, Aspergillus sclerotiorum, Monilinia fructigena, Penicillium expansum and Rhizopus stolonifer*.

Chapter 3: Materials and methods

3.1. Microbial maintenance

Lipopeptide production experiments, focusing on antifungal lipopeptides, were performed with *B. amyloliquefaciens* DSM 23117, which was previously identified as the most effective antifungal lipopeptide producer (refer to literature outlined in section 1.2.2). A freeze-dried culture of the bacterium was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

Cultures were preserved as glycerol stocks, 30% (v/v) glycerol in distilled water, at -18°C. Culture revival was achieved with the use of nutrient broth medium, of which 1 ml was added to a glycerol stock and incubated for 30 minutes at 30ºC in an orbital shaker (Labcon®). Nutrient agar plates were inoculated with the bacteria by aseptically spreading 100 µl of the incubated medium over the entire plate surface. The plates were then incubated (Labcon®) at 30ºC for another 24 hours and stored at 4°C until needed.

The antifungal efficacy was investigated and quantified by using target phytopathogens (refer to section 3.4.6), which were grown at 25ºC on plates of potato dextrose agar (PDA) (Biolab®) and stored at 4°C.

3.2. Culture media

3.2.1. Solid medium

Nutrient agar (Biolab) was used to create plates in order to culture and grow the bacterium, *B. amyloliquefaciens*. This was achieved by dissolving 31 g of powdered nutrient agar in 1 l distilled water. The medium was then sterilised, in a 1 l Schott bottle, by autoclaving it for 15 minutes at 100 kPa and 121°C (Speedy autoclave®).

3.2.2. Liquid media

Liquid medium which used ammonium nitrate as the nitrogen source, was prepared based on information obtained from literature (Pretorius *et al*., 2015). The composition is given in Table 3 below.

Table 3: Medium containing ammonium nitrate as the single nitrogen source (Pretorius *et al*., 2015)

Nutrient 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	-

3.2.3. Medium preparation

Sterilisation was achieved by autoclaving the medium at 100 kPa and 121°C for 15 minutes prior to use. However, due to the Maillard reaction that occurs between glucose and nitrogen at elevated temperatures and the potential of other unwanted reactions, the nutrients had to be prepared and autoclaved (Speedy autoclave®) separately.

This was achieved by splitting the nutrient components into different solutions. A certain volume of each solution was made, autoclaved and combined once room temperature was reached. The concentrations and volumes required for each solution are outlined in Table 4.

The medium was made up to a 10% higher concentration than required to account for the 10% volume addition from the inoculum. It is assumed that the inoculum addition contains almost no nutrients, and would only decrease the final concentration if not considered.

All cultures contained an inorganic nitrogen source(s) (NH_4NO_3 or NH_4Cl and $NaNO_3$) and yeast extract, as the organic nitrogen source containing approximately 10% organic nitrogen. Yeast extract is made up of various amino acids, vitamins, peptides and carbohydrates. The same amount of yeast extract was present in all cultures as its theorised that *Bacillus* can't produce some of the components found in yeast extract. The effect of inorganic nitrogen, both concentration and source, was the focus of the project. Nitrogen source/concentration refers to inorganic nitrogen.

3.2.3.1. Inoculum and medium with ammonium nitrate as sole nitrogen source

The concentrations and volume requirements of each solution to achieve the concentrations, as outlined in Table 3, is given in Table 4 below.

Table 4: Solution concentrations and volumes required for medium containing ammonium nitrate as the single nitrogen source. SF refers to shake flasks, both inoculum and tests

Solution	Nutrient component	Concentration (g/l)	V - SF (135 ml)	V – Bioreactor (900 ml)	V – Feed (8000 ml)
Carbon	Glucose	89.787	66.825	445.5	3960
Nitrogen and phosphates	NH_4NO_3	8.977	66.825	445.5	3960
	Na_2HPO_4	15.933			
	KH_2PO_4	15.275			
	Yeast extract	1.122			
Trace elements	$MgSO_4.H_2O$	36.89	1.35	9	80
	$MnSO_4.H_2O$	0.189			
	$FeSO_4.7H_2O$	0.222			
	$CaCl_2.4H_2O$	0.111			

In the case of the inoculum and shake flask cultures, autoclaving (Speedy autoclave®) was done in two 500 ml Erlenmeyer flasks. The carbon and trace element solution was transferred to one flask, the nitrogen and phosphate solution to the other. After the flasks reached room temperature, the contents of the one flask was aseptically transferred to the other, resulting in a single flask of sterile nutrient medium containing all components in the required concentration as outlined in Table 3.

A similar approach was followed for bioreactor experiments. However, one of the 500 ml Erlenmeyer flasks was replaced by the bioreactor itself. In this way, the bioreactor was sterilised along with the contents. The contents of the flask were aseptically transferred to the bioreactor once room temperature was reached. All Erlenmeyer flasks were closed off with non-absorbent cotton wool plugs and covered with tinfoil during autoclaving (Speedy autoclave®).

In the case of the feed medium for continuous culture experiments, 3960 ml of each of the individual carbon and the nitrogen and phosphate solutions was made up in 10 l Schott bottles, while only 80 ml of the trace element solution was required. These solutions would serve to make 8 l of feed medium. The three solutions were then autoclaved (Speedy autoclave®) separately and left to cool.

After the sterilised solutions reached room temperature, they were aseptically combined in one of the 10 l Schott bottles to form 8 l sterile feed medium. All solutions used for feed medium were autoclaved (Speedy autoclave®) at 100 kPa and 121°C for 30 minutes. The longer autoclaving time is due to the higher volume of medium.

3.2.3.2. Media with ammonium and nitrate as separate nitrogen sources

The contribution of nitrogen obtained from separate ammonium and nitrate sources was varied in order to investigate the effect of different ratios of nitrogen source on growth and production kinetics. Medium containing these different ratios had to be prepared for experiments. Ammonium nitrate was replaced by individual ammonium and nitrate sources, namely ammonium chloride (NH_4Cl) and sodium nitrate ($NaNO_3$). Total nitrogen was kept constant for all ratios and matched that in the medium containing ammonium nitrate as the single nitrogen source (1.4 g N/l). Furthermore, all other concentrations were kept the same as outlined in Table 3.

The concentrations and volume requirements of each solution to achieve the required concentrations, as outlined in Table 3, is given in Table 5 below.

Table 5: Solution concentrations and volumes required for media containing ammonium and nitrate as separate nitrogen sources. SF refers to shake flasks

Solution	Nutrient component	Concentration (g/l)	Volume - SF (135 ml)	Volume – Bioreactor (900 ml)
Carbon	Glucose	99.773	66.15	441
Nitrogen	Total Nitrogen	6.481	32.4	216
Phosphates	Na_2HPO_4	30.333	35.1	234
	KH_2PO_4	29.081		
	Yeast extract	2.137		
Trace element	$MgSO_4.H_2O$	36.889	1.35	9
	$MnSO_4.H_2O$	0.189		
	$FeSO_4.7H_2O$	0.222		
	$CaCl_2.4H_2O$	0.111		

Two separate nitrogen solutions, 30 g/l ammonium chloride and 80 g/l sodium nitrate, were used to make up the different ratios. This was achieved by combining different volumes of ammonium chloride, sodium nitrate and water, which are outlined for shake flasks Table 6 below.

Table 6: Make up of nitrogen solution for each ratio for shake flasks

Ratio (NH_4:NO_3)	Volume – NH_4Cl (ml)	Volume – $NaNO_3$ (ml)	Volume – Water (ml)
0:1	0	15.93	16.47
0.25:0.75	6.68	11.95	13.77
0.5:0.5	13.37	7.97	11.07
0.75:0.25	20.05	3.98	8.37
1:0	26.73	0	5.67

The different solutions, in Table 5, were sterilised and combined in the same manner as in the inoculum and ammonium nitrate experiments.

3.3. Experimental procedure

3.3.1. Inoculum development

The inoculum required for both shake flask and bioreactor studies was developed through a two-stage inoculation process, ensuring that the bacteria from the first stage were at optimum growth and activity within the exponential phase when being transferred to the second stage and, therefore, that a consistent final inoculum could be obtained. A *Bacillus* colony was transferred from a nutrient agar plate to 100 ml liquid medium, prepared as outlined in section 3.2.2, by means of an inoculation loop. This first stage was incubated at 30°C and 150 rpm in an orbital shaker (Labcon®) between 8 and 18 hours, which correlated to the duration of the exponential phase (as defined by an optical density from 2.3 to 10). The first stage inoculum was used to inoculate the second stage only when the cells were in the mid to end exponential phase at an optical density between 5 and 8.

The second stage was prepared by transferring 10 ml from the first stage to 90 ml liquid medium (10% by volume), prepared as outlined in section 3.2.2, and incubated between 4 and 12 hours. The second stage inoculum was used once it was in the mid to end exponential phase, which related to an optical density between 5 and 10. The second stage inoculum was used to inoculate both shake flasks and bioreactor cultures by transferring 10% by volume (15 ml to 135 ml medium in the case of shake flasks and the entire 100 ml to 900 ml in the bioreactor).

The incubation period for the first and second inoculation stages was obtained through experimental means. These time periods reflect when the inoculum would be in the exponential phase and thus the optimum time to inoculate the second stage and shake flask/bioreactor. The optical density measurements were obtained by diluting a sample with

distilled water, which ensured that the spectrophotometer reading fell within the linear range of the CDW standard curve, and factoring in the dilution when determining the actual optical density and CDW values (refer to section 3.4.1.2).

Growth data was obtained, through culture samples obtained at regular intervals, from the first stage inoculum during a 48-hour incubation period, at 30°C and 150 rpm in an orbital shaker (Labcon®). The same was done for the second stage inoculation. Refer to Appendix 1 for the experimental results and discussion.

The inoculum was prepared in 500 ml baffled Erlenmeyer flasks and all transfers were done aseptically by making use of the laminar flow cabinet and sterile equipment.

3.3.2. Shake flask cultures

Shake flask experiments were conducted in 500 ml baffled Erlenmeyer flasks, except for the unbaffled oxygen experiments. Baffled shake flasks consisted of four equally spaced indetations acting as baffles around the base of the flask; these baffles were created in unbaffled flasks by a glass blower. The medium was incubated at 30°C and 150 rpm for 48 hours in an orbital shaker (Labcon®).

Culture samples for growth, substrate and production analysis were taken throughout the 48-hour time span (refer to section 3.4). The experiments were done in either duplicate or triplicate in order to assess the reproducibility.

3.3.3. Bioreactor cultures

Bioreactor experiments were conducted in a 1.3 l bench-top fermenter (New Brunswick, BioFlo 110) with a working volume of 1 l. The bioreactor made use of temperature, pH and dissolved oxygen probes and controllers to monitor and adjust variables.

The operating conditions for the bioreactor were obtained from literature (Pretorius *et al*, 2015). The temperature was maintained at 30°C, the pH was controlled between 6 and 7 with the addition of 2M sodium hydroxide, while the agitation and aeration was constant at 250 rpm and 0.8 vvm respectively.

3.3.3.1. Bioreactor sterilisation and setup

The sterilisation procedure consisted of two steps, namely sterilising the bioreactor/medium and reconnecting the bioreactor.

The bioreactor was first cleaned with soap water. The glass vessel was then lowered into the vessel stand and filled with the required carbon and trace element solution. An Erlenmeyer flask with a bottom outlet, with a silicon tube attached, was used to hold the required nitrogen

and phosphate solution. The silicon tube made it possible to aseptically transfer the nitrogen and phosphate solution to the bioreactor after sterilisation and cool down. The required solution volume was experimentally dependent (refer to section 0).

The head plate was installed, all bolts and adapters were finger-tightened equally to balance the pressure exerted. The O-rings were inspected prior to installing the head plate and replaced and/or greased as required. The sampling assembly, consisting of the sampling tube and bottle, was connected to its addition port. The sparger and exhaust port filters were installed. The pH probe was inspected, calibrated and installed while the dissolved oxygen probe's membrane was inspected, replaced if necessary, and filled with electrolyte solution before installation. Care was taken to ensure that there was no interference with the probes. In order to protect the external connections to the motor and probes during autoclaving (Speedy autoclave®), these were covered with protective caps.

The addition bottles reserved for pH buffer and antifoam solutions were filled with water and attached to the addition port by means of silicon tubing. All tubes, except the exhaust port filter were clamped off. All filters and ports were wrapped with non-absorbent cotton wool and protective aluminium tinfoil. The entire assembly, bioreactor and addition bottles, along with the Erlenmeyer flask, were autoclaved for 30 minutes at 121°C and 100 kPa.

After the autoclaving process the bioreactor, addition bottles and the Erlenmeyer flask, along with their respective contents, were left to cool to room temperature. The silicon tube from the Erlenmeyer flask was attached to one of the unused addition ports of the bioreactor, after sterilising both with ethanol, allowing aseptic transfer of its contents to the bioreactor. The 2M sodium hydroxide solution was made by adding the required amount of sodium hydroxide to one of the sterilised addition bottles containing only water. Similarly, 10% by volume of 30% antifoam was added to the other sterile water containing addition bottle.

At this point the bioreactor could be reconnected, and all probes, water and air lines were connected. The temperature probe was inserted with glycerol present in the thermowell. The temperature control system was installed by connecting the cooling water loop and heating jacket. Once the set point temperature was reached, the pH probe's calibration was confirmed by aseptically drawing a sample of the culture and measuring its pH with an external probe. The dissolved oxygen probe had to be polarised by plugging it in and leaving it for 6 hours, after which it had to be calibrated.

Calibration was achieved by sparging the bioreactor sequentially with nitrogen and air in order to determine a point of minimum dissolved oxygen and a point of maximum dissolved oxygen. The bioreactor was first sparged with nitrogen until the meter reading stopped fluctuating,

which was used as the zero point. The same was done with air, but to set the span (100% dissolved oxygen) point.

The bioreactor was then inoculated from the second stage inoculum by transferring the entire 100 ml inoculum aseptically to the bioreactor, by means of a sterile Erlenmeyer flask with an attached silicon tube, bringing the entire bioreactor volume to 1 l.

3.3.3.2. Batch bioreactor operation

The bioreactor (Figure 5) sampling procedure was different from that of shake flasks, as the laminar flow chamber could not be used with the bioreactor. In order to ensure that the bioreactor remained sterile from other contaminants during and after sampling, a dedicated sampling assembly was used.

The sampling assembly was connected to an addition port on the bioreactor by silicone tubing which fed into the sampling bottle that screwed into the assembly and formed a seal with the use of an O-ring. The other side of the assembly consisted of an air filter attached to a syringe. The syringe was used to draw air through the filter, creating a pressure drop between the bioreactor and sampling bottle, resulting in the bottle being filled with a sample of the culture. The sampling bottle could then be removed and replaced by another sterile sampling bottle.

After each sample, some culture would remain in the tubing, which would first be removed by the same method described above, before taking the actual sample that would be used for analysis. This ensured that each sample taken for analysis originated from the bioreactor and represented what was happening in the culture at that time. All sampling bottles were autoclaved prior to use at 121°C and 100 kPa for 15 minutes.

Culture samples for growth, substrate and production analysis were taken throughout a 48-hour time span. The experiments were done in duplicate in order to assess the reproducibility.

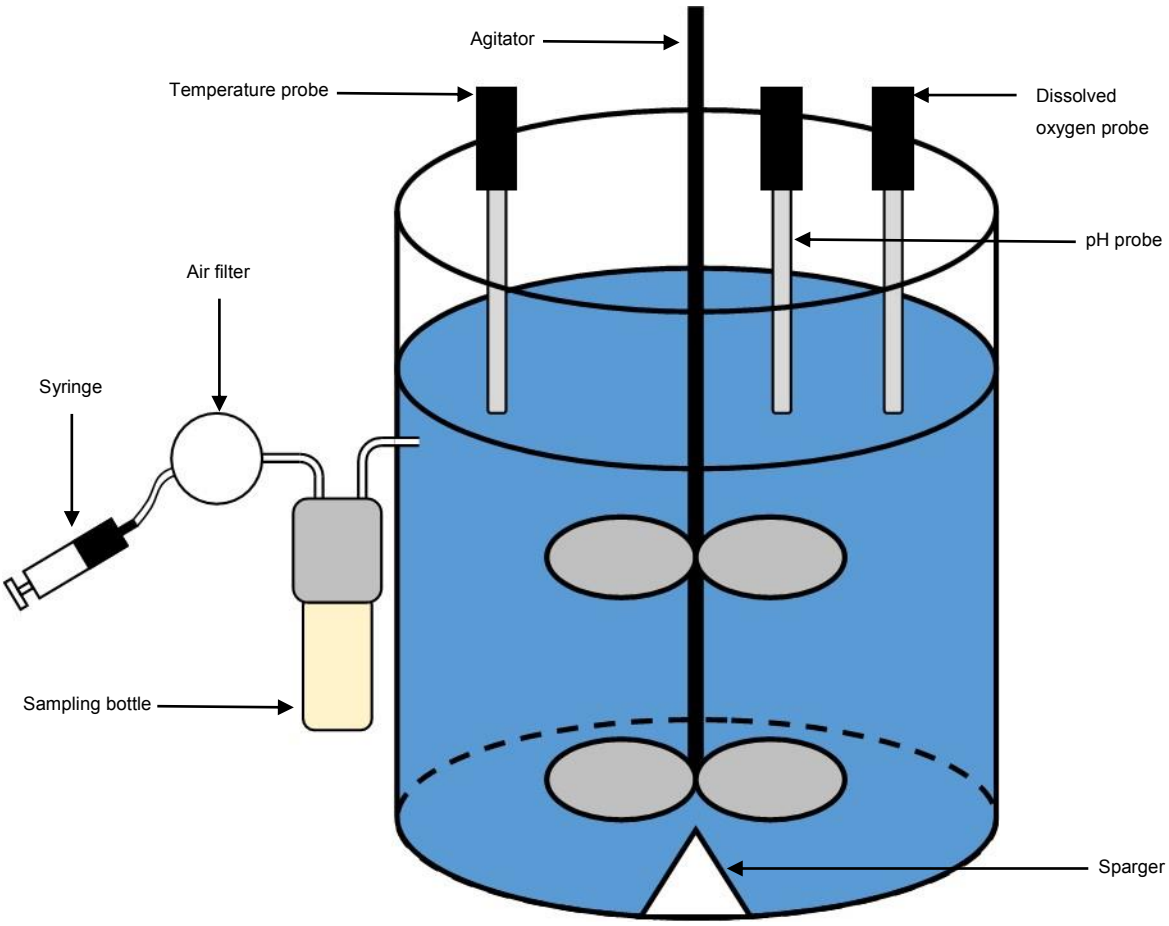

Figure 5: Graphical representation of batch bioreactor setup

3.3.3.3. Continuous bioreactor operation

Continuous bioreactor experiments (Figure 6) were initiated as batch experiments for 10 hours, which was when the bacteria would still be in the exponential phase. After 10 hours, the configuration was switched to continuous by turning on both the feed and waste pump in order to continuously add fresh nutrient medium and remove spent culture, at the same rate (0.1 l/h). This resulted in a constant volume system throughout the experiment. The experiment would continue until steady state was reached, at which point sampling would commence for 2 volume changes before the experiment would be halted.

Continuous culture experiments followed the same sampling procedure as batch experiments. Samples were also taken for growth, substrate and production analysis, but not over a 48-hour time span. The experiment time span depended on when steady state was reached.

Sampling for growth occurred at 1/3 volume change intervals, which depended on the dilution rate, and continued for at least two volume changes after steady state had been reached, as confirmed by both optical density and glucose concentrations by means of DNS (also took place at 1/3 volume change intervals). Once steady state was reached, samples were taken

for glucose, nitrate and lipopeptide analysis at 1/3 volume change intervals until two volume changes were completed.

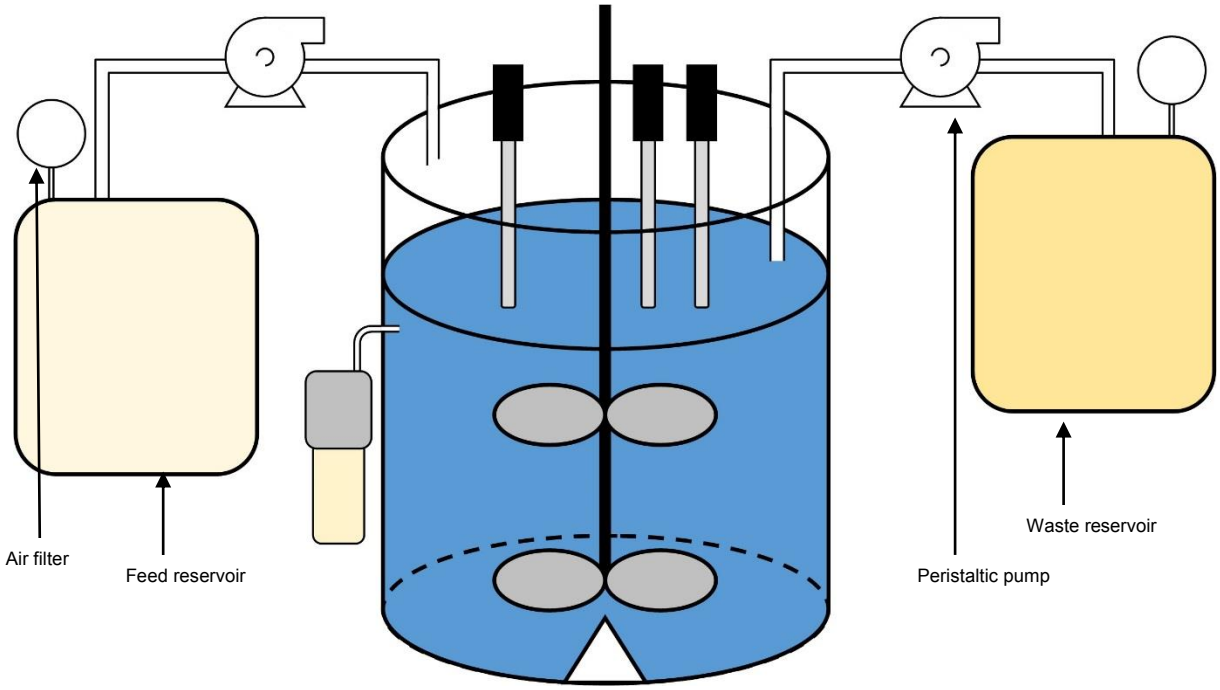

Figure 6: Graphical representation of continues bioreactor setup

3.4. Analytical techniques

3.4.1. Cell concentration

Cell concentration was used to quantify microbial growth patterns and kinetics. Two different methods are available to achieve this, namely direct and indirect methods. The direct method makes use of cell dry weight (CDW) while the indirect method uses optical density.

Optical density is the preferred method as it is fast, simple and requires small sample volumes as opposed to CDW which requires large volumes and is much more tedious. For this reason, a standard curve relating optical density to CDW was constructed so that CDW data could be obtained directly from optical density measurements without the need for ongoing CDW analysis.

3.4.1.1. Cell dry weight

To set up the CDW standard curve, CDW was determined using a Buchner vacuum filter (Millipore®) lined with a 0.2 µm filter paper disk (Anatech®). A 10 ml sample was filtered through the paper in order to separate cells from supernatant. The sample was taken from the second stage inoculum during the exponential phase to ensure active and viable cells. Prior to filtration, the paper disk was dried in an oven (Memmer®) at 60°C for 24 hours, cooled in a desiccator and weighed to four decimal places (Sauter AR 100 ®). After filtration, the

aforementioned steps were repeated and CDW was the difference between the weight measurements, divided by the volume filtered.

3.4.1.2. Optical density

Optical density was measured by the following procedure: a 1 ml culture sample was taken (from either shake flask or bioreactor) and placed in a micro-centrifuge tube (Eppendorf®) by means of a micro-pipet. The sample was then centrifuged (Eppendorf® Minispin Plus) for 5 minutes at 14500 rpm in order to obtain a cell pellet. The supernatant was removed and 1 ml distilled water was added to the pellet followed by vortexing in order to re-suspend the pellet. The optical density was measured with a spectrophotometer (Varian®) at 620 nm against a blank of distilled water. The samples were diluted as needed to ensure that the measurements remained below 0.8, which correlates with the linear region of the standard curve.

The standard curve was determined by measuring the optical density and CDW of a series of dilutions made up from the second stage inoculum. These optical density measurements plotted against the CDW data gave rise to the standard curve as shown in Figure 78 in Appendix 3.

The standard curve showed a good R^2 value (0.993), indicating a good linear fit for the data, with a regression equation model by Equation 1 below.

Equation 1: CDW standard curve regression equation

$$y = 1.9638x$$

3.4.2. Glucose concentration

Glucose concentrations were determined by both colorimetric and high performance liquid chromatography (HPLC) analysis.

3.4.2.1. Colorimetric analysis

The dinitrosalicylic acid (DNS) method is a fast and inexpensive method that is used to determine the concentration of reducing sugars like glucose. It is for these reasons that it was used to monitor the concentration of glucose during continuous culture experiments.

The method required two solutions, namely the DNS reagent and 40% potassium sodium tartrate solution. Both solutions were made up in water to the desired volume. The solution compositions are given in Table 7 below.

Table 7: DNS solution composition

Components	Concentration (g/l)
Dinitrosalicylic acid reagent solution	
3,5-Dinitrosalicylic acid	10
Phenol	2
Sodium metabisulphite	0.5
Sodium Hydroxide	10
40% Potassium Sodium Tartrate	
Potassium Sodium Tartrate	400

A culture sample of 1 ml was taken aseptically and diluted in water. The extent of the dilution depended on the glucose concentration and multiple attempts to get within the linear region of the standard curve were required. The diluted sample was then transferred aseptically to a micro-centrifuge tube (Eppendorf®) and centrifuged in a minispin plus (Eppendorf® Minispin Plus) for 5 minutes at 14500 rpm in order to separate the cells from the supernatant.

A sample of 1 ml was taken from the cell free supernatant and transferred to a test tube(s), followed by 1 ml of DNS reagent. The test tube(s) was placed in a bath of boiling water for six minutes, after which the water was replaced every two minutes with freshly boiled water to ensure that the test tube(s) remained in water at approximately 100°C. At the end of the six minutes, 0.33 ml of 40% potassium sodium tartrate was added to the test tube(s), which was then immediately quenched in ice water. A sample of the mixture was then taken and the optical density measured at 540 nm against a blank, which was prepared in the same manner with water instead of cell free supernatant. A standard curve was used to convert optical density values to glucose concentrations.

The standard curve was constructed by preparing a series of glucose solutions of known concentrations, which ranged from 0.05 to 0.9 g/l. The optical density of these dilutions was determined by the DNS method and plotted against the known glucose concentrations. The standard curve is given in Figure 79 in Appendix 3.

The DNS glucose standard curve showed a high R^2 value (0.972), indicating a good linear fit between data points, with a regression model defined by Equation 2 below.

Equation 2: DNS glucose standard curve regression equation

$$y = 0.0022x$$

3.4.2.2. High performance liquid chromatography

All experiments (including continuous experiments) made use of HPLC to determine glucose concentrations. The specifications for glucose analysis by HPLC are given in Table 8 below.

Table 8: HPLC specifications for determining glucose concentrations

HPLC specifications	
Column	Aminex HPX-87H column equipped with a Cation-H Micro-Guard Cartridge (Bio-Rad, Johannesburg, South Africa). Column temperature at 65°C
Mobile phase	5 mM sulphuric acid
Sample volume	30 µL
Flow rate	0.6 mL/min
Detector	RI detector (Shodex, RI-101)

A culture sample of 1 ml was transferred aseptically to a micro-centrifuge tube (Eppendorf®) and centrifuged in a minispin plus (Eppendorf® Minispin Plus) for 5 minutes at 14500 rpm in order to separate the cells from the supernatant. The supernatant was diluted 4-fold with water by combining 500 µl supernatant with 1500 µl water. The supernatant water mixture was then filtered through a 0.22 µm syringe filter (Anatech®), of which 1500 µl was sent for HPLC analysis of which 30 µl was injected into the column.

Prior to glucose analysis, a glucose standard was injected into the column and eluted with the mobile phase. Glucose concentrations were determined by comparing the absorption peaks of the supernatant with that of the standard.

A glucose standard curve was constructed in order to relate peak areas, from HPLC, to concentration values. This was achieved by preparing and analysing known concentrations of glucose and is shown in Figure 80 in Appendix 3.

The HPLC glucose standard curve showed R^2 value of 1, indicating exceptional linear fit between data points, with a regression model defined by Equation 3 below.

Equation 3: HPLC glucose standard curve regression equation

$$y = 5.1946x$$

3.4.3. Nitrogen concentration

Nitrogen concentration in terms of both nitrate and ammonium was measured. Nitrate was determined by ion chromatography while ammonium was determined through a colorimetric method.

3.4.3.1. Nitrate concentration

Nitrate concentrations were quantified by means of ion chromatography (Dionex®) with a conductivity detector. The specifications are given in Table 9 below.

Table 9: Ion chromatography specifications for determining nitrate concentrations

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

A sample of 1 ml was transferred aseptically to a micro-centrifuge tube (Eppendorf®) and centrifuged in a minispin plus (Eppendorf® Minispin Plus) for 5 minutes at 14500 rpm in order to separate the cells from the supernatant. A two-fold dilution was made by combining 500 µl supernatant with equal amounts of buffer (5 mM sodium carbonate), followed by a 50-fold dilution where 500 µl supernatant-buffer was mixed with 24500 µl of water. The mixture was then filtered through a 0.22 µm syringe filter (Anatech®). A 1500 µl sample was sent for ion chromatography (Dionex®) analysis of which 1000 µl was injected into the column.

Prior to nitrate analysis, a nitrate standard was injected into the column and eluted with the mobile phase. Nitrate concentrations were determined by comparing the absorption peaks of the supernatant with that of the standard.

Similar to glucose analysis, a nitrate standard was constructed and is shown in Figure 81 in Appendix 3. The standard curve showed a high R^2 value (0.988), indicating a good linear fit between data points, with a regression model defined by Equation 4 below.

Equation 4: Nitrate standard curve regression equation

$$y = 5 \times 10^{6x}$$

3.4.3.2. Ammonium concentration

The concentration of ammonium during varied nitrogen source experiments was quantified by a colorimetric analysis. This method makes use of two solutions, namely reagent A (5 g phenol and 25 mg nitroprusside added to 500 ml distilled water), and reagent B (2.5 g sodium hydroxide and 6 ml sodium hypochlorite (household bleach with 3.5% chlorine)) added to 500 ml distilled water.

A culture sample of 1 ml was taken aseptically and diluted in water, if required. The diluted sample was then transferred aseptically to a micro-centrifuge tube (Eppendorf®) and centrifuged in a minispin plus (Eppendorf® Minispin Plus) for 5 minutes at 14500 rpm in order to separate the cells from the supernatant.

A sample of 350 µl was taken from the cell free supernatant and transferred to a test tube(s), followed by 1.4 ml of both reagents A and B. The test tube(s) were left for 15 minutes at room temperature, followed by optical density measurements at 600 nm against a blank, which was prepared in the same manner but with water instead of cell free supernatant. A standard curve was used to convert optical density values to ammonium concentrations.

The standard curve was constructed by preparing a series of ammonium chloride solutions of known concentrations, which ranged from 0 to 2 mM. The optical density of these dilutions was determined and plotted against the known concentrations. The standard curve is given in Figure 82 in Appendix 3. The standard curve showed a R^2 value of 1, indicating good linear fit between data points, with a regression model defined by Equation 5.

Equation 5: Ammonium standard curve regression equation

$$y = 0.0525x$$

3.4.4. Lipopeptide concentration

3.4.1.1. Surfactin concentration

Surfactin concentrations were determined by means of HPLC. The HPLC specifications are given in Table 10 below.

Table 10: Lipopeptide HPLC specifications

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
Optical density	210 nm

A sample of 1 ml was transferred aseptically to a micro-centrifuge tube (Eppendorf®) and centrifuged in a minispin plus (Eppendorf® Minispin Plus) for 5 minutes at 14500 rpm in order to separate the cells from the supernatant. A 2-fold dilution was made by combining 1500 µl supernatant with 1500 µl acetonitrile mixture which consisted of 80% acetonitrile and 0.05% trifluoracetic acid. The mixture was then filtered through a 0.22 µm syringe filter and sent for analysis where 50 µl was injected into the column. The optical density of the eluent was measured at 210 nm.

Prior to surfactin analysis, a surfactin standard was injected into the column and eluted with the mobile phase. Surfactin concentrations were determined by comparing the absorption peaks of the supernatant with that of the standard.

The surfactin standard curve is given in Figure 83 in Appendix 3. The standard curve showed a R^2 value of 1, indicating good linear fit between data points, with a regression model defined by Equation 6 below.

Equation 6: Surfactin standard curve regression equation

$$y = 71.478x$$

3.4.4.2. Antifungal concentration

Antifungal concentrations followed the same sample preparation and HPLC method as surfactin. Antifungals were quantified based on the area of the peaks obtained from HPLC, as no standard was available for fengycin while the iturin standard available was not valid for the

entire homologues and was thus not used. The area of the iturin and fengycin peaks were grouped together as antifungal area. These areas can be separated into iturin and fengycin concentrations in terms of g/l once standards become available.

3.4.5. Analytical repeatability

Experiments were conducted in either duplicate or triplicate. Each experiment yielded two or three samples (each from a repeated experiment) at any given time. Each of these samples were analysed once and the average of the analysed values, along with standard deviation, were used as the results.

3.4.6. Antifungal efficacy

The antifungal efficacy was determined by means of radial diffusion plate assays. PDA was prepared, sterilised, poured into petri-dishes and allowed to set. The back end of a sterile pipette tip was used to cut four small and equally spaced circular wells into the agar, aseptically. The petri-dishes were inoculated with a target phytopathogen, three of the wells were filled aseptically with 100 µL antifungal lipopeptides and one with sterile water to act as control. The petri-dishes were then incubated at 25°C and monitored over the course of a few days and record was kept, after visual inspection, of any signs of growth inhibition.

One set of plates were used with the three supernatant containing wells acting as the repeatability factor of the tests. Efficacy tests were conducted with partially purified supernatant obtained from resolubilised acid precipitated crude supernatant (refer to Appendix 4).

Chapter 4: Results and Discussion

Antifungal lipopeptide production for use against fungal phytopathogens was investigated by using kinetic studies. The effect of nitrogen concentration, nitrogen source (from ammonium and nitrate) and dissolved oxygen levels on bacterial growth and product formation was investigated using shake flask and controlled bioreactor cultures. The use of continuous bioreactor cultures was assessed as the next step in kinetic optimisation. The antifungal lipopeptides were tested against six different fungal phytopathogens in efficacy tests to assess their effectiveness as an alternative for chemically derived fungicides.

4.1. Effect of nitrogen concentration on growth and production kinetics

Literature (Davis et al. 1999; Rangarajan et al. 2015) suggests that lipopeptide production is nitrate utilisation dependent. Production starts once nitrate is utilised, which occurs either when nitrate is the only available nitrogen source, or when the culture becomes oxygen limited, since nitrate is utilised as an alternative electron acceptor. Increasing the duration of nitrate utilisation, by adding more nitrate to the culture, could increase lipopeptide production.

The effect of two nitrogen concentrations on bacterial growth and lipopeptide production kinetics was investigated, using 4 g/l NH_4NO_3 and 8 g/l NH_4NO_3. The investigation was carried out in both shake flask and batch bioreactor cultures. The results shown below are based on the average between triplicates for shake flasks and duplicates for the bioreactor, unless otherwise stated. Error bars indicate the standard deviations between repeats.

4.1.1. Growth, substrate utilisation and product formation

The growth curve with 4 g/l NH_4NO_3 in shake flasks in Figure 7 started with a slight lag phase of 2 hours, followed by the exponential phase which ranged from 2 to 10 hours. A maximum CDW of 7.18 g/l was achieved at the start of the stationary phase (14 hours).

Glucose utilisation started immediately after inoculation until completely consumed at 23 hours, coinciding with the stationary phase which ranged from 14 to 34 hours. Rapid glucose utilisation occurred from 5 to 13 hours, which was during the exponential phase and was therefore to be expected. Nitrate utilisation ranged from 0 to 33 hours, after which approximately 1.3 g/l nitrate remained.

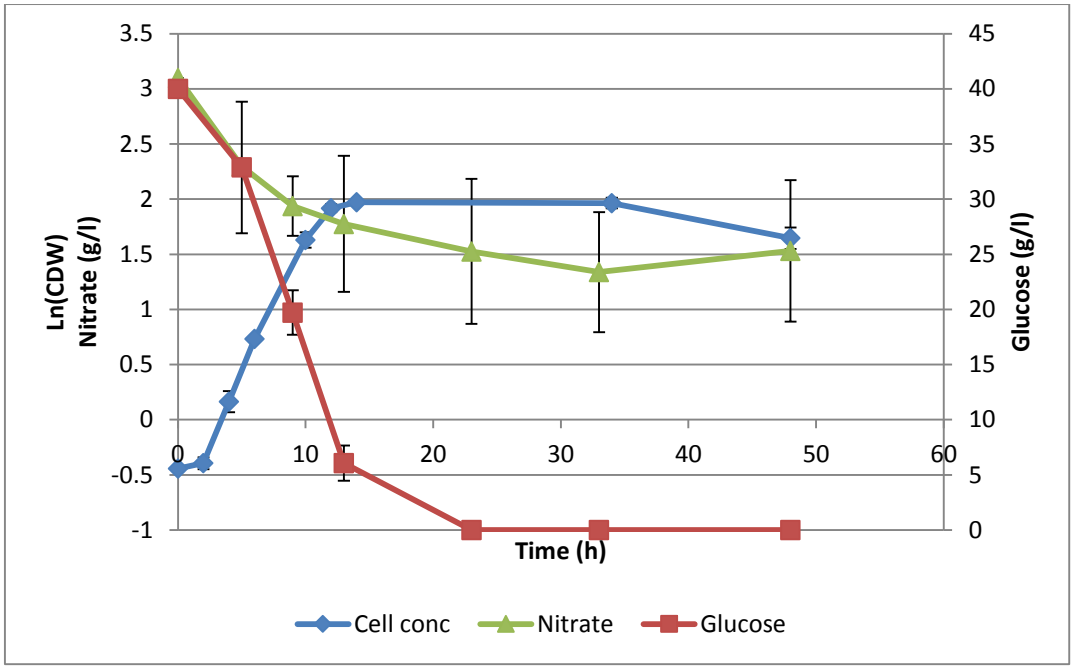

Figure 7: Growth and substrate utilisation of *B. amyloliquefaciens* in 4 g/l NH_4NO_3 shake flask cultures

Surfactin production (Figure 8) was most rapid during the exponential phase and achieved a maximum concentration of 539 mg/l at the end of the phase, after which it plateaued. Antifungal production was most rapid from mid to end exponential phase (5 to 13 hours), after which it reached a maximum of 404.9 mAU*min at 48 hours. Antifungal production plateaued after nitrate utilisation ceased, which indicated that production might be associated with the nitrate utilisation period as suggested in literature (Davis et al. 1999; Rangarajan et al. 2015). Surfactin production ceased slightly earlier while nitrate was still being utilised. It is possible that production during the stationary phase could be more selective towards antifungals than surfactin.

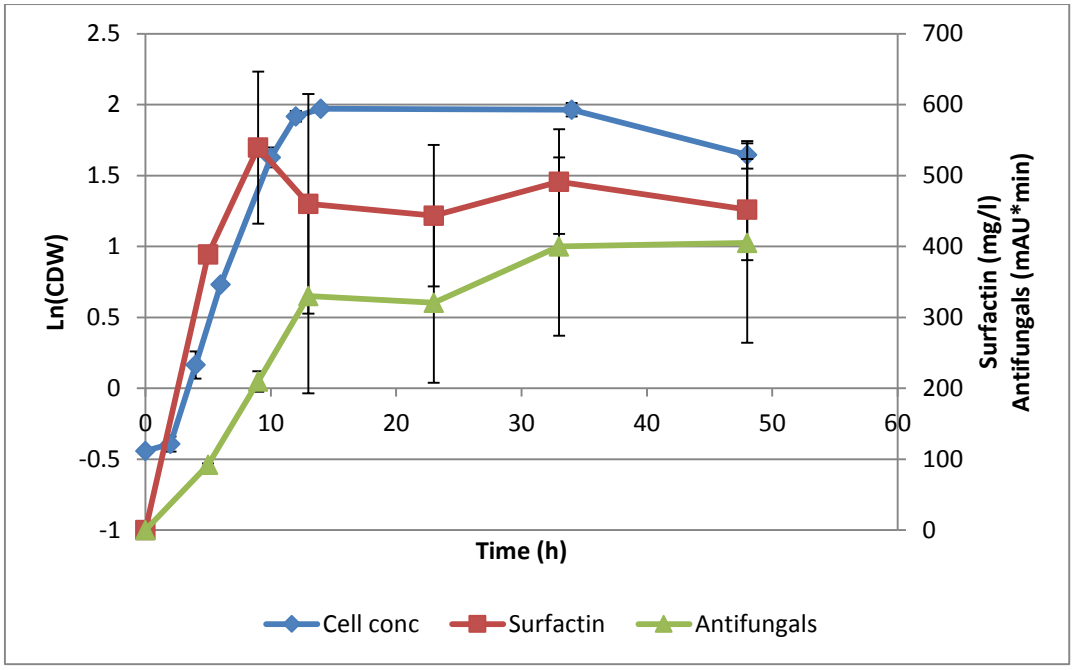

Figure 8: Growth and product formation of *B. amyloliquefaciens* in 4 g/l NH_4NO_3 shake flask cultures

The growth curve with 8 g/l NH_4NO_3 in shake flasks in Figure 9 started with an acceleration phase of 4 hours, followed by the exponential phase which ranged from 6 to 14 hours. A maximum CDW of 8.15 g/l was achieved at the start of the stationary phase (24 hours).

From Figure 9 it seems like there was a 2-hour delay in glucose utilisation, this is however not the case as the glucose concentration at time 0 was an estimate based on the 40 g/l that the culture medium was made up of. The starting glucose concentration was possibly slightly higher than 40 g/l due to the residual glucose left in the inoculum. Thus, glucose utilisation most likely started directly after inoculation and continued until depleted at 23 hours. The end of glucose utilisation occurred within the stationary phase, which ranged from 14 to 34 hours. Nitrate utilisation ranged from 0 to 33 hours, after which approximately 3.2 g/l nitrate remained.

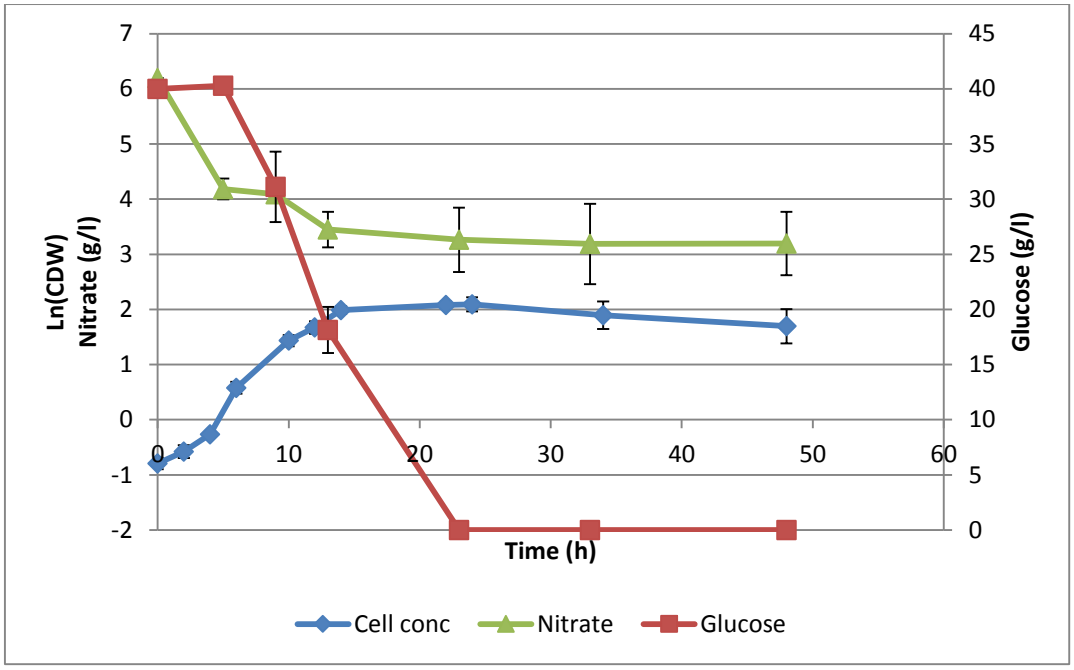

Figure 9: Growth and substrate utilisation of *B. amyloliquefaciens* in 8 g/l NH_4NO_3 shake flask cultures

Surfactin production (Figure 10) achieved a maximum of 518 mg/l at the end of the exponential phase, after which it plateaued. Antifungal production slowed down significantly from 33 hours onward, which was when nitrate utilisation ceased. This trend coincided with what was observed in Figure 8 and in literature (Pretorius *et al.*, 2015). A maximum antifungal concentration of 605 mAU*min at 48 hours was achieved, which was approximately 200 mAU*min above that of the 4 g/l NH_4NO_3 experiment.

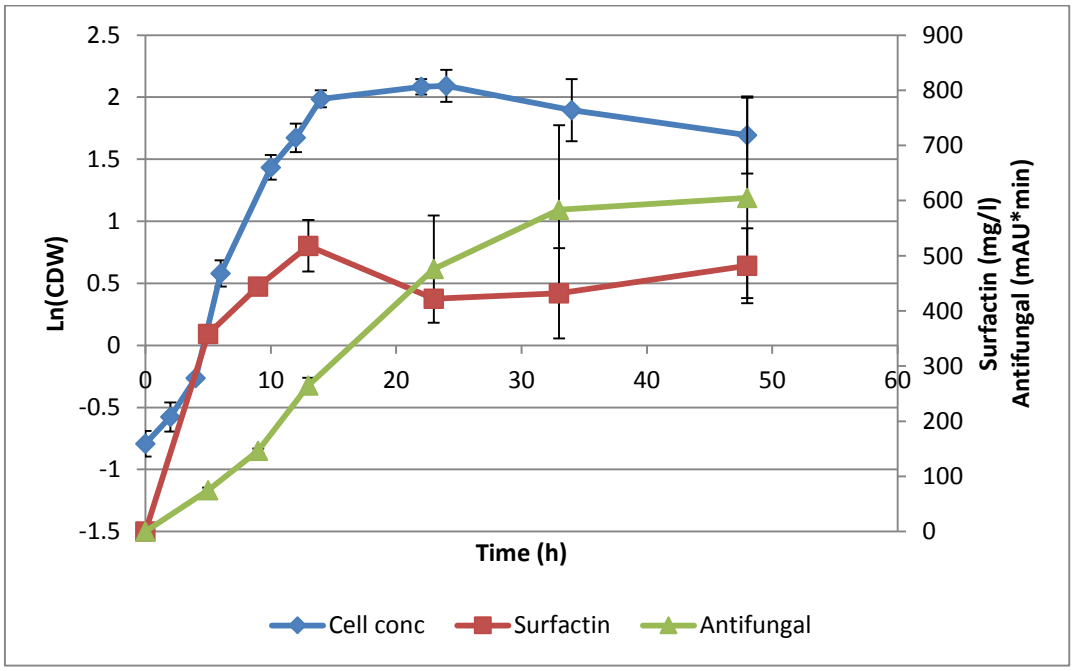

Figure 10: Growth and product formation of *B. amyloliquefaciens* in 8 g/l NH_4NO_3 shake flask cultures

The growth curve in Figure 11, with 4 g/l NH_4NO_3 in controlled bioreactor culture, showed a slight lag phase from 0 to 2 hours, followed by the exponential phase that ranged from 2 to 12 hours. The stationary phase ranged from 14 hours onward. A maximum CDW of 7.55 g/l was reached during the stationary phase, at 22 hours.

The dissolved oxygen levels decreased rapidly from 100% to 17% in the first 4 hours, after which it continued to decrease at a slower rate until it became oxygen-limited at 22 hours. Glucose utilisation continued until 23 hours, at which point glucose was depleted. Nitrate utilisation increased rapidly at 5 hours as the dissolved oxygen level was almost 0%, and continued until 23 hours, where glucose was depleted, after which it plateaued with approximately 0.4 g/l remaining.

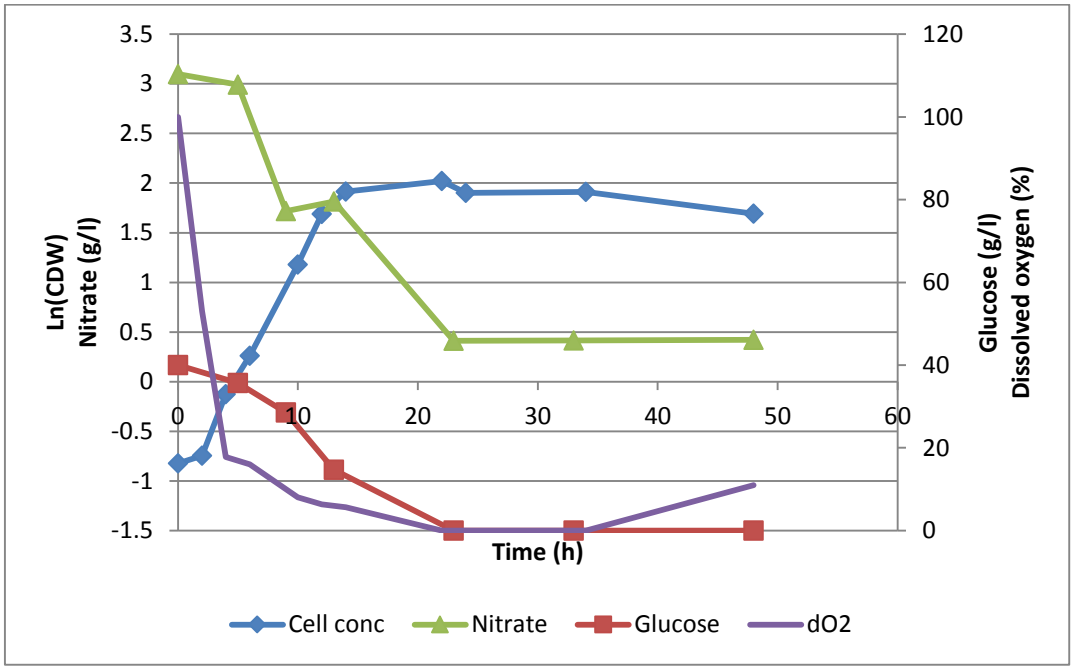

Figure 11: Growth and substrate utilisation of *B. amyloliquefaciens* in 4 g/l NH_4NO_3 controlled bioreactor cultures

Surfactin and antifungal production in controlled bioreactor culture (Figure 12) achieved a maximum of 626 mg/l and 247 mAU*min at 13 hours, after which both decreased significantly. Lipopeptide production was expected to decrease slightly, or to plateau after nitrate utilisation ceased at 22 hours, however the decrease was extreme and did not follow the same trend observed in shake flask cultures (Figure 8 and Figure 10). The reason behind this rapid decrease is unclear as nothing out of the ordinary was observed during the experiment. It is possible that antifoam accumulation during the bioreactor culture interfered with lipopeptide production or stability in some way. The 4g/l NH_4NO_3 bioreactor experiment was not done in duplicate due to time constraints.

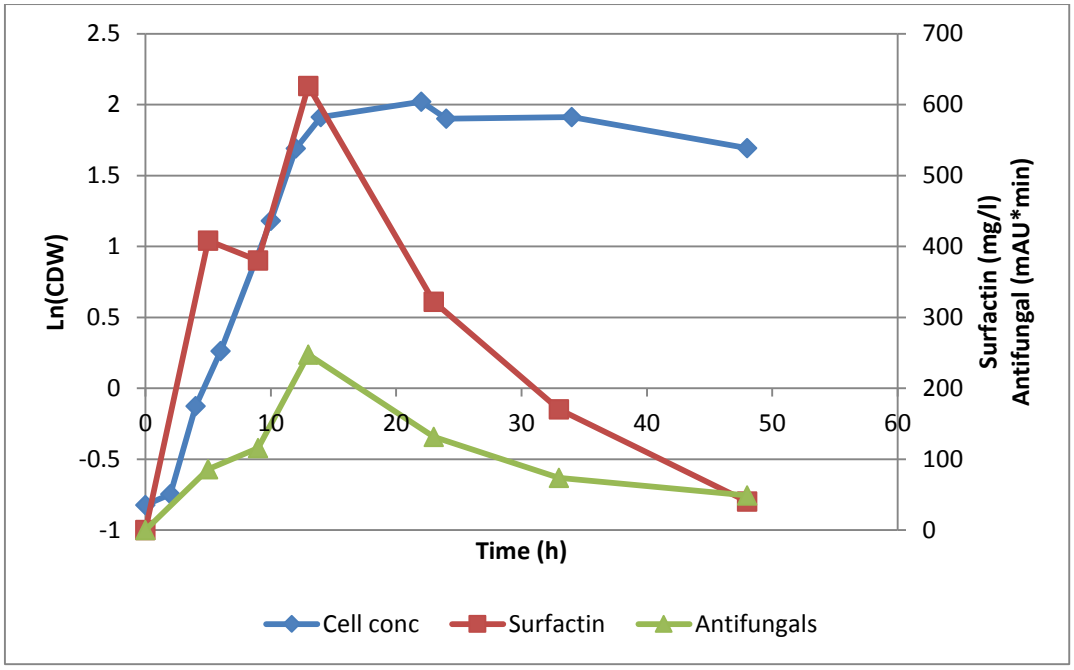

Figure 12: Growth and product formation of *B. amyloliquefaciens* in 4 g/l NH_4NO_3 controlled bioreactor cultures

The growth curve of Figure 13, with 8 g/l NH_4NO_3 in controlled bioreactor culture, showed an exponential phase of 14 hours with the absence of a notable lag phase. A maximum CDW of 5.90 g/l was achieved at 22 hours, which was during the stationary phase which ranged from 22 to 48 hours.

The dissolved oxygen decreased rapidly from 100% to 6.4% in 4 hours, decreasing slightly until reaching 0% at 8 hours. Glucose utilisation occurred rapidly until 23 hours, and was completely utilised by 33 hours. Rapid nitrate utilisation occurred when the dissolved oxygen levels were almost 0% at 5 hours and continued until 23 hours, after which it plateaued and then decreased slightly once more.

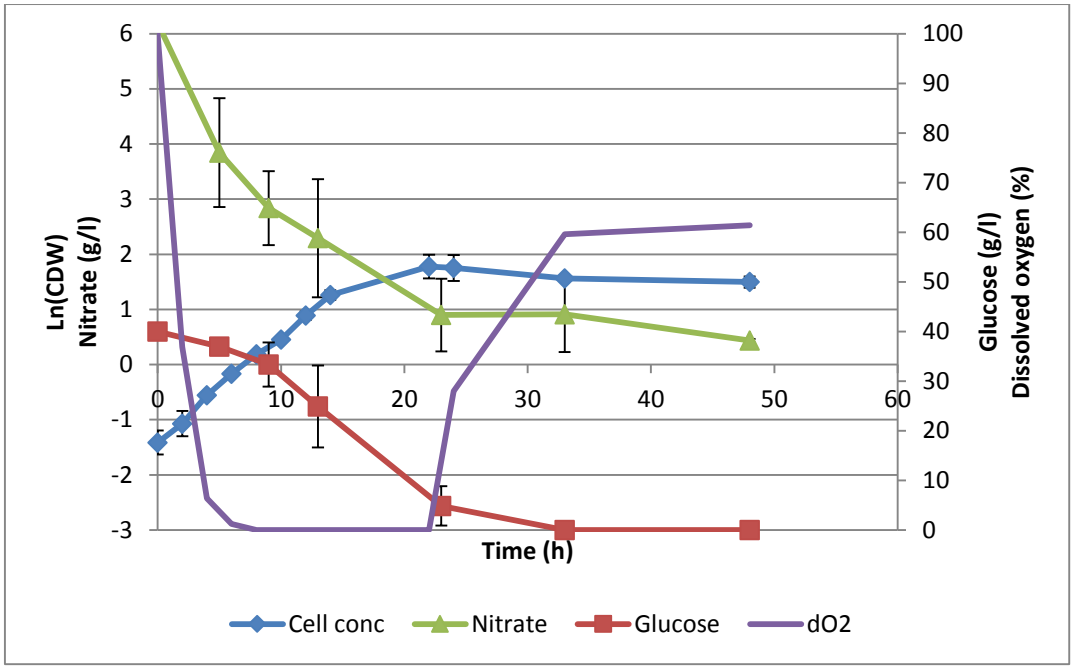

Figure 13: Growth and substrate utilisation of *B. amyloliquefaciens* in 8 g/l NH_4NO_3 controlled bioreactor cultures

Surfactin and antifungal production (Figure 14) achieved a maximum of 450 mg/l and 340 mAU*min after 14 hours. Surfactin production plateaued after its maximum, which is possibly due to the decrease in nitrate utilisation, which ceased after 23 hours. Antifungals decreased after its maximum at 13 hours, which is due to the decrease in nitrate utilisation even though the point of maximum antifungals are expected to be achieved somewhere in the stationary phase, as observed in shake flask cultures (Figure 8 and Figure 10).

Error bars for cell growth and glucose utilisation are extremely small while the other trends exhibited more significant error bars. This was possibly due to the fact that the bioreactor experiments could not be done in duplicate at the same time and the different inocula used for each could have contributed to the difference in lipopeptide values.

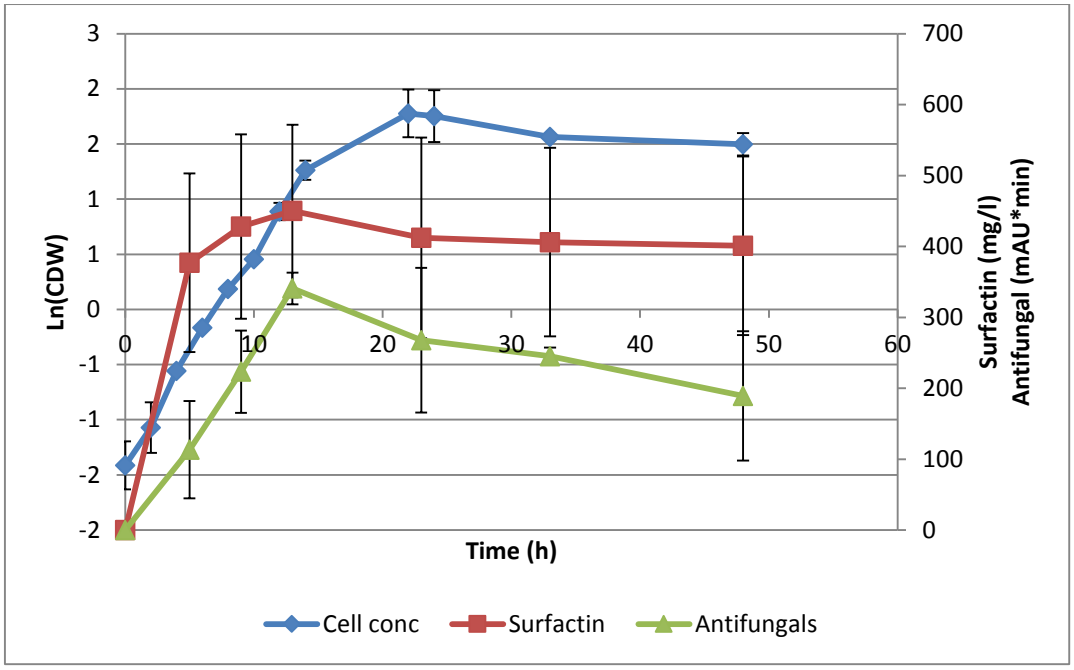

Figure 14: Growth and product formation of *B. amyloliquefaciens* in 8 g/l NH_4NO_3 controlled bioreactor cultures

4.1.2. Comparison of growth and substrate utilisation trends

The growth curves of both the 4 g/l and 8 g/l NH_4NO_3 shake flask cultures and the 4 g/l NH_4NO_3 bioreactor culture (Figure 15) were all very close to one another, while the 8 g/l NH_4NO_3 bioreactor culture exhibited the same trend.

The shake flask cultures achieved higher maximum CDW values than bioreactor cultures. In the case of shake flasks, the 8 g/l NH_4NO_3 culture achieved the highest maximum CDW of 8.15 g/l, compared to 7.18 g/l from the 4 g/l NH_4NO_3 culture. The opposite was the case with the bioreactor with CDW of 7.55 g/l in the 4 g/l NH_4NO_3 culture and 5.90 g/l in the 8 g/l NH_4NO_3 culture.

All four experiments, except the 8 g/l NH_4NO_3 bioreactor culture, improved on the CDW concentrations of Pretorius *et al*. (2015), who obtained 4.6 g/l and 6.7 g/l in similar 4 g/l and 8 g/l NH_4NO_3 bioreactor cultures. Furthermore, the error bars that are present are quite small, showing that the difference is likely to be statistical significant as no error bars overlap with one another.

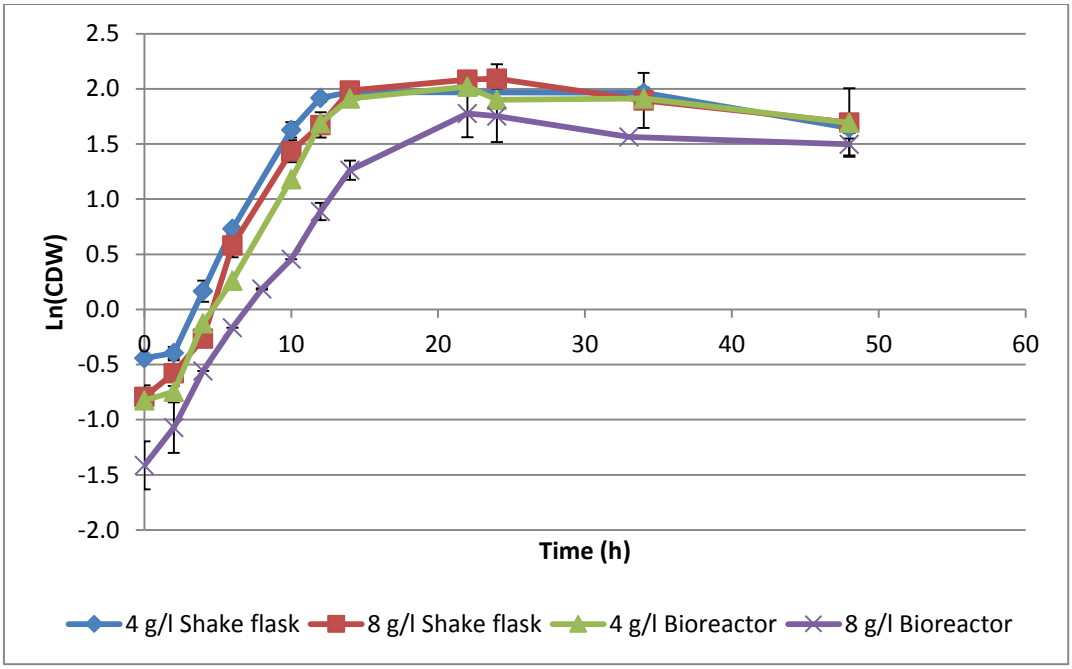

Figure 15: Comparison of *B. amyloliquefaciens* growth in 4 g/l and 8 g/l NH_4NO_3 shake flask and batch bioreactor cultures

The increased NH_4NO_3 concentration marginally decreased the maximum specific growth rate (Figure 16), indicating slightly slower growth at 8 g/l compared to 4 g/l NH_4NO_3. In the case of shake flask cultures, μ_{max} decreased form 0.252 h^{-1} to 0.175 h^{-1}, and from 0.237 h^{-1} to 0.19 h^{-1} for the bioreactor cultures. All four experiments showed high R^2 values, indicating good linear fit between the data points identified as the exponential phase.

The μ_{max} values were significantly lower than that of Pretorius *et al*. (2015), who achieved 0.43 h^{-1} and 0.54 h^{-1} for the 4 g/l and 8 g/l bioreactor cultures. The reason for the lower growth rates is not known.

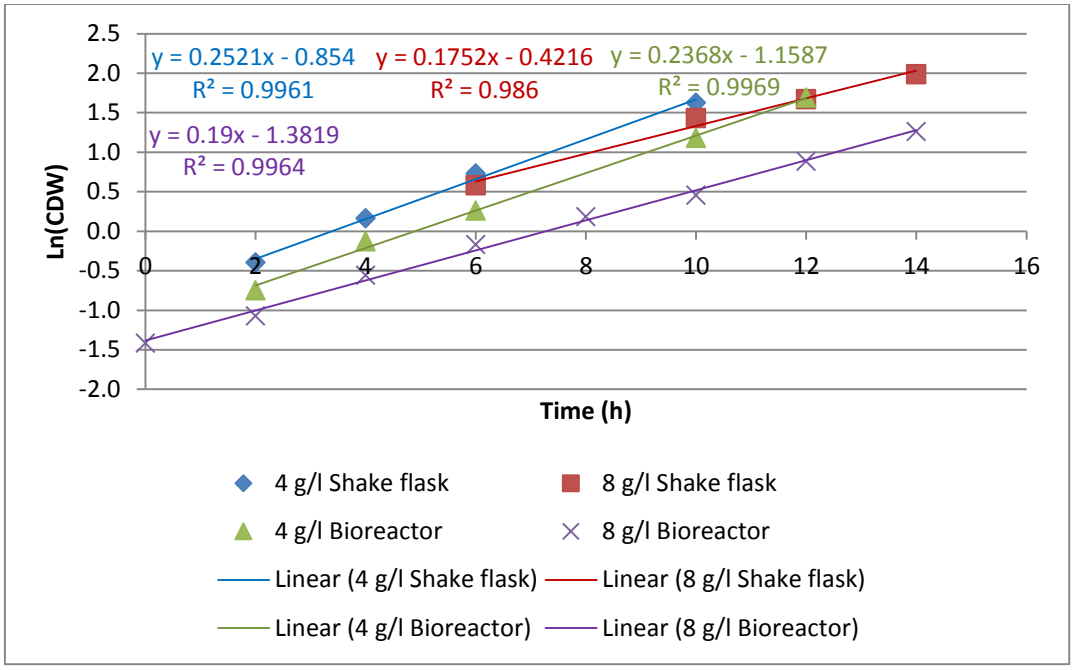

Figure 16: Comparison of *B. amyloliquefaciens* growth rates in 4 g/l and 8 g/l NH_4NO_3 shake flask and batch bioreactor cultures

Glucose utilisation (Figure 17) in the 4 g/l and 8 g/l NH_4NO_3 shake flask and bioreactor cultures continued until 23 hours, at which point glucose was depleted. Utilisation was extended during the 8 g/l NH_4NO_3 bioreactor experiment and glucose was only depleted at 33 hours as opposed to 23 hours.

The comparison between glucose utilisation is likely to be statistically significant due to the small error bars observed between compared cultures. The 8 g/l bioreactor culture did however obtain higher error bars, and it should be kept in mind when comparing the cultures.

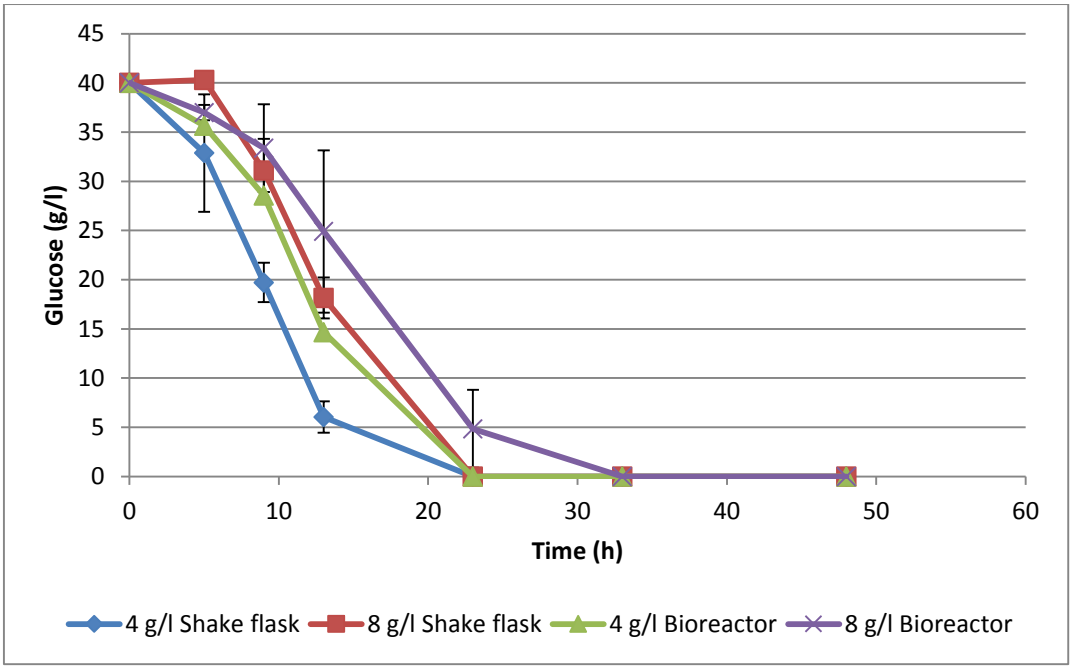

Figure 17: Comparison of *B. amyloliquefaciens* glucose utilisation in 4 g/l and 8 g/l NH_4NO_3 shake flask and batch bioreactor cultures

Nitrate (Figure 18) was utilised more efficiently during bioreactor cultures than during shake flask cultures, as approximately 13% and 14.5% nitrate remained in bioreactor cultures as opposed to 45% and 51% in shake flask cultures.

In both bioreactor cases nitrate utilisation increased rapidly when the dissolved oxygen levels were close to, or at 0%, which was expected as nitrate would be used as an alternative electron acceptor in the absence of oxygen. Nitrate utilisation continued until glucose became limiting, at 23 hours for the 4 g/l NH_4NO_3 culture and at 33 hours for the 8 g/l NH_4NO_3 culture.

The comparison between nitrate utilisation shows overlapping error bars with the 4 g/l shake flask and the 8 g/l bioreactor culture, showing that the difference is likely not significant.

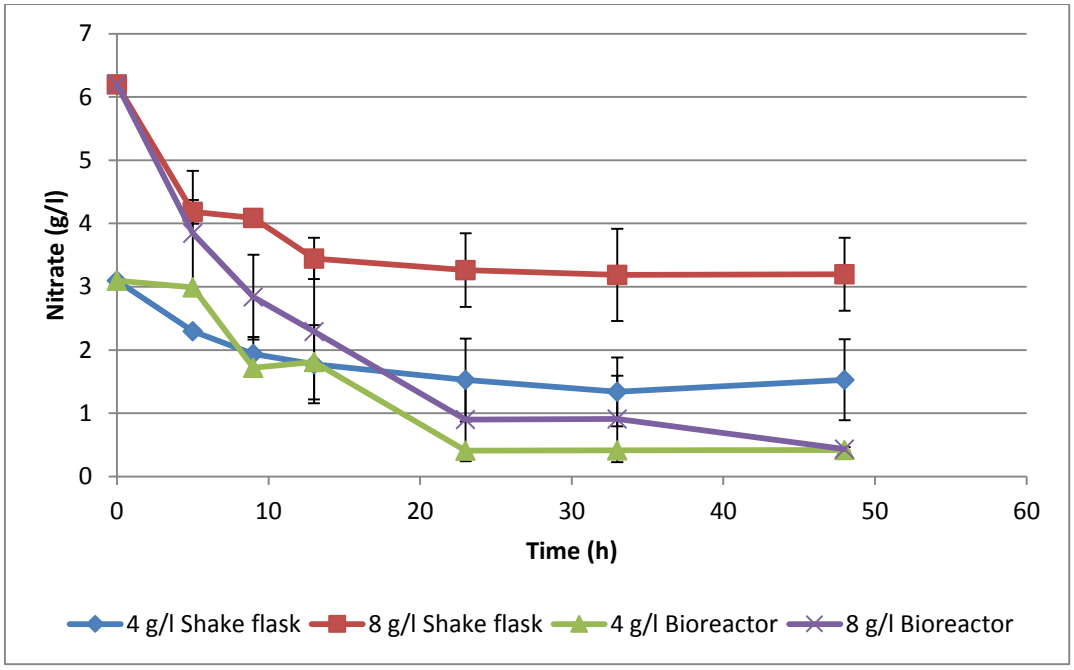

Figure 18: Comparison of *B. amyloliquefaciens* nitrate utilisation in 4 g/l and 8 g/l NH_4NO_3 shake flask and batch bioreactor cultures

4.1.3. Comparison of lipopeptide production trends

Surfactin production profiles at the different NH_4NO_3 concentrations are compared in Figure 19 below.

The increased NH_4NO_3 concentration from 4 g/l to 8 g/l NH_4NO_3 did not result in a significant difference in surfactin production. Figure 19 showed all experiments, except the 4 g/l NH_4NO_3 bioreactor culture, followed the same trend with values very close to one another. The shake flask and bioreactor cultures showed maximum production during the 4 g/l NH_4NO_3 cultures. The decrease in lipopeptide concentration for the NH_4NO_3 bioreactor cultures after 13 hours (Figure 12) was possibly due to the accumulation of antifoam which was not added to the shake flask cultures, and/or the loss of lipopeptides in the foam. However, further investigation would be required to confirm this.

The production profiles of Figure 19 did not represent the same trends obtained by Pretorius *et al*. (2015), however, production was significantly higher for both NH_4NO_3 concentrations. Pretorius *et al.* (2015) obtained a maximum surfactin concentration of 68 mg/l for the 4 g/l NH_4NO_3 bioreactor culture and 302 mg/l for the 8 g/l NH_4NO_3 bioreactor culture. The maximum surfactin concentrations achieved were 626 mg/l for the 4g/l NH_4NO_3 bioreactor culture, an increase of almost 90%, and 450 mg/l for the 8 g/l NH_4NO_3 bioreactor culture, a 30% increase. The shake flask experiments achieved a maximum of 539.3 mg/l and 518 mg/l at the 4 g/l and 8 g/l NH4NO3 cultures, which was an 87% and 42% increase respectively.

The significant increase in surfactin production could be attributed to the optimised inoculum, which ensured that a highly active inoculum was used (refer to Appendix 1).

Overlapping error bars are observed for all surfactin trends, except for the 4 g/l bioreactor culture which was not done in duplicate. The comparison between surfactin production is likely not statistically significant.

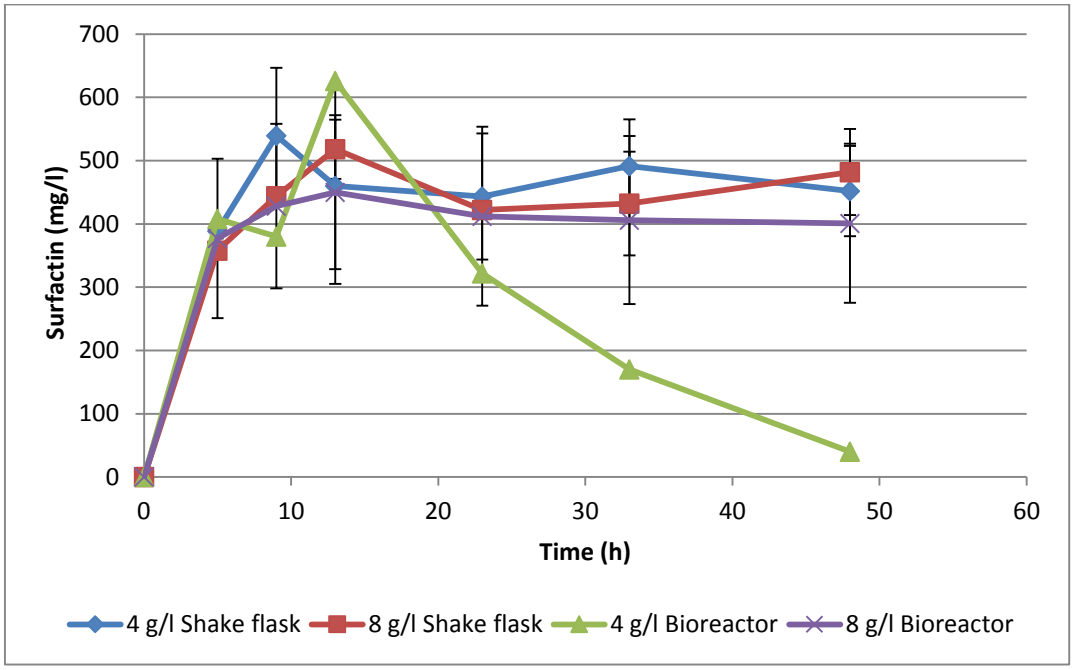

Figure 19: Comparison of surfactin production by *B. amyloliquefaciens* in 4 g/l and 8 g/l NH_4NO_3 shake flask and batch bioreactor cultures

Antifungal lipopeptide production profiles at different NH_4NO_3 concentrations are given in Figure 20 below. The increased NH_4NO_3 concentration favoured antifungal production as both shake flask and bioreactor cultures each achieved greater production with 8 g/l NH_4NO_3 than with 4 g/l NH_4NO_3. The maximum production in shake flasks increased from 404.9 mAU*min to 604.7 mAU*min, a 33% increase. Similarly, bioreactor production increased from 247.2 mAU*min to 340.7 mAU*min, a 27% increase.

The production profiles in Figure 20 showed the same trends as obtained by Pretorius *et al*. (2015), but gave higher concentrations for all NH_4NO_3 concentrations and production strategies. Compared to Pretorius *et al*. (2015), the maximum antifungal concentration increased from 114.6 mAU*min to 247 mAU*min for the 4 g/l NH_4NO_3 bioreactor culture, and from 285.7 mAU*min to 340.7 mAU*min for the 8 g/l NH_4NO_3 bioreactor culture, which relates to an increase of 53% and 16% respectively.

The error bars for antifungal production overlap between compared cultures. The difference may not be statistically significant.

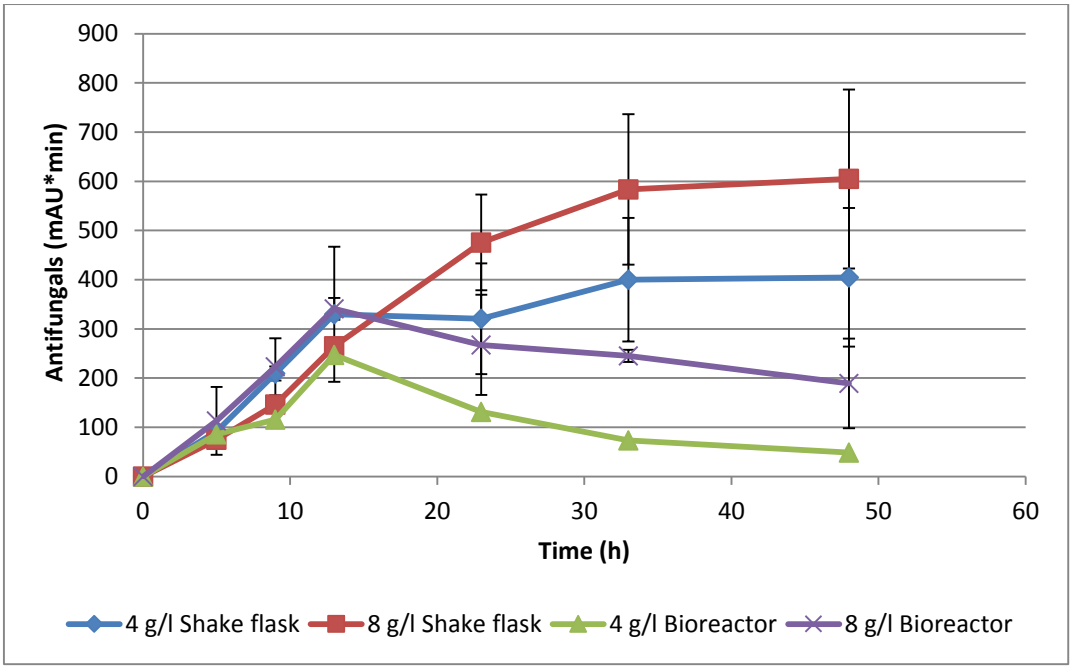

Figure 20: Comparison of antifungal production by *B. amyloliquefaciens* in 4 g/l and 8 g/l NH_4NO_3 shake flask and batch bioreactor cultures

4.1.4. Comparison of normalised cell and lipopeptide concentrations and associated kinetic parameters

A range of normalised kinetic parameters, with regards to bacterial growth, antifungal and surfactin production, was used to assess the process performance of increased NH_4NO_3 concentrations. All kinetic parameters were calculated at the time where antifungal production reached its maximum for each experiment. This was due to the fact that antifungal production was the focus of the study and would be harvested for downstream processing at the maximum point. The normalised kinetic parameters are summarised in Figure 21 below.

The increased NH_4NO_3 concentration did not lead to an increased maximum specific growth rate, while CDW increased with shake flasks but decreased with the bioreactor.

Increased NH_4NO_3 concentrations increased antifungal production for both shake flask and bioreactor cultures, which reflected the trend observed in Figure 20 as both production strategies considered the point of maximum antifungal production. Surfactin production increased slightly for shake flask cultures with increased NH_4NO_3 concentration, however, for the bioreactor experiments the 4 g/l NH_4NO_3 culture achieved a much greater concentration than the 8 g/l NH_4NO_3 culture. This agrees for the most part with the trend observed in Figure 19. All experiments had a high antifungal selectivity with both 8 g/l NH_4NO_3 shake flask and bioreactor cultures having the highest, which was desirable as the focus of production was aimed at antifungals.

Substrate utilisation, specifically glucose, with regards to growth, antifungal and surfactin production, was evaluated by means of the yield on substrate parameters, $Y_{x/s}$, $Y_{p/s}$ (p=antifungals) and $Y_{p/s}$ (s=surfactin). These parameters showed the degree to which cells and lipopeptides were formed per unit substrate consumed. The 8 g/l NH_4NO_3 bioreactor culture showed higher lipopeptide yields ($Y_{p/s}$) than cell yields($Y_{x/s}$), while the opposite was the case for all other experiments. The highest cell yield ($Y_{x/s}$) was obtained by the 4 g/l NH_4NO_3 bioreactor culture, while the highest antifungal and surfactin yields ($Y_{p/s}$) came from the 8 g/l NH_4NO_3 bioreactor culture.

Specific antifungal and surfactin production was evaluated by means of $Y_{p/x}$ (p=antifungals) and $Y_{p/x}$ (s=surfactin). These parameters showed the extent of antifungals and surfactin production per cell. The 8 g/l NH_4NO_3 bioreactor culture achieved the highest yield for both antifungals and surfactin.

Productivity relates lipopeptide formation per unit of time. Antifungal productivity was significantly higher at 26.21 mAU*min/h during the 8 g/l NH_4NO_3 bioreactor culture, while the 4 g/l NH_4NO_3 bioreactor culture was the highest for surfactin productivity at 34.62 mAU*min/h.

Based on the kinetic parameters summarised in Figure 21, the increased NH_4NO_3 concentration was advantageous for bioreactor-based antifungal production. Not only did the maximum antifungal concentration (340.69 mAU*min) increase with 8 g/l NH_4NO_3, but also antifungal productivity (26.207 mAU*min/h), specific antifungal production (124.35 mAU*min/g/l) and antifungal selectivity (92%). This coincides with the findings of Pretorius *et al*. (2015).

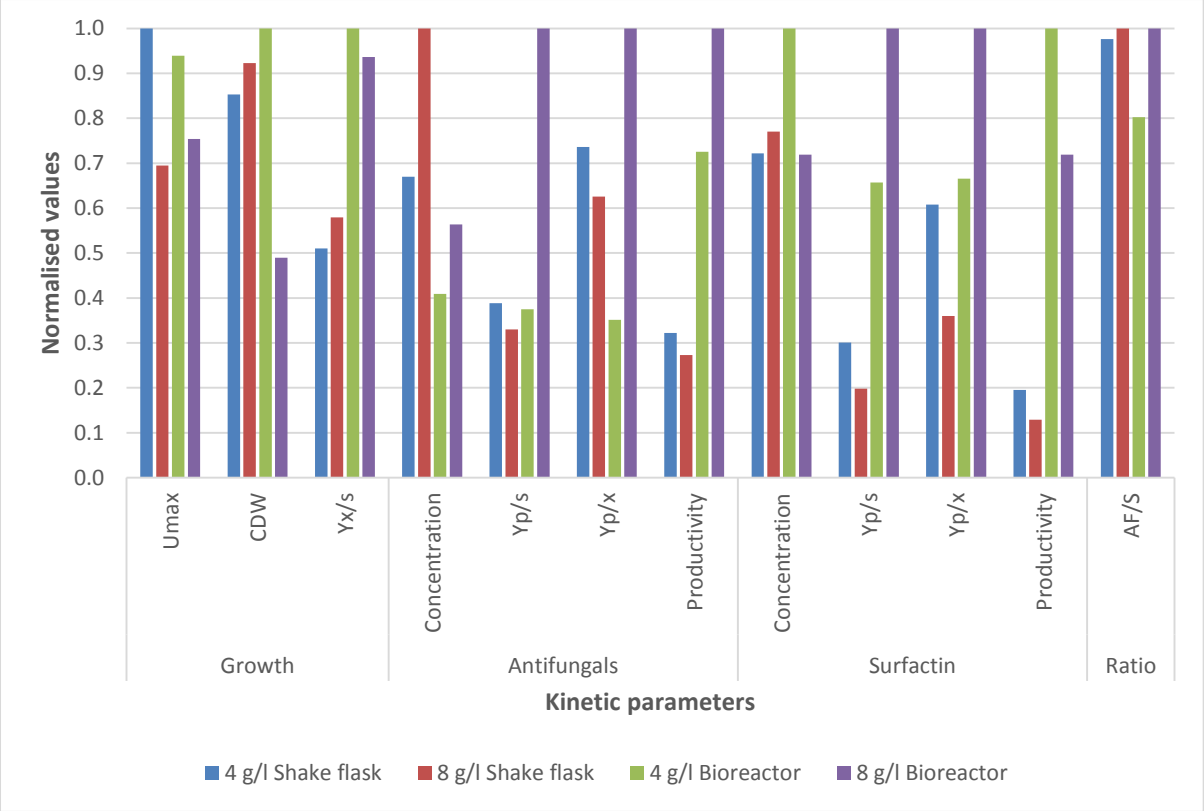

Figure 21: Summary of *B. amyloliquefaciens* growth and lipopeptide normalised kinetic parameters at maximum antifungal concentration in 4 g/l and 8 g/l NH_4NO_3 shake flask and batch bioreactor cultures

4.2. Effect of nitrogen sources on growth and production kinetics

Different nitrogen sources can be used for microbial growth and production, of which ammonium and nitrate are most commonly used. Ammonium is primarily used for cell growth while nitrate is involved in lipopeptide production and utilised as an alternative electron acceptor during anaerobic respiration. Nitrogen sources in the form of ammonium and nitrate were considered and varied through different ratios of nitrogen, supplied by ammonium, and nitrogen, supplied by nitrate.

Ammonium is used by the bacteria for cell and/or lipopeptide synthesis while nitrate is used for lipopeptide production, or as an alternative electron acceptor in oxygen limiting conditions (refer to literature in section 1.4.3.). The effect of various nitrogen sources (ammonium to nitrate) on the process kinetics is unclear.

The effect of varying the nitrogen source was investigated and assessed through quantification of bacterial growth and lipopeptide production kinetics using different ratios of nitrate and ammonium salts (refer to Appendix 2). The ratios of nitrogen from the ammonium to nitrate sources that were considered were: 0:1, 0.25:0.75, 0.5:0.5, 0.75:0.25 and 1:0. These ratios were calculated to match the total amount of nitrogen present in 4 g/l NH_4NO_3. The optimum,

8 g/l NH_4NO_3, identified in the previous section, was not used since it was desired to have a concentration that would result in nutrient limiting conditions.

The investigation was carried out in shake flasks, which were made up in triplicate. The results shown below (Figure 22 to Figure 41) are based on the average between these triplicates. Error bars indicate the standard deviations between repeat experiments.

4.2.1. Growth, substrate utilisation and product formation

The growth curve of the culture with nitrate as the sole nitrogen source, in Figure 22, showed no lag or acceleration phase. The exponential phase started directly after inoculation and ranged from 0 to 12 hours. A maximum CDW of 8.17 g/l was achieved at 48 hours.

Glucose utilisation started immediately after inoculation, coinciding with the exponential phase, and lasted until almost completely consumed at 48 hours with 0.8 g/l remaining. Glucose would become depleted shortly after 48 hours, which would result in the onset of the stationary phase, which is not observed in the 48-hour time span.

Glucose concentration at 0 hours was shown to be less than at 5 hours since glucose at 0 hours was assumed to be that of the growth medium composition, which did not consider any glucose still present in the inoculum. Due to the absence of a glucose HPLC sample at 0 hours, the constant rate of glucose utilisation seems to range from 5 to 48 hours, however, it would have started at 0 hours.

Nitrate utilisation occurred from 0 to 48 hours, at which point approximately 1.9 g/l remained. Nitrate utilisation was most rapid during the first 5 hours, which was expected as no ammonium was present. Utilisation at 48 hours ceased since glucose was essentially depleted.

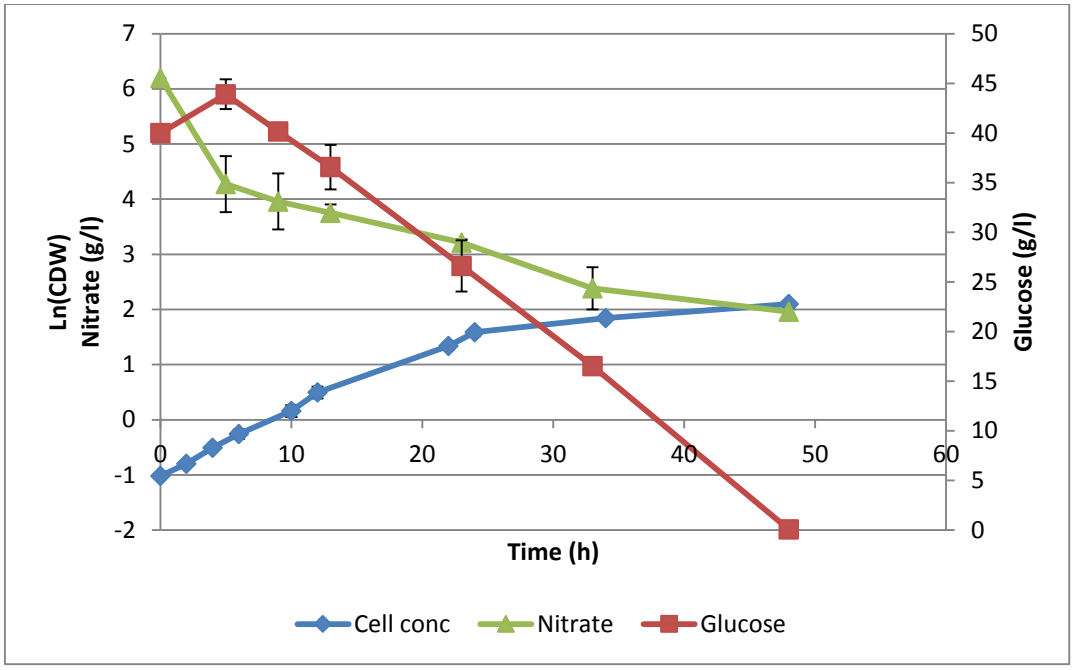

Figure 22: Growth and substrate utilisation of *B. amyloliquefaciens* during the culture with nitrate as the sole nitrogen source

Surfactin production (Figure 23) was rapid during the first 5 hours, which related to the initial section of the exponential phase and rapid nitrate utilisation, after which the production rate decreased. Surfactin production reached its maximum of 706.7 mg/l at 48 hours.

Antifungal production was most rapid from 13 to 33 hours, which coincided with the end exponential and deceleration phase. Antifungals reached its maximum of 977.3 mAU*min at 48 hours. Surfactin and antifungal production were expected to plateau after 48 hours since glucose would be depleted and nitrate utilisation would cease.

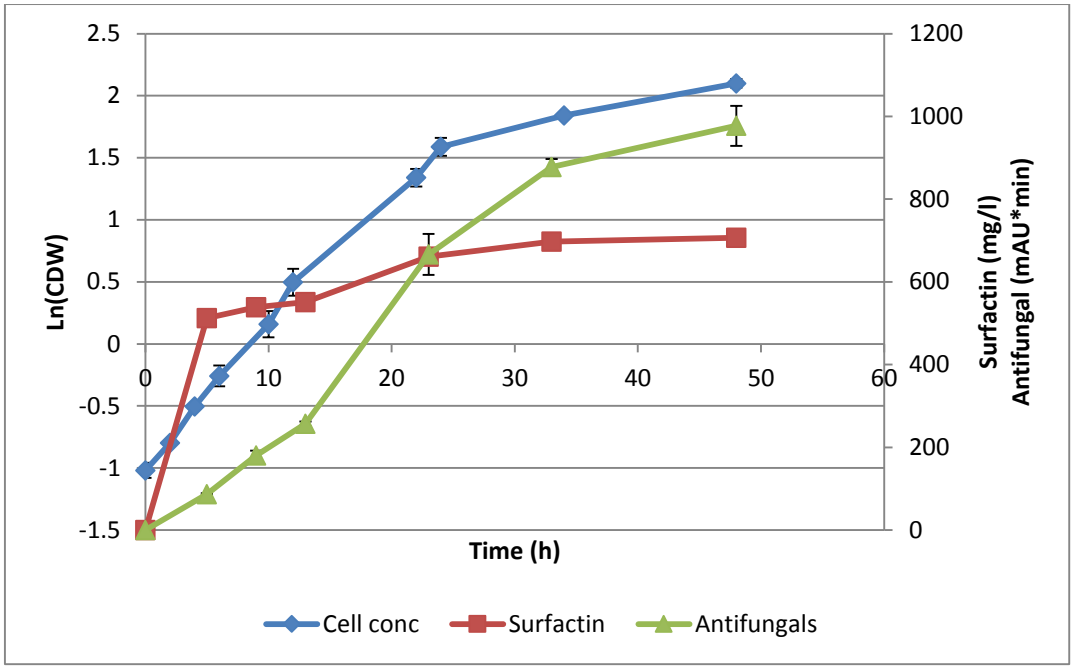

Figure 23: Growth and product formation of *B. amyloliquefaciens* during the culture with nitrate as the sole nitrogen source

The growth curve of the culture with a 0.25:0.75 nitrogen ratio from ammonium and nitrate sources respectively, in Figure 24, started immediately after inoculation, with an exponential phase from 0 to 12 hours. The stationary phase started at 34 hours. A maximum CDW of 6.54 g/l was reached at 48 hours.

Glucose utilisation started directly after inoculation and continued until depletion at 48 hours. As with Figure 22, the concentration of glucose at 0 hours was shown to be less than at 5 hours due to the assumption, which did not take the residual glucose form the inoculum into account, that the concentration is that of the growth medium composition. The absence of a glucose HPLC sample at 0 hours, caused it to seem that utilisation started at 5 hours whereas it would have started at 0 hours.

Nitrate utilisation started rapidly from 0 to 5 hours, after which it seemed to remain relatively constant until 13 hours. Nitrate utilisation continued once again from 13 to 48 hours, at which point utilisation ceased as glucose became limiting. Ammonium started with a concentration of 450 mg/l (not shown), which was utilised at a rapid rate during the first 10 hours, then at a slower rate until essentially depleted at 22 hours.

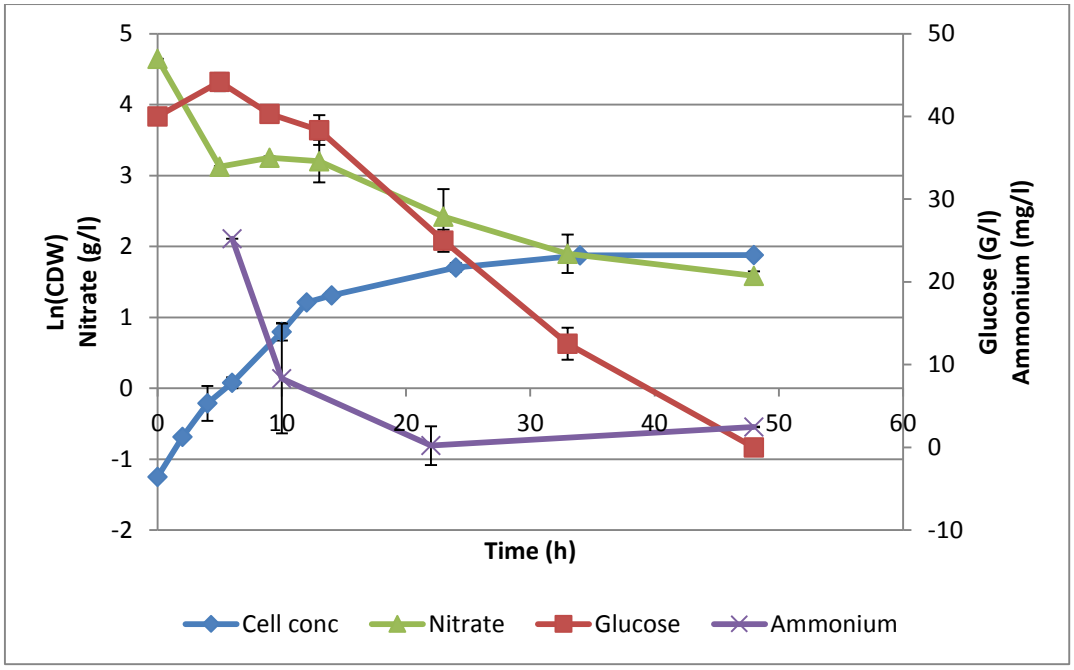

Figure 24: Growth and substrate utilisation of *B. amyloliquefaciens* during the culture with a 0.25:0.75 nitrogen ratio from ammonium and nitrate sources respectively

Rapid surfactin production (Figure 25) occurred during the first 13 hours, which coincided with the exponential phase and rapid ammonium utilisation phase. After 13 hours, the rate of surfactin production plateaued until 48 hours, where production was expected to stop as glucose was exhausted. The maximum surfactin concentration was reached at 33 hours with a value of 781 mg/l.

Rapid antifungal production (Figure 25) occurred from 5 to 33 hours, which coincided in part with the nitrate utilisation phase and mid exponential phase up until the stationary phase. During the stationary phase, the rate of antifungal production decreased and was expected to stop after 48 hours as nitrate utilisation ceased due to glucose being limiting. The maximum antifungal concentration achieved was 724 mAU*min at 33 hours.

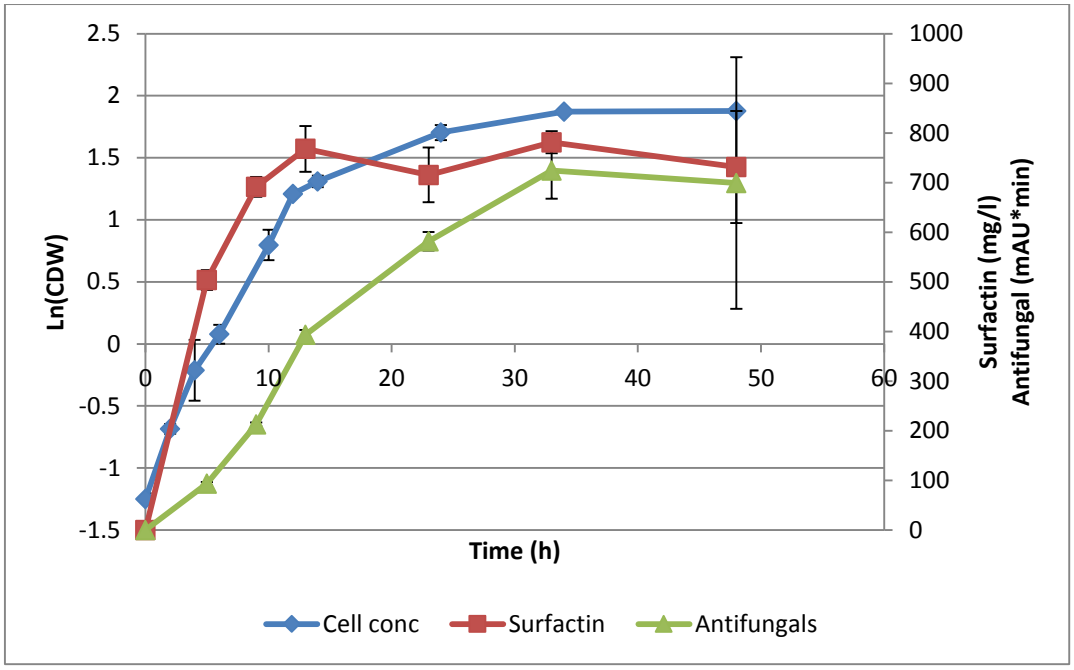

Figure 25: Growth and product formation of *B. amyloliquefaciens* during the culture with a 0.25:0.75 nitrogen ratio from ammonium and nitrate sources respectively

The growth curve of the culture with a 0.5:0.5 nitrogen ratio from ammonium and nitrate sources respectively, in Figure 26, had no lag phase and an exponential phase from 0 to 14 hours. A maximum CDW of 7.38 g/l was reached at 14 hours.

Glucose was utilised from the start until depletion at 23 hours, which coincided with the stationary phase indicating that glucose became limiting. Nitrate utilisation occurred during the same period as glucose utilisation, however at 23 hours there was approximately 1.2 g/l nitrate left. Nitrate utilisation ceased after 23 hours due to limiting glucose. Ammonium started with a concentration of 901 mg/l (not shown), which was rapidly utilised to 18.69 mg/l in the first six hours, after which it was utilised at a constant rate until 34 hours. Further ammonium utilisation was minimal until depletion at 48 hours.

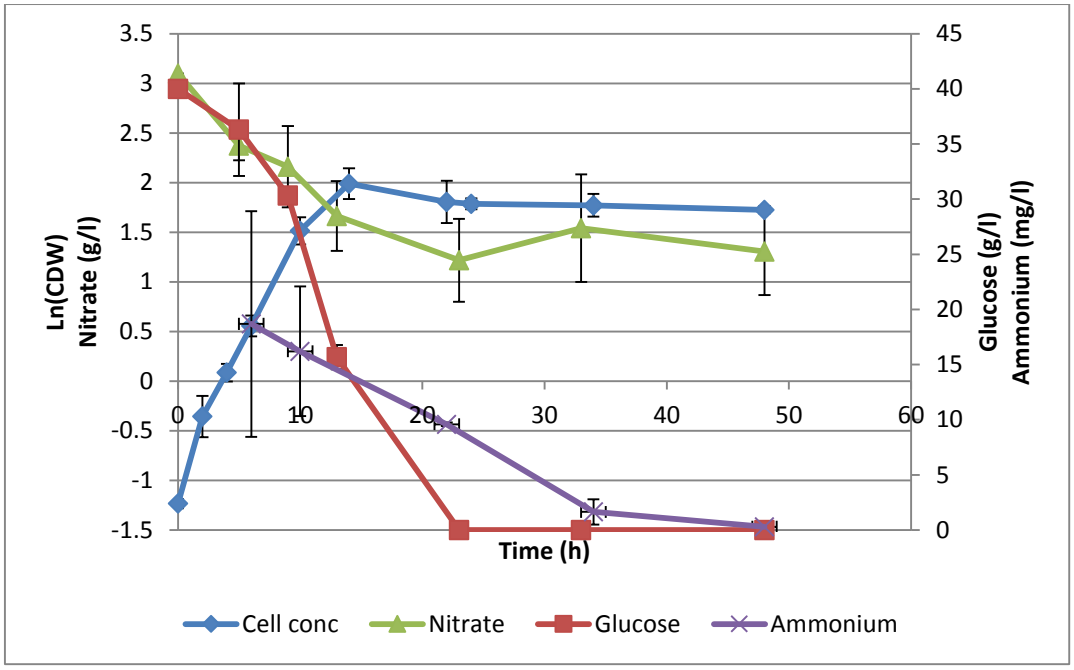

Figure 26: Growth and substrate utilisation of *B. amyloliquefaciens* during the culture with a 0.5:0.5 nitrogen ratio from ammonium and nitrate sources respectively

Rapid surfactin production (Figure 27) occurred from 0 to 13 hours, which coincided with the exponential growth and ammonium and nitrate utilisation phases. Maximum surfactin of 809 mg/l was reached at the end of the exponential phase (13 hours). Antifungal production was most rapid from 9 to 33 hours, which coincided with mid exponential to stationary phase, nitrate and, in this case, ammonium utilisation. A maximum antifungal concentration of 888 mAU*min was reached at 33 hours.

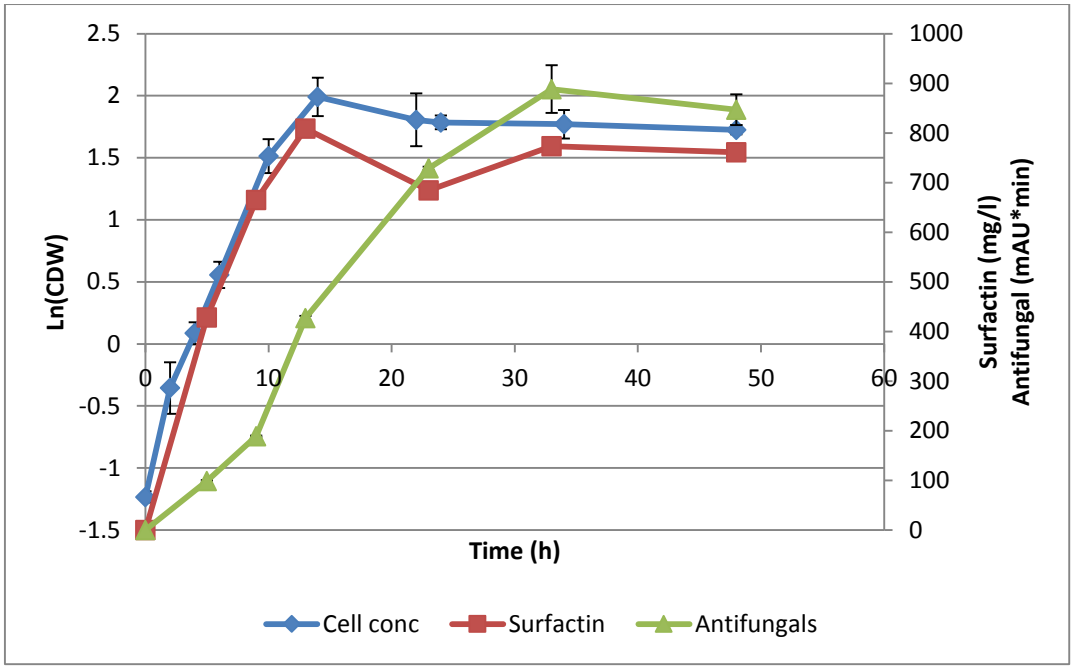

Figure 27: Growth and product formation of *B. amyloliquefaciens* during the culture with a 0.5:0.5 nitrogen ratio from ammonium and nitrate sources respectively

The growth curve of the culture with a 0.75:0.25 nitrogen ratio from ammonium and nitrate sources respectively, in Figure 28, had an exponential phase from 0 to 12 hours. Stationary phase was observed from about 14 hours.

Glucose utilisation ranged from the start of the experiment until depletion at 23 hours, which was within the stationary phase. As with Figure 22, glucose concentration was shown to be lower at 0 hours than at 5 hours as it was assumed the inoculum contained no residual glucose, which was not the case.

Nitrate concentration remained constant for the first 5 hours, where after it was utilised at a low rate until 13 hours. Rapid utilisation occurred from 13 to 23 hours, where utilisation ceased with approximately 0.7 g/l remaining due to glucose being limiting in the stationary phase.

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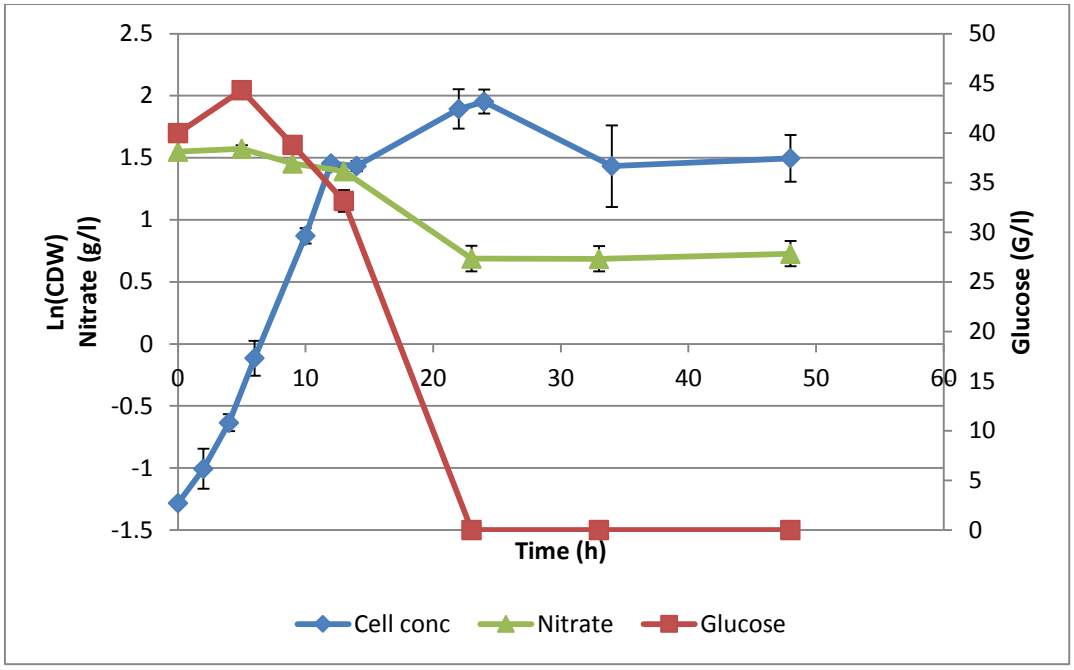

Figure 28: Growth and substrate utilisation of *B. amyloliquefaciens* during the culture with a 0.75:0.25 nitrogen ratio from ammonium and nitrate sources respectively

The ammonium utilisation profile is given in Figure 29 below as its axis range does not fall within the range in Figure 28 and would result in compression of other trends.

Ammonium started with a concentration of 1350 mg/l (not shown) which was utilised rapidly in the first 10 hours, and coincided with the exponential phase. Utilisation decreased after the exponential phase until it ceased completely at 22 hours due to glucose limitation.

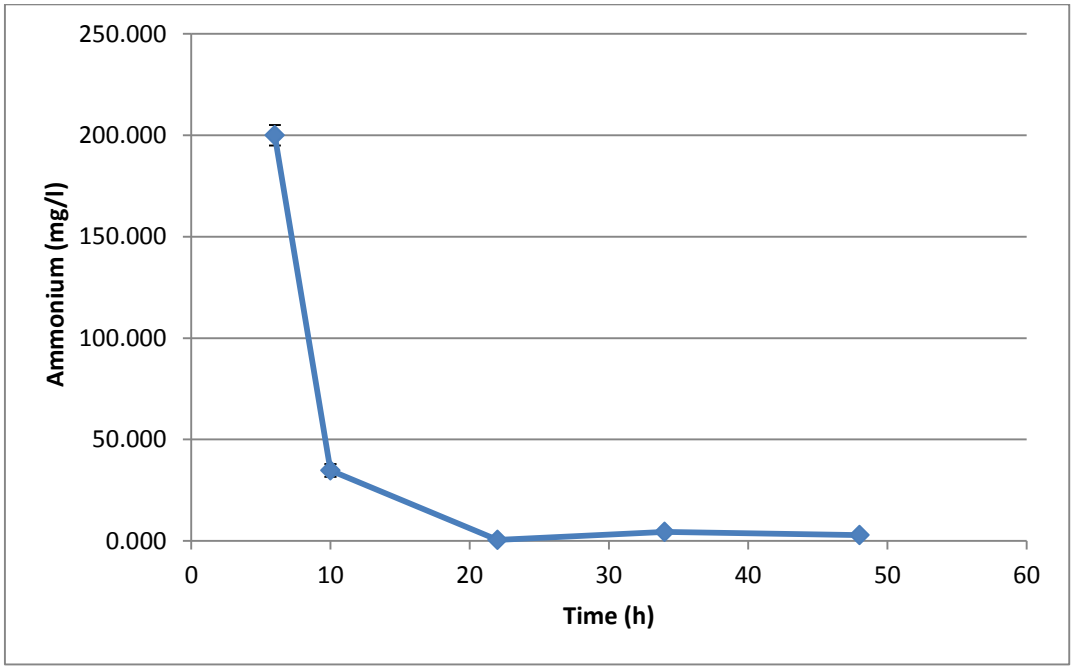

Figure 29: Ammonium utilisation of *B. amyloliquefaciens* during the culture with a 0.75:0.25 nitrogen ratio from ammonium and nitrate sources respectively

Surfactin production (Figure 30) was most rapid during the early exponential phase, which was also when ammonium was utilised most rapidly and nitrate was not being utilised at all. After 5 hours, the rate of surfactin production decreased significantly. Surfactin concentration was almost constant for the rest of the experiment with a maximum concentration of 598 mg/l being reached at 48 hours.

Antifungal production was most rapid from mid exponential phase, 5 to 23 hours, which coincided with part of the rapid nitrate utilisation and rapid ammonium utilisation phase. Antifungal concentration achieved a maximum of 674 mAU*min at 48 hours.

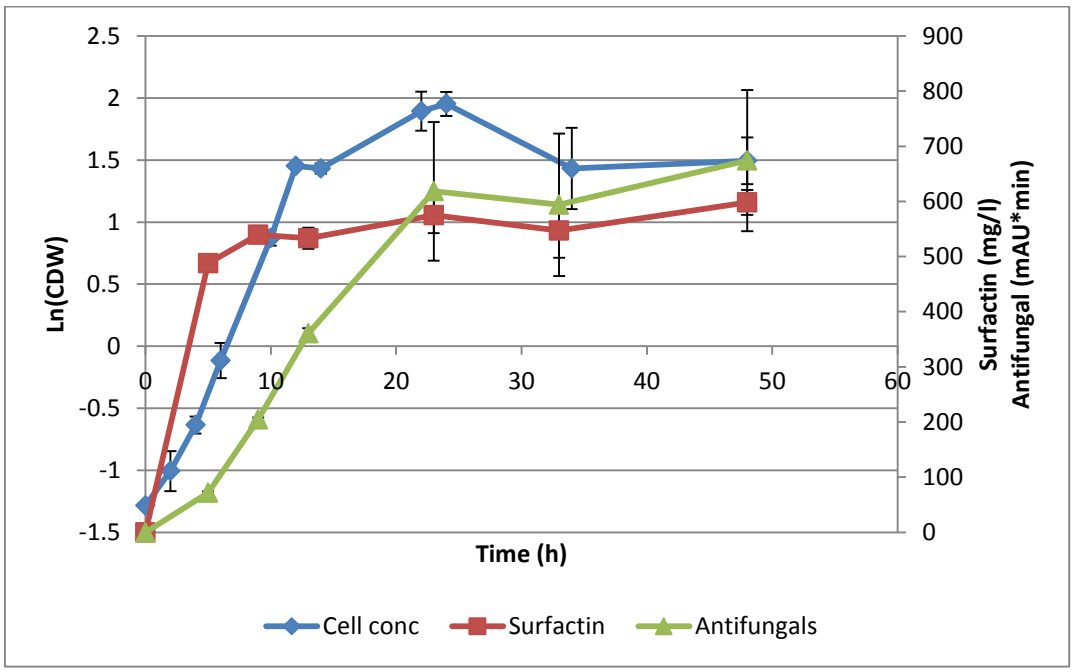

Figure 30: Growth and product formation of *B. amyloliquefaciens* during the culture with a 0.75:0.25 nitrogen ratio from ammonium and nitrate sources respectively

The growth curve of the culture with ammonium as the sole nitrogen sources, in Figure 31, had a lag phase of 2 hours, followed by an exponential phase that ranged from 2 to 12 hours. A maximum CDW of 5.76 g/l was reached at 24 hours.

Glucose was utilised slowly for the first 13 hours during which approximately 4 g/l was used, followed by rapid utilisation from 13 to 23 hours. Utilisation plateaued at 23 hours, which coincided with the stationary phase. Having approximately 23 g/l glucose remaining at the stationary phase suggests that a different nutrient than glucose was limiting.

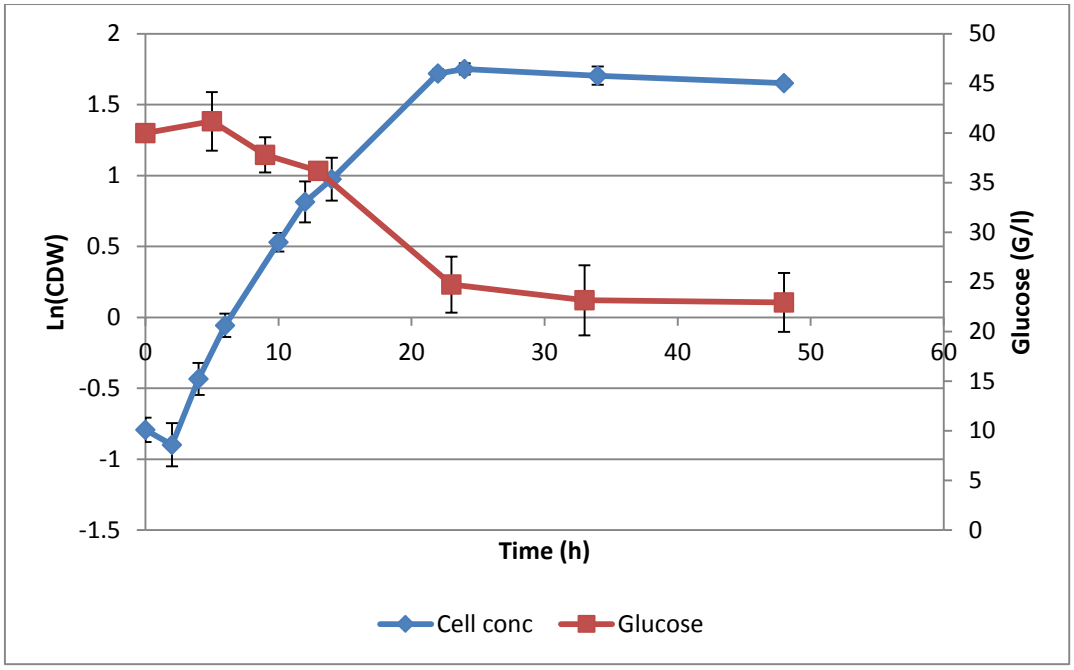

Figure 31: Growth and substrate utilisation of *B. amyloliquefaciens* during the culture with ammonium as the sole nitrogen sources

The ammonium utilisation profile is given in Figure 32 below. Ammonium started with a concentration of 1803 mg/l (not shown), which was utilised rapidly during the first 10 hours, coinciding with the exponential phase. Utilisation was slower from 10 to 23 hours, at which point ammonium was essentially depleted and thus the likely limiting nutrient.

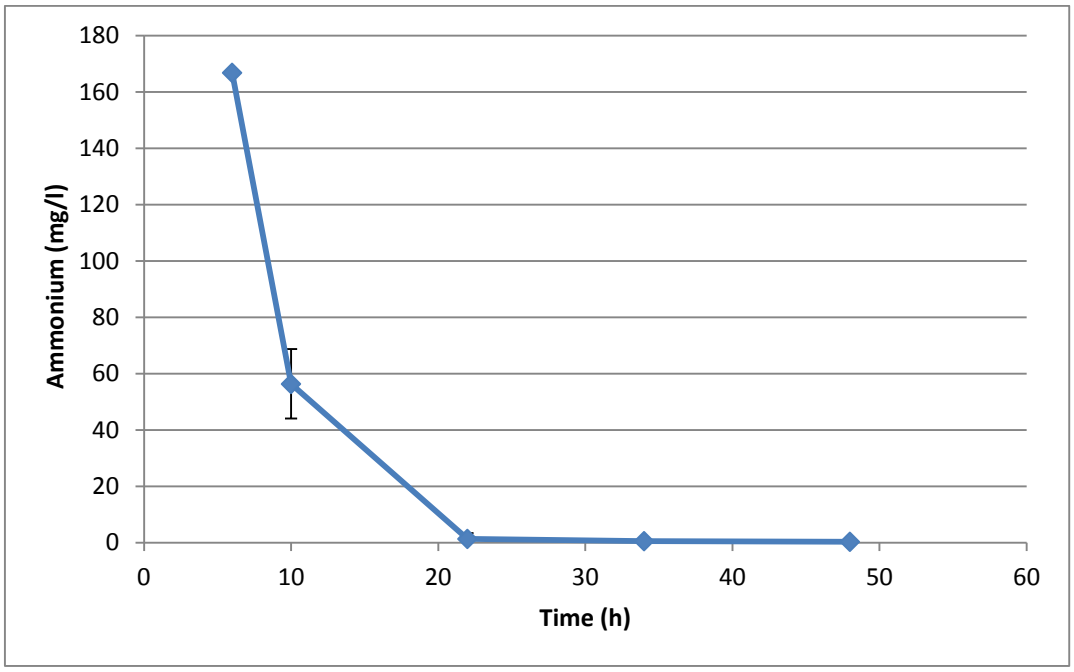

Figure 32: Ammonium utilisation of *B. amyloliquefaciens* during the culture with ammonium as the sole nitrogen sources

Surfactin production (Figure 33) was most rapid during the initial exponential phase and decreased in rate after 5 hours. A maximum of 530.7 mg/l was reached at 13 hours, after which surfactin concentration decreased rapidly until plateauing at 33 hours. A similar trend was observed for antifungals, except that most rapid production occurred between 5 and 13 hours. A maximum of 377.2 mAU*min was reached at 13 hours.

The decrease in lipopeptide concentrations from 13 hours onwards was potentially due to the absence of nitrate and the insufficient ammonium concentration left in the medium. Thus, there was no nitrogen source after 13 hours to continue and maintain lipopeptide production. Furthermore, it is theorised that the bacteria would dissimilate the produced lipopeptides in order to sustain growth and cell maintenance.

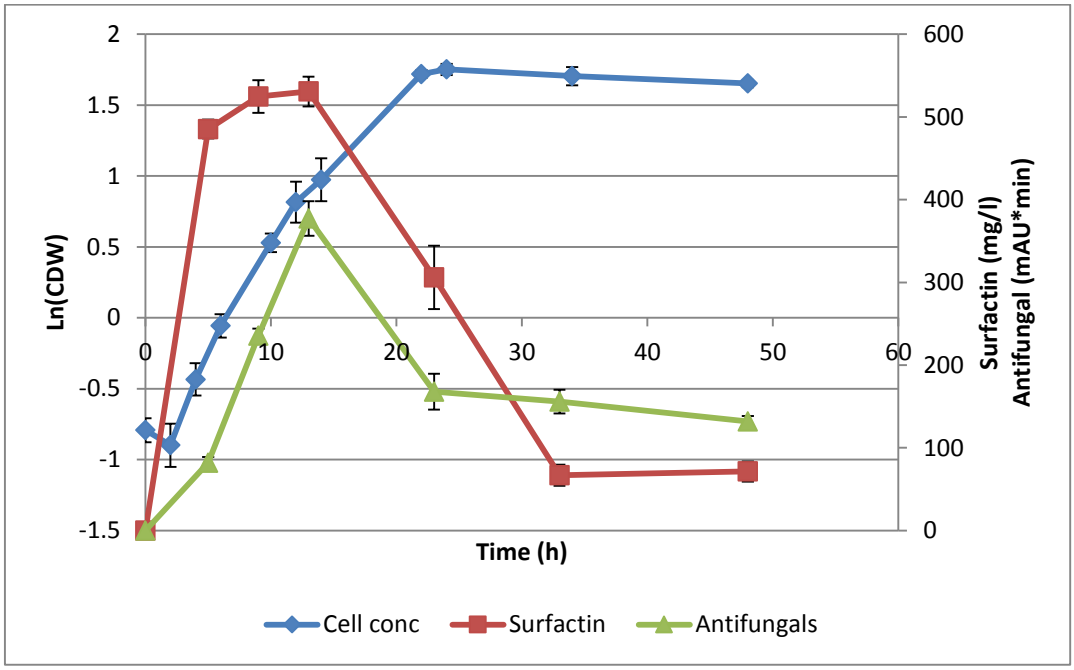

Figure 33: Growth and product formation of *B. amyloliquefaciens* during the culture with ammonium as the sole nitrogen source

4.2.2. Comparison of growth and substrate utilisation trends

The effect of the different nitrogen sources on growth is compared in Figure 34 below. The growth curves follow the same basic trend with differences in the slope of the exponential phase, the duration of the exponential phase and where the stationary phase started. The highest maximum CDW was achieved during the culture containing nitrate as the sole nitrogen source with 8.17 g/l at 48 hours, while the lowest CDW was reached during the culture containing ammonium as the sole nitrogen source with 5.76 g/l at 24 hours. The 0.5:0.5 nitrogen ratio culture achieved the centre value of 7.38 g/l at 14 hours, which was slightly higher than using 4 g/l NH_4NO_3 of 7.18 g/l, also at 14 hours.

Small error bars are observed between compared cultures. The differences in cell growth, between cultures, is likely statistically significant.

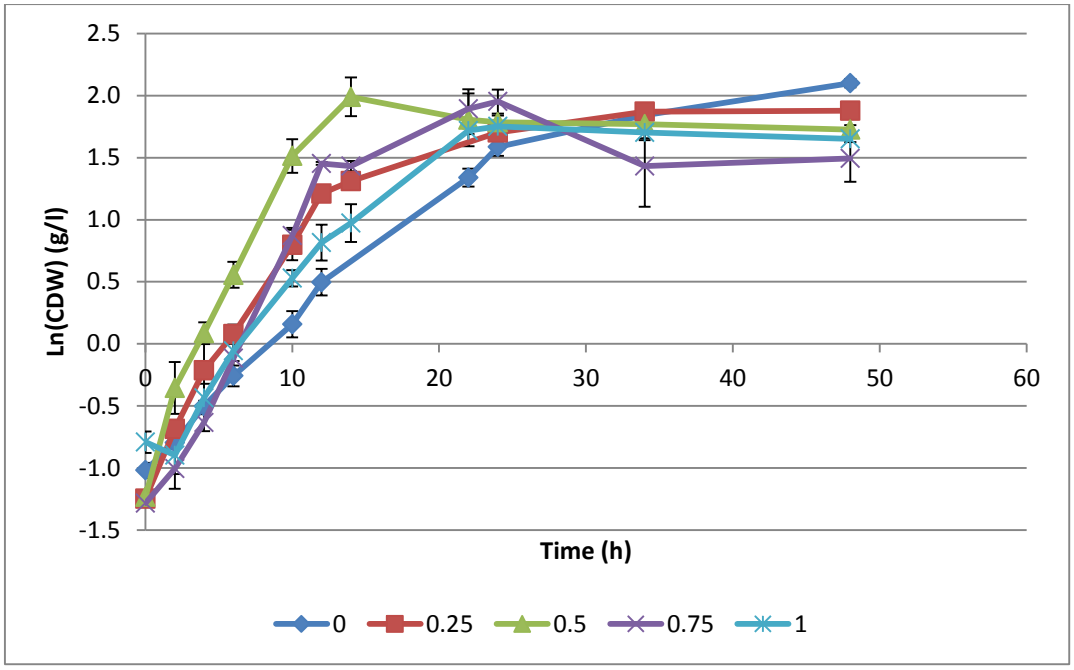

Figure 34: Comparison of *B. amyloliquefaciens* growth at all nitrogen ratios. The legend refers to the fraction of nitrogen from the ammonium source relative to the nitrate source.

The maximum specific growth rate (Figure 35) increased as the fraction of nitrogen from the ammonium source increased from 0 to 0.75 and then decreased to just above the culture containing only nitrate. The growth rate of the different ratios is given in Figure 35 below.

The maximum specific growth rate increased from 0.1238 h^{-1} to 0.2581 h^{-1} with the culture containing only nitrate to the 0.75:0.25 nitrogen ratio culture, and then decreased to 0.1542 h^{-1} with the culture containing only ammonium. The optimum ratio for maximum specific growth rate was the 0.75:0.25 nitrogen ratio culture, with a growth rate of 0.2581 h^{-1}. High R^2 values were present for all ratios, indicating good linear fit to the exponential phase data points.

The 0.5:0.5 nitrogen ratio culture achieved a growth rate of 0.2345 h^{-1}, which was slightly lower than the maximum specific growth rate of 4 g/l NH_4NO_3 culture. The 4 g/l NH_4NO_3 culture also consists of a 0.5:0.5 ammonium to nitrate ratio (refer to Appendix 2).

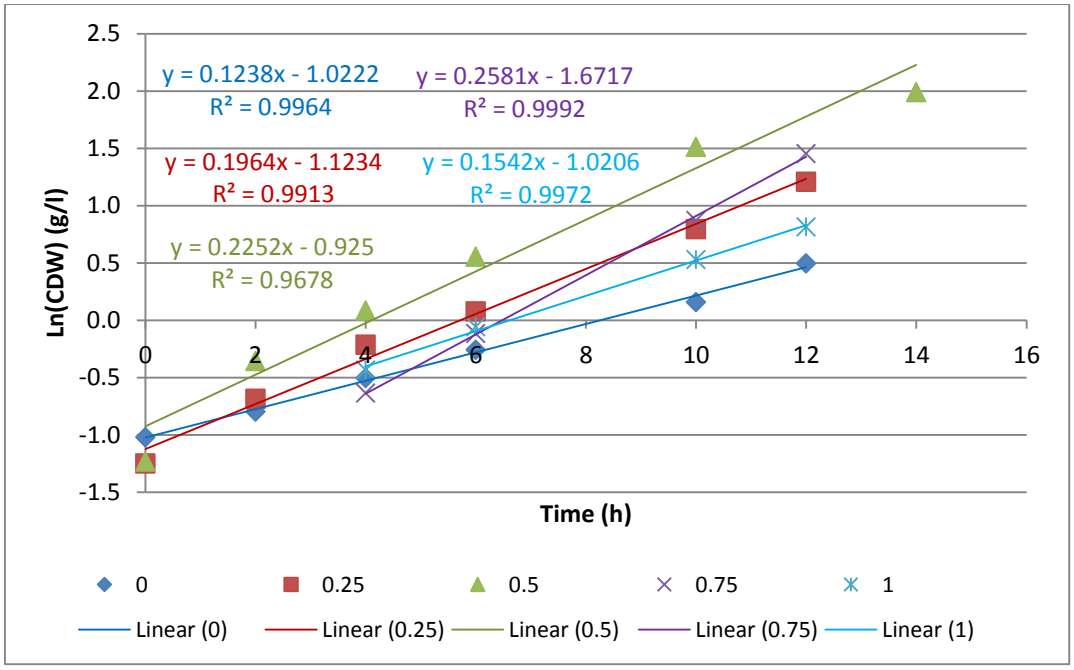

Figure 35: Comparison of *B. amyloliquefaciens* growth rates at all nitrogen ratios. The legend refers to the fraction of nitrogen from the ammonium source relative to the nitrate source.

Glucose (Figure 36) was utilised until depletion and the limiting factor in four of the five ratios. The culture containing only ammonium did not utilise all available glucose since ammonium was limiting. The culture containing only nitrate and the 0.25:0.75 nitrogen ratio culture had almost identical glucose utilisation curves. Both had rapid utilisation for the entire period and were depleted or close to depleted at 48 hours. The 0.5:0.5 and the 0.75:0.25 nitrogen ratio cultures were both depleted of glucose by 23 hours although the glucose utilisation occurred at different rates.

Glucose utilisation between the different nitrogen ratio cultures have small error bars. The small error bars show that the difference is likely statistically significant as no error bars overlap with one another.

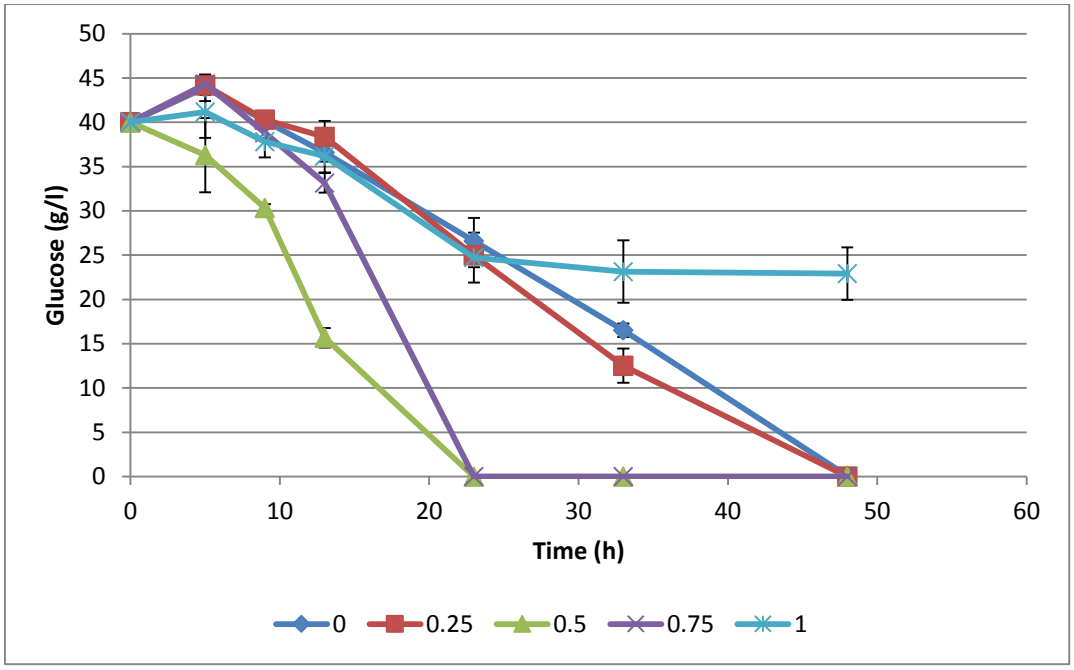

Figure 36: Comparison of *B. amyloliquefaciens* glucose utilisation at all nitrogen ratios. The legend refers to the fraction of nitrogen from the ammonium source relative to the nitrate source.

Nitrate utilisation ceased once glucose became limiting. This was true for all four ratios represented in Figure 37. As with glucose utilisation, the culture containing only nitrate and the 0.25:0.75 nitrogen ratio culture followed similar nitrogen utilisation trends, with utilisation ending at 48 hours. The 0.5:0.5 and the 0.75:0.25 nitrogen ratio cultures ceased utilisation at 23 hours. In all four ratios, nitrate utilisation ceased due to glucose becoming limiting.

Small error bars are observed with the nitrate only and the 0.5:0.5 nitrogen ratio cultures, while the other ratios had an absence of error bars. The error bars that are present do not overlap, indicating that the difference is likely statistically significant.

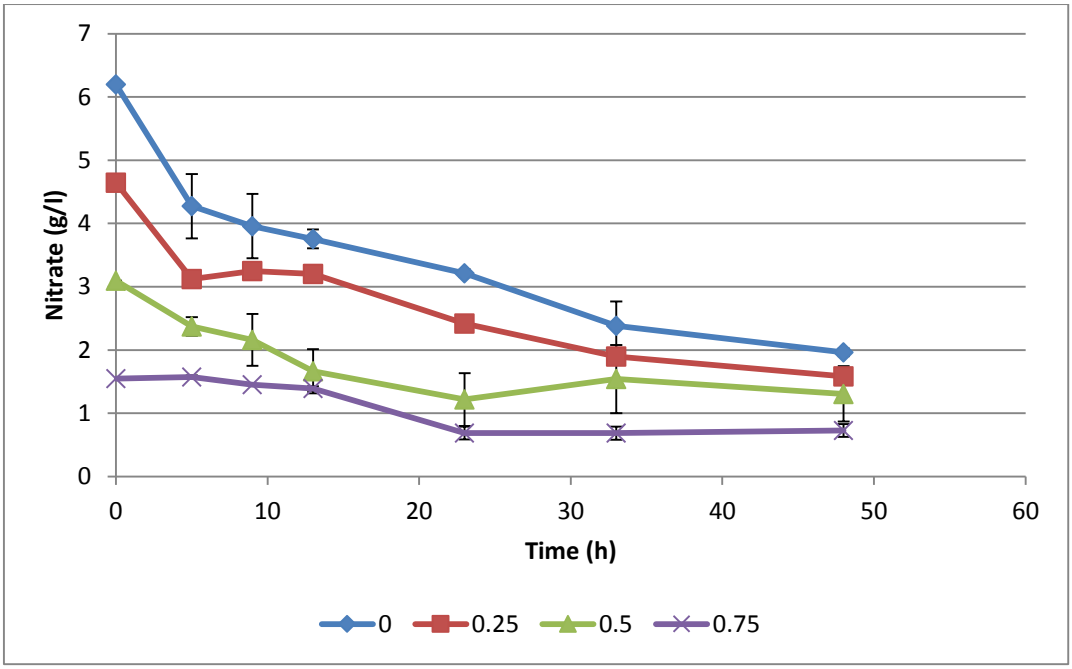

Figure 37: Comparison of *B. amyloliquefaciens* nitrate utilisation at all nitrogen ratios. The legend refers to the fraction of nitrogen from the ammonium source relative to the nitrate source.

Rapid ammonium utilisation (Figure 38) occurred almost exclusively during the exponential phase for all ratios except with the 0.5:0.5 nitrogen ratio culture, which utilised ammonium until glucose was depleted and the stationary phase reached. When both ammonium and nitrate sources were present, nitrate utilisation took place after ammonium utilisation, except with the 0.5:0.5 nitrogen ratio culture.

The 0.5:0.5 nitrogen ratio culture showed high error bars with while the other ratios had much smaller error bars. The difference in ammonium utilisation may not be statistically significant as error bars overlap between all utilisation trends.

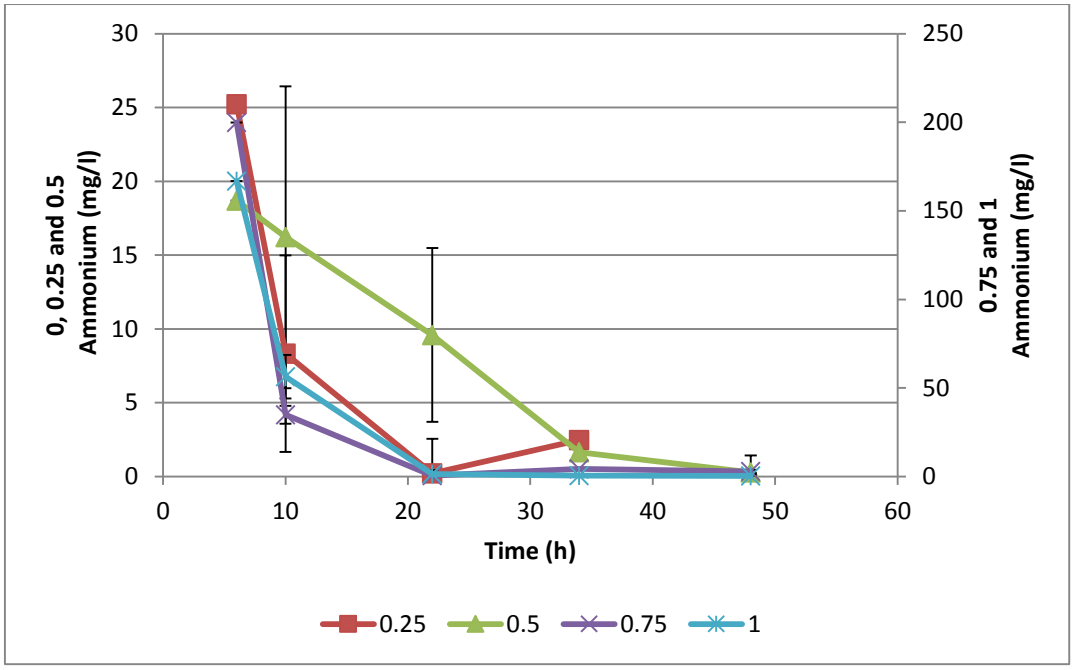

Figure 38: Comparison of *B. amyloliquefaciens* ammonium utilisation at all nitrogen ratios. The legend refers to the fraction of nitrogen from the ammonium source relative to the nitrate source.

4.2.3. Comparison of lipopeptide production trends

Surfactin production with the different nitrogen sources was compared in Figure 39 below. Surfactin production trends were similar between the 0.25:0.75 and 0.5:0.5 nitrogen ratio cultures. Surfactin production was most rapid during the exponential phase, which coincided with rapid ammonium utilisation, after which the rate of production decreased and the concentration plateaued except in the absence of nitrate, where a sharp decrease in surfactin was observed. The nitrogen ratios that achieved the greatest maximum surfactin production was the 0.25:0.75 and 0.5:0.5 nitrogen ratio cultures as well as the culture containing only nitrate, which achieved maximum concentrations of 781.3, 809 and 706.7 mg/l respectively.

The maximum surfactin production with 4 g/l and 8 g/l NH_4NO_3 was 539.3 mg/l and 518 mg/l. All five nitrogen ratio cultures outperformed the 8 g/l NH_4NO_3 culture while all except the culture containing only ammonium outperformed the 4 g/l NH_4NO_3 culture. The 0.5:0.5 nitrogen ratio culture achieved an increase of 33.3% in surfactin production when compared with the culture using 4 g/l NH_4NO_3, both these cultures contain a 0.5:0.5 ratio of nitrogen supplied by ammonium and nitrogen supplied by nitrate (refer to Appendix 2).

Nitrate was more important than ammonium for surfactin production, as the culture containing only nitrate achieved one of the highest concentrations while the culture containing only ammonium showed the worst production. Production improved when both ammonium and nitrate was present, as shown by the 0.25:0.75 and 0.5:0.5 nitrogen ratio cultures. Increasing the nitrogen ratio further, to a ratio containing more than a 0.5 fraction ammonium and nitrate

decreased production once more. The optimum nitrogen source configuration for maximum surfactin production was with the 0.25:0.75 and 0.5:0.5 nitrogen ratio culture.

The error bars obtained for surfactin production with the different nitrogen ratios are all very small. The only point where error bars overlap is at 48 hours due to higher error bars at this point with the 0.25:0.75 nitrogen ratio. The difference in production between these ratios is likely significant.

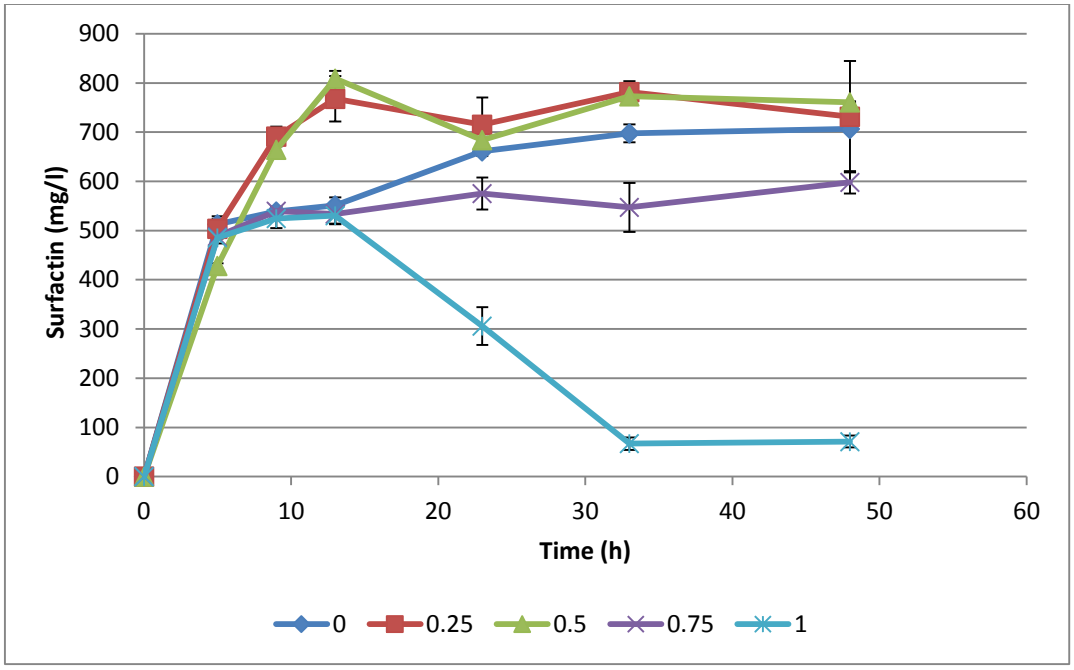

Figure 39: Comparison of surfactin production by *B. amyloliquefaciens* at all nitrogen ratios. The legend refers to the fraction of nitrogen from the ammonium source relative to the nitrate source.

Antifungal production with different nitrogen sources is given in Figure 40 below.

Antifungal production trends were similar between the culture containing only nitrate and the 0.5:0.5 nitrogen ratio culture, and between the 0.25:0.75 and the 0.75:0.25 nitrogen ratio cultures. The culture containing only ammonium achieved poor production, significantly less than the others. Production was most rapid from mid exponential to stationary phase, which coincided with rapid nitrate utilisation, except for the culture containing only ammonium which was due to the absence of nitrate. The nitrogen ratios that achieved the greatest maximum antifungal production were the cultures containing only nitrate, the 0.5:0.5, 0.25:0.75, and 0.75:0.25 nitrogen ratio cultures, achieving 977.3, 888.3, 724.1 and 674 mAU*min.

The maximum antifungal production with 4 g/l and 8 g/l NH_4NO_3 was 404.9 and 604.7 mAU*min. The 4 g/l NH_4NO_3 culture was outperformed by all the nitrogen ratios except the culture containing only ammonium, while the 8 g/l NH_4NO_3 culture was outperformed by the culture containing only nitrate, the 0.5:0.5 and the 0.25:0.75 nitrogen ratio cultures. The 0.5:0.5

nitrogen ratio culture achieved an increase of 54% in antifungal production when compared with the 4 g/l NH_4NO_3 culture.

Antifungal production required nitrate for maximum production, confirmed by the poor production with the culture containing only ammonium. Ammonium was not required for antifungal production as the culture containing only nitrate achieved the greatest antifungal concentration. The optimum nitrogen source configuration was with the culture containing only nitrate and the 0.5:0.5 nitrogen ratio culture. The culture containing only nitrate showed the best production as *B. amyloliquefaciens* can convert excess nitrate to ammonia.

The antifungal production trends showed similar small error bars except at 48 hours with the 0.25:0.75 nitrogen ratio. All other error bars are quite small and do not overlap with one another. Thus, the difference in antifungal production is likely statistically significant.

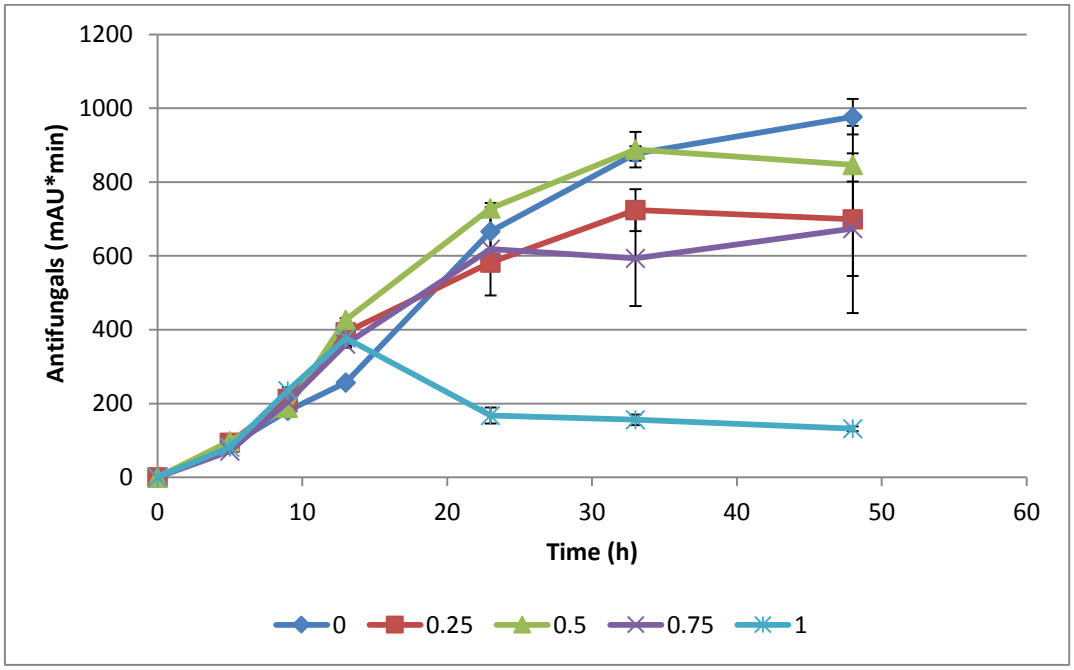

Figure 40: Comparison of antifungal production by *B. amyloliquefaciens* at all nitrogen ratios. The legend refers to the fraction of nitrogen from the ammonium source relative to the nitrate source.

4.2.4. Comparison of normalised cell and lipopeptide concentrations and associated kinetic parameters

The same kinetic parameters employed in section 4.1.4 were used to assess the process performance of the different nitrogen source configurations. Kinetic parameters were calculated at the point of maximum antifungal production. The normalised kinetic parameters are summarised in Figure 41 below.

The highest μ_{max} was achieved with the 0.75:025 nitrogen ratio culture, while the highest maximum CDW was achieved with the culture containing only nitrate.

The optimum nitrogen source configuration for maximum antifungal production was achieved with the culture containing only nitrate, followed by the 0.5:0.5 nitrogen ratio culture. The optimum configuration for maximum surfactin production was achieved with the 0.25:0.75 and 0.5:0.5 nitrogen ratios, which were practically identical, followed by the culture containing only nitrate. Antifungal to surfactin production ratios were in the 0.9 mark for all nitrogen ratios, indicating that selectivity towards antifungals was much greater than towards surfactin.

Cell and lipopeptide formation per unit substrate consumed is reflected by the kinetic parameters, $Y_{x/s}$, $Y_{p/s}$ (p=antifungals) and $Y_{p/s}$ (s=surfactin). The culture containing only ammonium was not considered in any of the discussions that follow as its high kinetic performance was attributed to the small amount of glucose utilised, which due to the nature of the kinetic equations (refer to Appendix 2) resulted in the high kinetic values observed. The ratios all showed higher cell yields ($Y_{x/s}$) than lipopeptide yields ($Y_{p/s}$). The 0.25:0.75 nitrate ratio culture achieved the highest cell ($Y_{x/s}$) and lipopeptide yields ($Y_{p/s}$).

Specific antifungal and surfactin production are reflected by the kinetic parameters $Y_{p/x}$ (p=antifungals), and $Y_{p/x}$ (s=surfactin). The highest specific antifungal production was achieved with the 0.5:0.5 and 0.75:0.25 nitrogen ratio cultures, while the culture containing only nitrate and the 0.25:0.75 nitrogen ratio culture achieved similar values. The highest specific surfactin production was achieved with the 0.75:0.25, 0.5:0.5 and 0.25:0.75 nitrogen ratio cultures, in that order, while the culture containing only nitrate performed the worst.

Antifungal productivity was much higher than that of surfactin for all ratios. The 0.5:0.5 nitrogen ratio culture outperformed all others with regards to antifungal productivity, achieving 26.92 mAU*min/h while the 0.25:0.75 and 0.5:0.5 nitrogen ratio cultures achieved the highest surfactin productivity at 23.68 mg/l/h and 23.42 mg/l/h.

The optimum nitrogen source configuration for antifungal production was between the culture containing only nitrate and the 0.5:0.5 nitrogen ratio culture. The 0.5:0.5 nitrogen ratio culture was the optimum as it had the second highest antifungal concentration (888.3 mAU*min), the highest specific antifungal production (158.2 mAU*min/g/l), highest antifungal productivity (26.92 mAU*min/h) and competitively high antifungal selectivity (86.7%). The culture containing only nitrate had the highest antifungal concentration, very competitive specific antifungal production and productivity, and the second highest antifungal selectivity. The 0.5:0.5 nitrogen ratio culture was also the optimum for surfactin production. Furthermore, the

0.5:0.5 nitrogen ratio culture outperformed the 4 g/l NH_4NO_3 culture in every kinetic parameter except maximum specific growth rate.

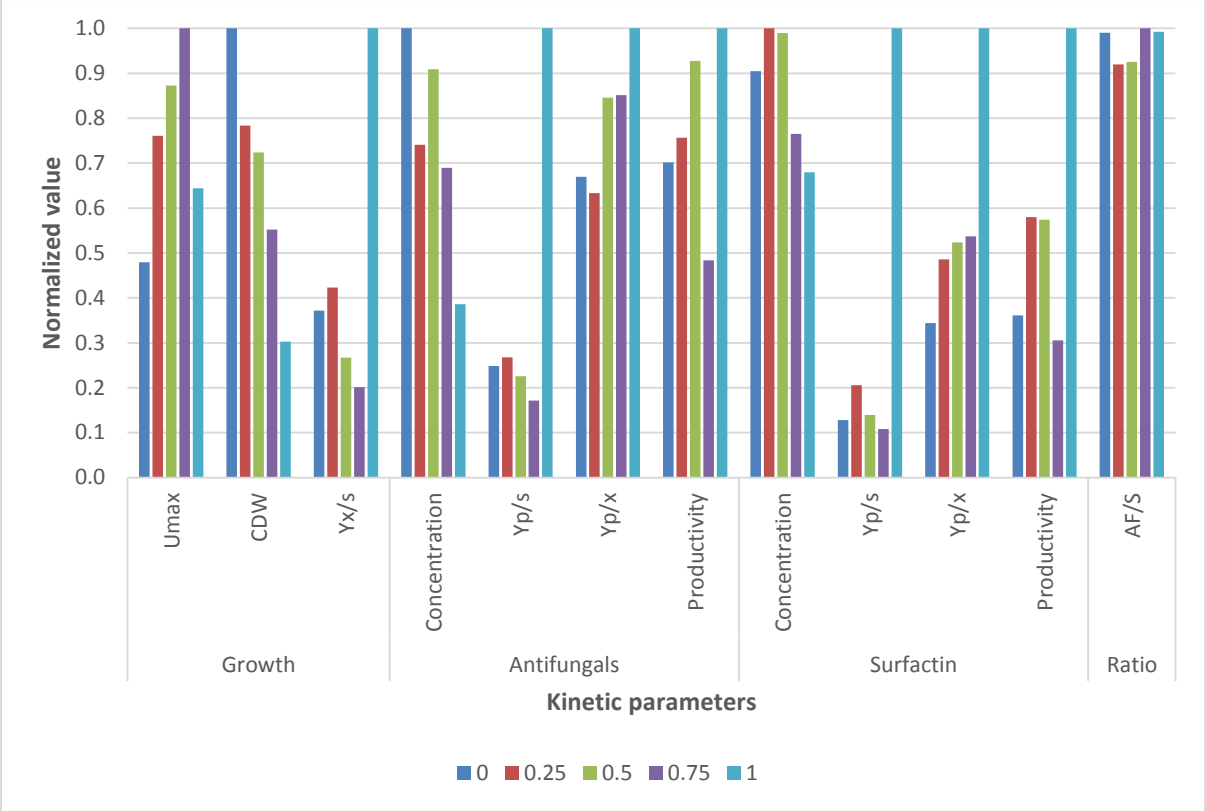

Figure 41: Summary of *B. amyloliquefaciens* growth and lipopeptide related kinetic parameters at maximum antifungal concentration for different nitrogen ratios. The legend refers to the fraction of nitrogen from the ammonium source relative to the nitrate source.

4.3. Effect of dissolved oxygen level on growth and production kinetics

Davis et al. (1999) suggests that a decrease in dissolved oxygen improves lipopeptide production. The optimum nitrogen source configurations identified as the 0.5:0.5 nitrogen ratio culture and culture containing only nitrate was used to investigate the effect of dissolved oxygen. Baffled and unbaffled shake flasks were used to achieve this. The baffles promote oxygen transfer and thus have higher concentrations of oxygen as opposed to unbaffled flasks.

4.3.1. Growth, substrate utilisation and product formation

The growth curve of the unbaffled culture with a 0.5:0.5 nitrogen ratio from ammonium and nitrate sources respectively, in Figure 42, had an acceleration phase of 2 hours followed by the exponential phase which ranged from 0 to 12 hours. A maximum CDW of 5.36 g/l at 22 hours was achieved.

Glucose utilisation started after inoculation until depletion at 33 hours. Nitrate utilisation ceased when the stationary phase was reached at 23 hours, at which point approximately 0.6 g/l nitrate remained. The extensive rapid utilisation of nitrate, from 0 to 23 hours, can be partly explained by the relatively low dissolved oxygen concentration present in the culture due to the absence of baffles in the shake flasks. Nitrate was thus used as the electron acceptor.

Ammonium concentration started with 901 mg/l (not shown) which was utilised almost to completion within the first 10 hours, with less than 1 mg/l remaining. Ammonium utilisation occurred during the exponential phase with rapid utilisation during the first 6 hours.

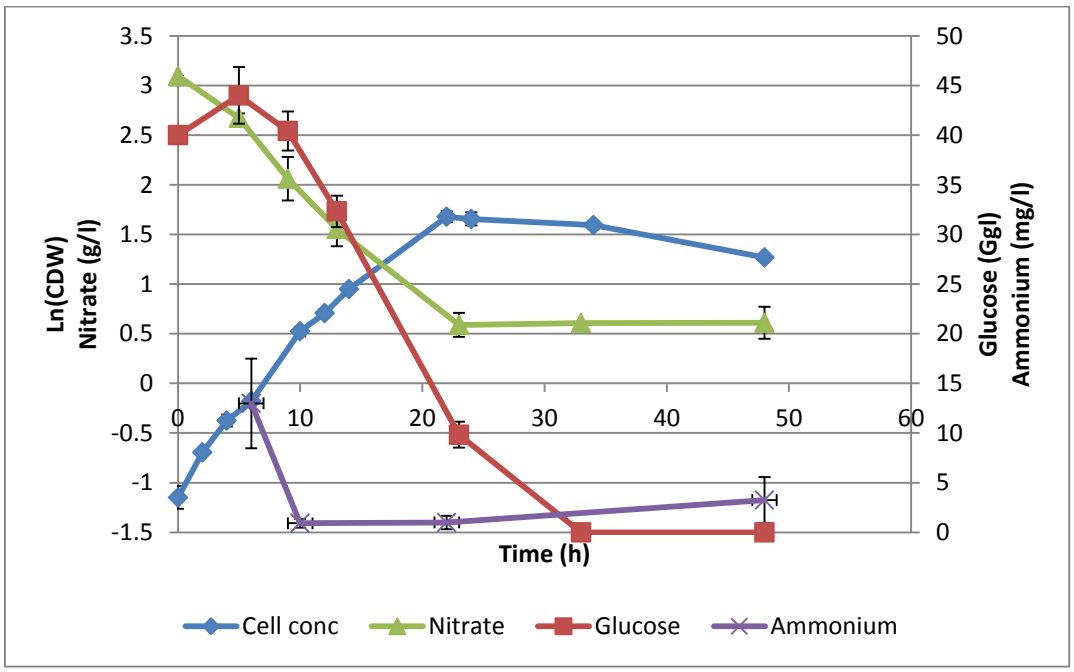

Figure 42: Growth and substrate utilisation of *B. amyloliquefaciens* during the unbaffled culture with a 0.5:0.5 nitrogen ratio from ammonium and nitrate sources respectively

Rapid surfactin production (Figure 43) occurred during rapid ammonium utilisation, during which time nitrate was also utilised. Production continued but at a slower rate until it plateaued at 23 hours. A maximum surfactin concentration of 660 mg/l was achieved at 33 hours, just after nitrate utilisation ceased at 23 hours. Antifungal production occurred after ammonium utilisation ceased, at end exponential to stationary phase. Production plateaued during the stationary phase and a maximum of 490 mAU*min was reached at 48 hours.

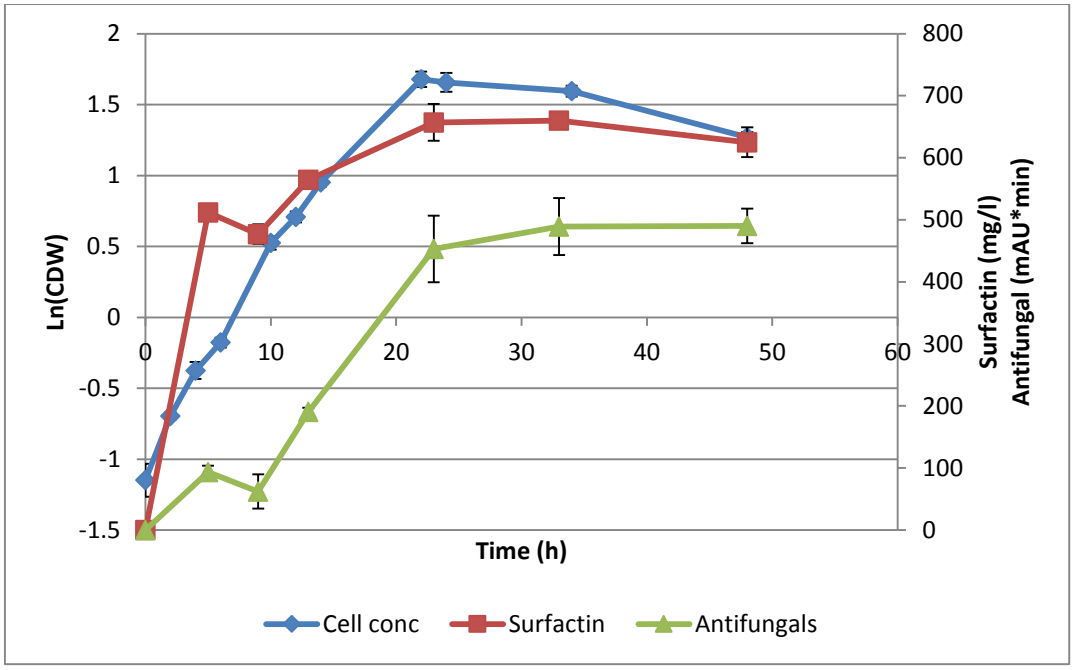

Figure 43: Growth and product formation of *B. amyloliquefaciens* during the unbaffled culture with a 0.5:0.5 nitrogen ratio from ammonium and nitrate sources respectively

The growth curve of the unbaffled culture containing only nitrate, in Figure 44, had a lag phase of 2 hours, followed by the exponential phase which ranged from 2 to 14 hours. A maximum CDW of 6.65 g/l at 24 hours was achieved.

Glucose utilisation occurred slowly for the first 9 hours, less than 4 g/l was used, after which rapid utilisation occurred until 23 hours. Glucose became depleted after 33 hours. Rapid nitrate utilisation ranged from 0 to 5 hours and then at a slightly lower rate from 9 to 23 hours and ceased at 33 hours with approximately 0.4 g/l remaining. The depletion of glucose and ceased usage of nitrate at 33 hours indicated the start of the stationary phase.

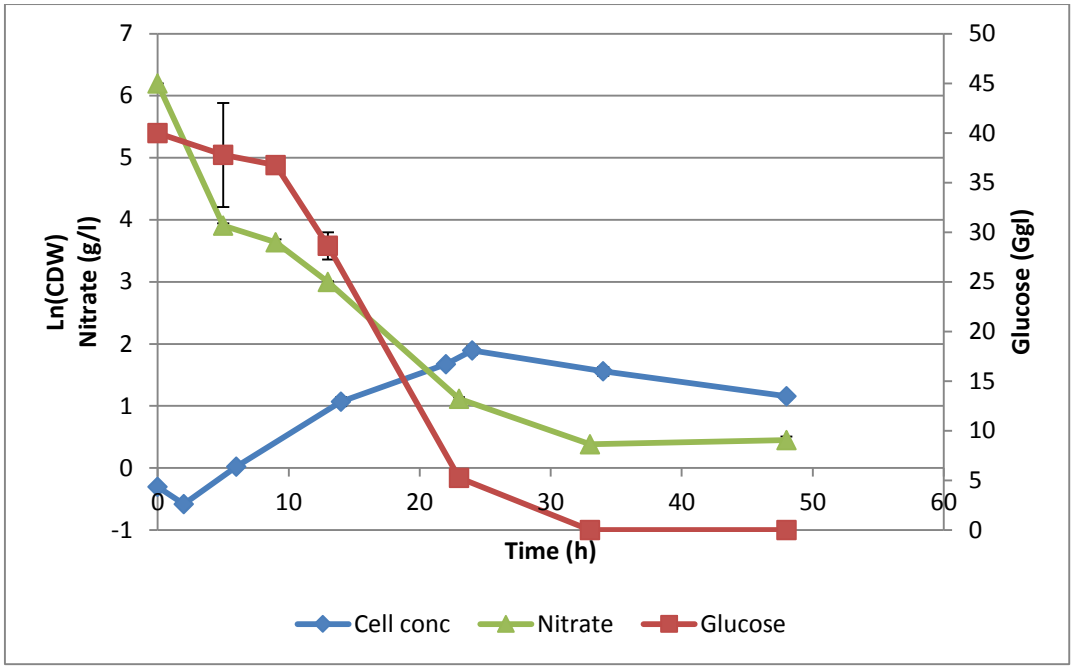

Figure 44: Growth and substrate utilisation of *B. amyloliquefaciens* during the unbaffled culture with nitrate as the sole nitrogen source

Poor surfactin production (Figure 45) was observed with the unbaffled culture containing only nitrate, reaching a maximum of 148 mg/l at 33 hours. Antifungal production occurred at an almost constant rate until its maximum of 563 mAU*min at 33 hours, after which it decreased since nitrate utilisation ceased.

The poor surfactin production was due to the combination of no ammonium and low oxygen availability since the unbaffled culture with a 0.5:0.5 nitrogen ratio (low oxygen with ammonium) and the baffled culture containing only nitrate (high oxygen with no ammonium) achieved a maximum surfactin concentration of 660 mg/l and 706.7 mg/l. This suggests that, in the absence of sufficient oxygen, ammonium availability was crucial for surfactin production, after which nitrate was used to maintain production.

A decrease in oxygen availability decreased both surfactin and antifungal concentrations, regardless of ammonium availability, as both the culture containing only nitrate and the 0.5:0.5 nitrogen ratio culture achieved greater surfactin and antifungal production with baffled shake flasks. Antifungal production decreased from 732 to 490 mAU*min with the 0.5:0.5 nitrogen ratio culture and from 977 to 563 mAU*min with the culture containing only nitrate.

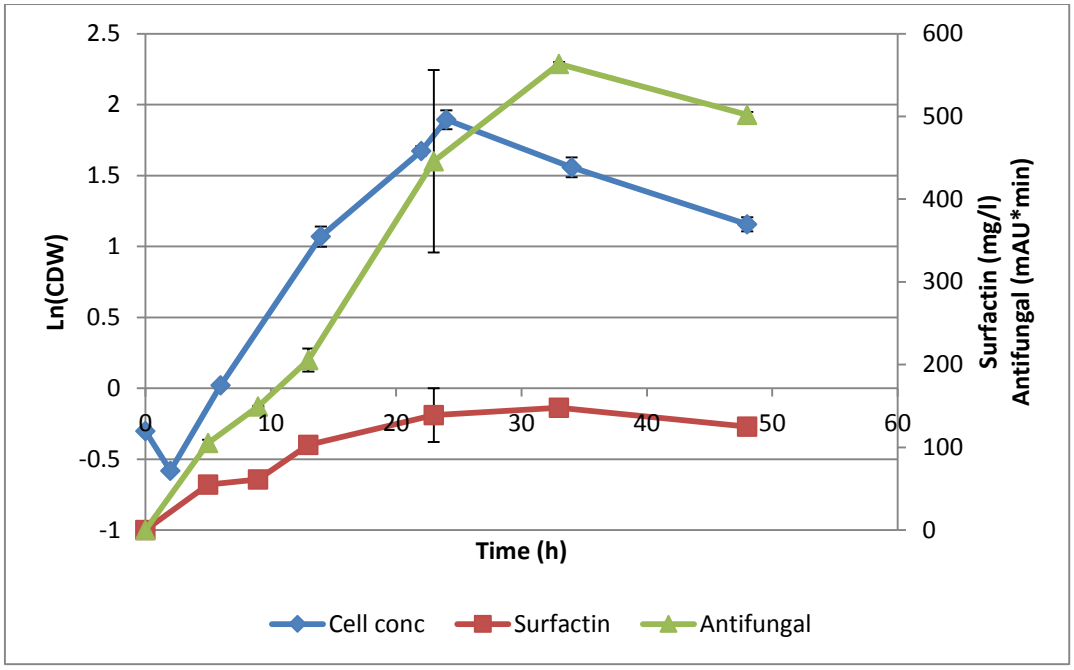

Figure 45: Growth and product formation of *B. amyloliquefaciens* during the unbaffled culture with nitrate as the sole nitrogen source

4.3.2. Comparison of growth and substrate utilisation trends

The effect of dissolved oxygen on growth is compared in Figure 46 below. The legend refers to the fraction of nitrogen from the ammonium source relative to the nitrate source.

Similar growth curves were observed for all experiments, except with the unbaffled 0.5:0.5 nitrogen ratio culture which has a greater slope during the exponential phase and thus a greater maximum specific growth rate. Higher maximum CDW values were achieved with the baffled cultures, 8.17 g/l compared to 6.65 g/l for the culture containing only nitrate, and 7.38 g/l compared to 5.36 g/l for the 0.5:0.5 nitrogen ratio.

Error bars are almost completely absent between the two baffled and unbaffled cultures. The differences in cell growth is likely statistically significant.

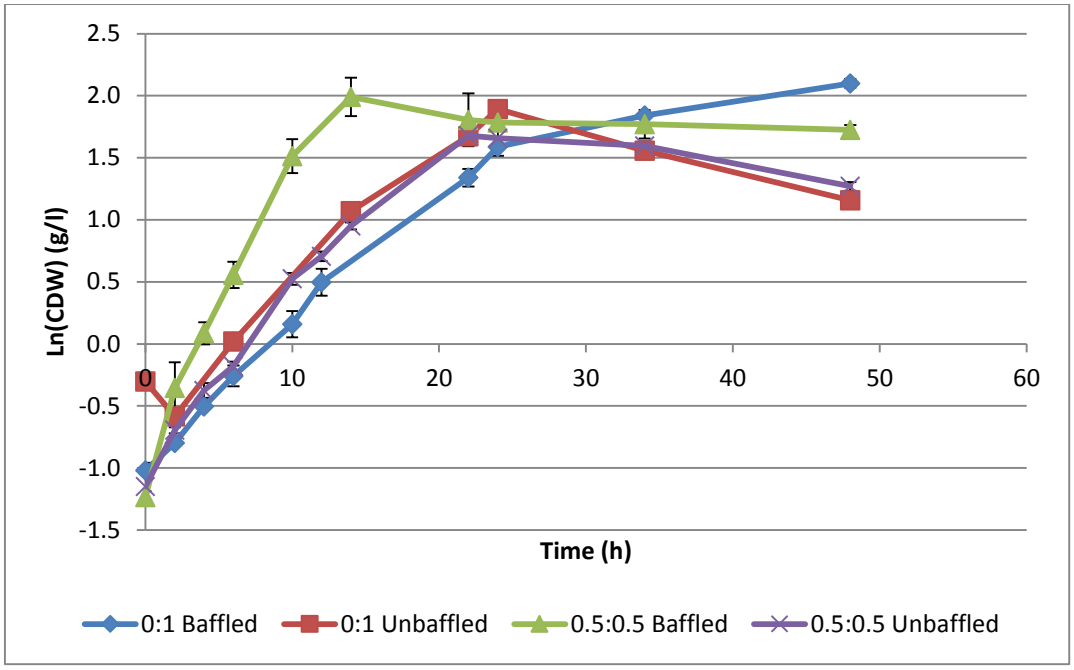

Figure 46: Comparison of *B. amyloliquefaciens* growth at optimum nitrogen ratios in baffled and unbaffled shake flasks.

The maximum specific growth rate was slightly greater for the unbaffled culture containing only nitrate than with the baffled culture. The opposite was the case with the 0.5:0.5 nitrogen ratio culture where the maximum specific growth rate was much higher with baffles than without. The growth rate of the different configurations is given in Figure 47 below.

The 0.5:0.5 nitrogen ratio achieved a greater maximum specific growth rate in the presence of high oxygen levels than in the absence thereof (0.235 h^{-1} compared to 0.124 h^{-1}). The opposite was the case with the culture containing only nitrate, albeit a slight difference between the baffled and unbaffled specific growth rates.

In the absence of sufficient oxygen availability, the specific growth rates were similar between the 0.5:0.5 nitrogen ratio culture and the culture containing only nitrate, however, the ammonium containing ratio still had a slightly higher growth rate (0.144 h^{-1}compared to 0.137 h^{-1}). The much higher maximum specific growth rate, under sufficient oxygen availability, with the 0.5:0.5 nitrogen ratio suggests that ammonium influenced the rate of cell growth and is thus required to achieve high growth rates. High R^2 values were present for all cultures, indicating good linear fit to the exponential phase data points.

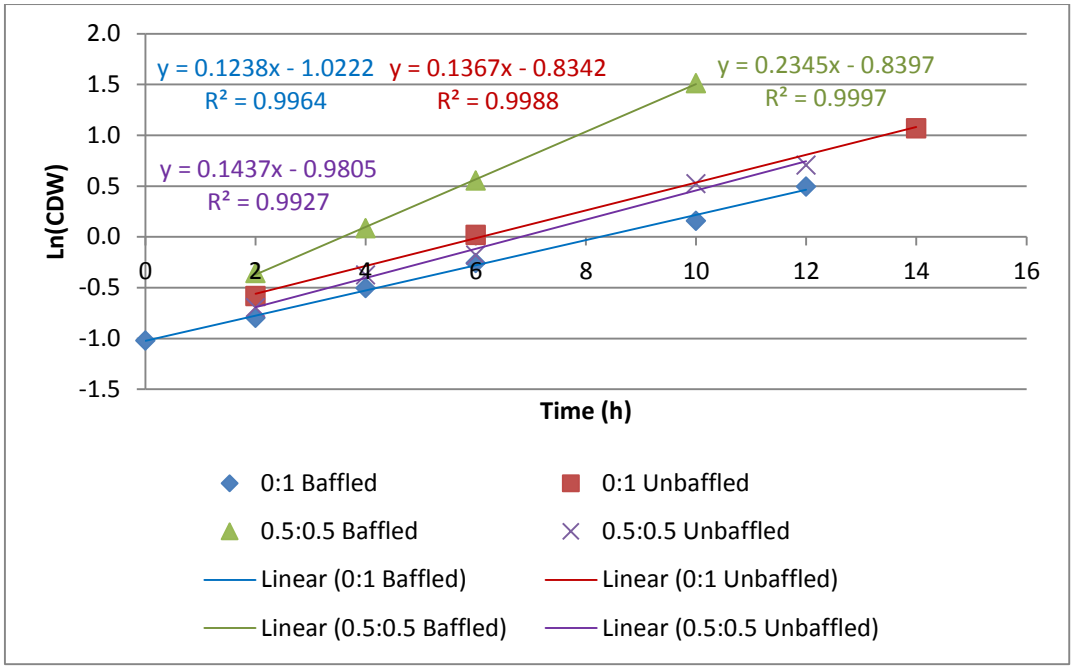

Figure 47: Comparison of *B. amyloliquefaciens* growth rates at optimum nitrogen ratios in baffled and unbaffled shake flasks

The effect of dissolved oxygen on glucose utilisation is compared in Figure 48 below. The unbaffled cultures were depleted of glucose at 33 hours, the baffled culture containing only nitrate at 48 hours and the 0.5:0.5 nitrogen ratio baffled culture at 23 hours. Conflicting results were observed as the unbaffled culture containing only nitrate depleted glucose earlier than the baffled culture, while the opposite was observed for the 0.5:0.5 nitrogen ratio culture.

Absent and small error bars are observed with glucose utilisation curves between the different cultures. The small error bars show that the difference is likely statistically significant as no error bars overlap with one another.

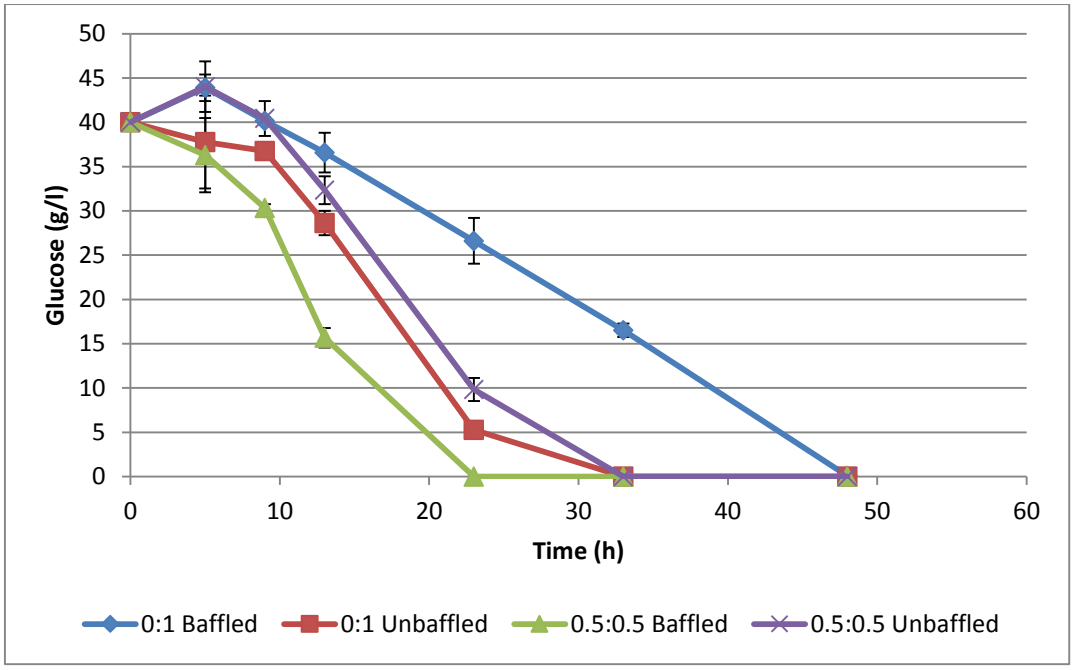

Figure 48: Comparison of *B. amyloliquefaciens* glucose utilisation at optimum nitrogen ratios in baffled and unbaffled shake flasks

The effect of dissolved oxygen on nitrate utilisation is compared in Figure 49 below.

Nitrate was utilised more effectively during the unbaffled cultures than during the baffled ones, as more nitrate was utilised in the 48-hour time span and utilisation ceased at lower nitrate concentrations, 0.451 g/l vs. 1.96 g/l with the culture containing only nitrate and 0.610 g/l vs.1.31 g/l with the 0.5:0.5 nitrogen ratio culture. This was due to the fact that oxygen became depleted earlier in the unbaffled flasks and nitrate was subsequently used as the electron acceptor in the absence of oxygen.

The nitrate utilisation curves showed small error bars between the cultures. These error bars do however, overlap at multiple instances since the utilisation curve are so similar to one another. The difference in utilisation may not be statistically significant.

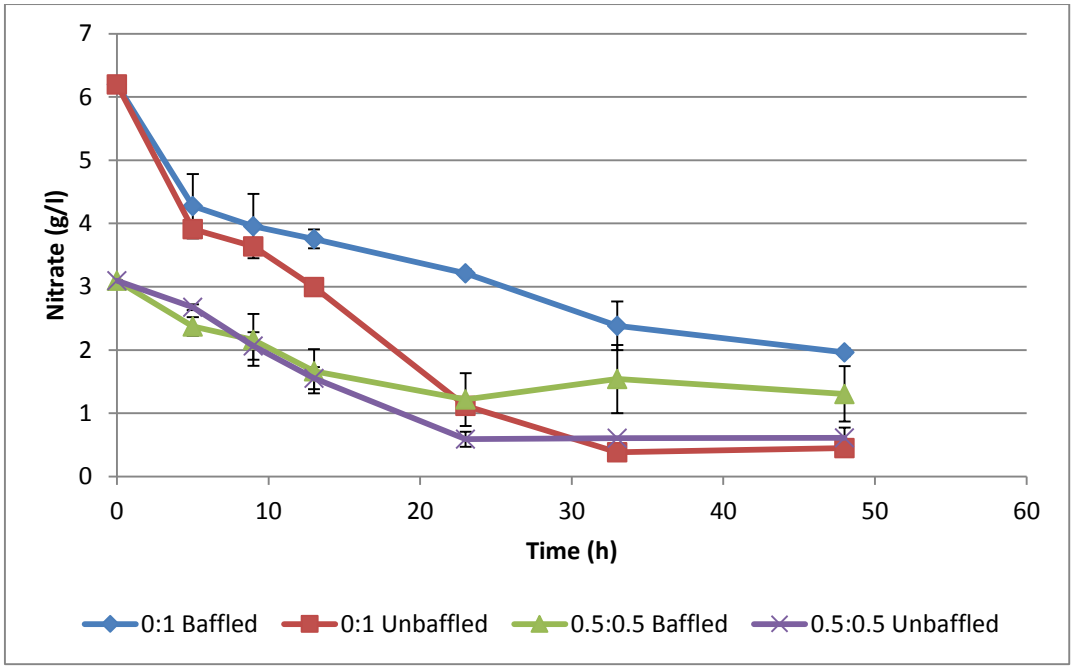

Figure 49: Comparison of *B. amyloliquefaciens* nitrate utilisation at optimum nitrogen ratios in baffled and unbaffled shake flasks

The effect of dissolved oxygen on ammonium utilisation is compared in Figure 50 below. Ammonium utilisation was much more rapid and depleted much earlier during the unbaffled cultures.

Error bars were high for the ammonium utilisation, especially with the 0.5:0.5 baffled culture. Despite the high error bars, there was no overlap with any other error bars indicating that the difference is likely statistically significant.

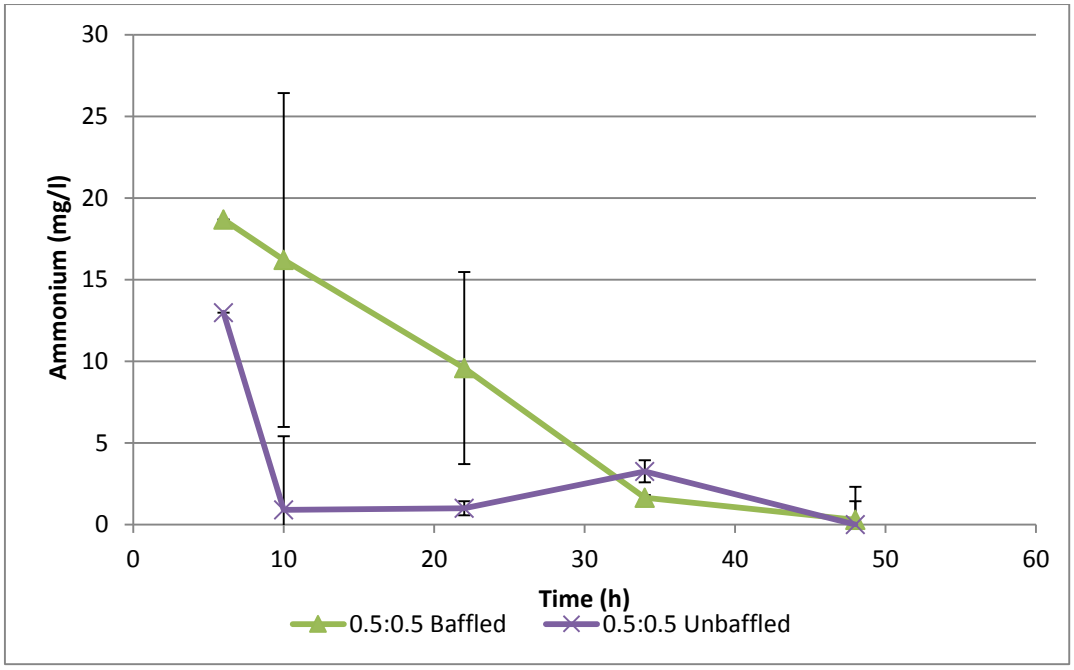

Figure 50: Comparison of *B. amyloliquefaciens* ammonium utilisation at optimum nitrogen ratios in baffled and unbaffled shake flasks

4.3.3. Comparison of lipopeptide production trends

Surfactin production for the 0.5:0.5 nitrogen ratio culture and the culture containing only nitrate in baffled and unbaffled flasks is given in Figure 51 below.

Surfactin production was higher with baffled cultures than with unbaffled cultures. Rapid surfactin production was observed during the exponential phase of the baffled 0.5:0.5 nitrogen ratio culture and during the early exponential phase (0 to 5 hours) of the unbaffled 0.5:0.5 nitrogen ratio culture. The baffled culture containing only nitrate also showed rapid surfactin production during the early exponential phase, while the unbaffled culture containing only nitrate showed poor production.

The greatest surfactin production, with a maximum concentration of 809 mg/l at 13 hours, was achieved with the baffled 0.5:0.5 nitrogen ratio culture while the worst production, with a maximum of 148 mg/l at 33 hours, was achieved with the unbaffled culture containing only nitrate. The baffled culture containing only nitrate and the unbaffled 0.5:0.5 nitrogen ratio culture achieved a maximum surfactin concentration of 706.7 mg/l at 48 hours and 660 mg/l at 33 hours.

The surfactin production trends showed a distinct lack of error bars, the few that are present are quite small. The error bars do not overlap with any of the other error bars present, thus it the differences is likely statistically significant.

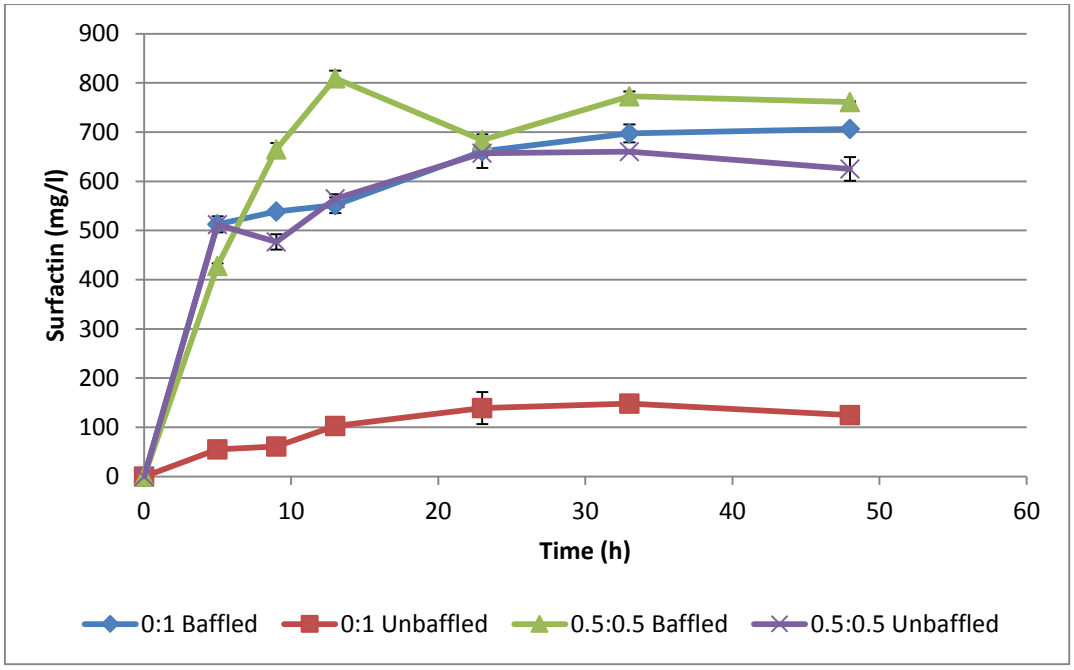

Figure 51: Comparison of surfactin production by *B. amyloliquefaciens* at optimum nitrogen ratios in baffled and unbaffled shake flasks

Antifungal production for the 0.5:0.5 nitrogen ratio culture and the culture containing only nitrate in baffled and unbaffled flasks are given in Figure 52 below. Antifungal production followed the same trend as observed with surfactin in the sense that the baffled ratios achieved higher production than the unbaffled ratios throughout.

Antifungal production was most rapid from end exponential to stationary phase, which coincided with rapid nitrate utilisation. The baffled cultures outperformed their unbaffled counterparts. The greatest antifungal production, with a maximum concentration of 977.25 mAU*min at 48 hours, was achieved with the baffled culture containing only nitrate while the worst production, with a maximum concentration of 490.31 mAU*min at 48 hours, was achieved with the unbaffled 0.5:0.5 nitrogen ratio culture. The baffled 0.5:0.5 nitrogen ratio culture and the unbaffled culture containing only nitrate achieved a maximum antifungal concentration of 888.30 mAU*min and 563.28 mAU*min at 33 hours.

Oxygen availability is of primary importance for lipopeptide production, as higher concentrations were achieved in the baffled flasks which have greater oxygen availability. The poor performance in the nitrate only unbaffled flasks suggests that less nitrate was available for conversion in to ammonia as it was being diverted for use as an electron acceptor.

The small error bars associated with antifungal production trends overlap at some points, specifically error bars between the two baffled cultures and error bars between the two unbaffled cultures. There are no error bars that overlap any of the baffled cultures with any of

the unbaffled cultures. Thus, effect of oxygen on antifungal production is likely statistically significant.

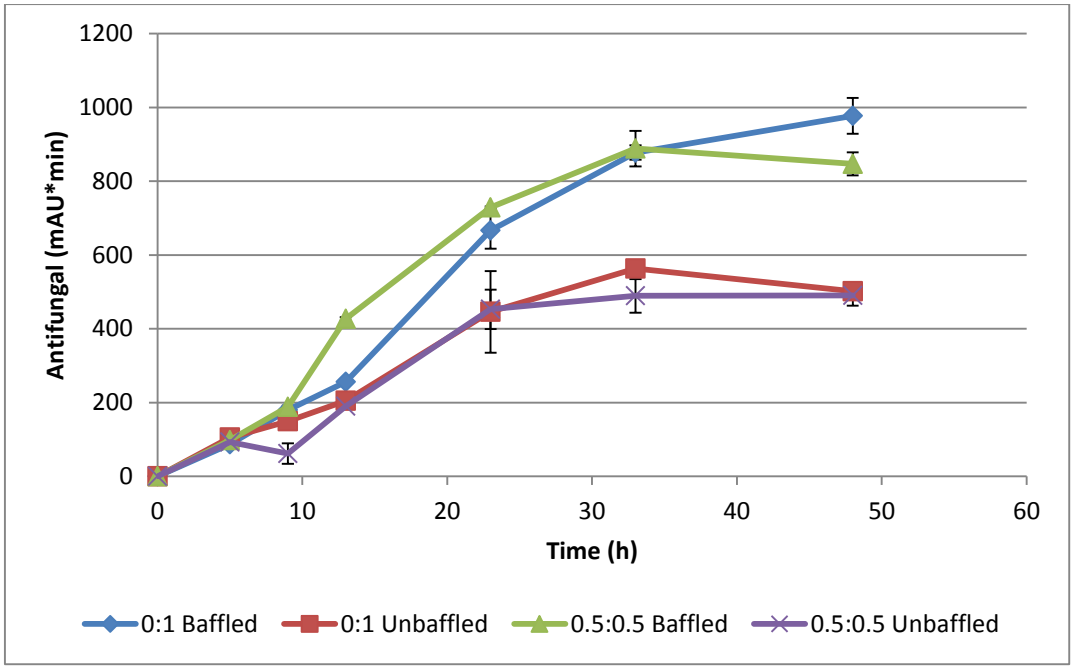

Figure 52: Comparison of antifungal production by *B. amyloliquefaciens* at optimum nitrogen ratios in baffled and unbaffled shake flasks

4.3.4. Comparison of normalised cell and lipopeptide concentrations and associated kinetic parameters

Kinetic parameters were used to assess the process performance of different dissolved oxygen levels. These parameters were calculated at the point of maximum antifungal production. The normalised kinetic parameters are summarised in Figure 53 below.

The availability of oxygen influenced the rate of cell growth and the extent of growth. The baffled cultures achieved higher maximum CDW values than unbaffled cultures. The culture containing only nitrate achieving a higher CDW than the 0.5:0.5 nitrogen ratio culture, 6.65 g/l vs. 5.36 g/l. The growth rate was also influenced by oxygen as a much greater maximum specific growth rate was achieved during the baffled 0.5:0.5 nitrogen ratio culture than during the analogous unbaffled culture. The baffled and unbaffled cultures containing only nitrate baffled showed similar maximum specific growth rates values.

Antifungal and surfactin concentrations were much greater during baffled cultures than during unbaffled cultures. The presence of ammonium did not have a significant effect on antifungal production, regardless of oxygen availability. This was however not the case with surfactin, as the absence of ammonium (culture containing only nitrate) showed a significant decrease in surfactin production during low dissolved oxygen levels. When oxygen was available,

ammonium was not required for production. The maximum antifungal concentration in an oxygen limiting environment was achieved with the culture containing only nitrate, which was expected as a higher nitrate concentration would allow longer nitrate utilisation and thus an increased period for production. The maximum surfactin concentration in an oxygen limiting environment was achieved with the 0.5:0.5 nitrogen ratio culture since a readily available source of ammonium was present, which was required for surfactin production.

Cells and lipopeptides formed per unit of substrate consumed are reflected by the kinetic parameters $Y_{x/s}$, $Y_{p/s}$ (p=antifungals) and $Y_{p/s}$ (s=surfactin). These parameters were greater with baffled cultures than with unbaffled cultures. The culture containing only nitrate showed that cell yields ($Y_{x/s}$) was greater than surfactin yields ($Y_{p/s}$), while the opposite was the case with the 0.5:0.5 nitrogen ratio culture. Antifungal yields ($Y_{p/s}$) could not be compared with the other two parameters as the units differed however, high antifungal yields ($Y_{p/s}$) were observed, especially for baffled cultures.

The specific antifungal production for baffled and unbaffled cultures were similar for both the 0.5:0.5 nitrogen ratio culture and the culture containing only nitrate. The baffled 0.5:0.5 nitrate ratio culture was slightly higher than the unbaffled culture while the opposite was the case with the culture containing only nitrate. The greatest specific antifungal production was achieved with the baffled and unbaffled 0.5:0.5 nitrogen ratio cultures (158.2 and 151.2 mAU*min/g/l). The unbaffled 0.5:0.5 nitrogen ratio culture outperformed all cultures with regards to specific surfactin production (0.193 g surfactin/g glucose) while the unbaffled culture containing only nitrate performed the worst (0.035 g surfactin/g glucose).

Productivity, for both antifungals and surfactin, was greater with baffled cultures than with unbaffled cultures. When comparing unbaffled cultures, the culture containing only nitrate achieved the higher antifungal productivity (17 vs. 10.2 mAU*min/h) during low oxygen availability, while the 0.5:0.5 nitrogen ratio culture achieved the higher surfactin productivity (13.02 vs. 4.46 g/l/h) during low oxygen availability. This was possibly due to a greater requirement for ammonium in an oxygen limited environment for surfactin production than for antifungal production. The baffled 0.5:0.5 nitrogen ratio culture achieved the greatest productivity, for both antifungals and surfactin, of all the cultures (26.92 mAU*min/h and 23.4 g/l/h).

Antifungal selectivity (AF/S ratio) was shown to be in the 0.9 range for all cultures, indicating greater selectivity towards antifungal than towards surfactin production. Antifungal selectivity is shown to decrease as the level of dissolved oxygen decreased with the culture containing only nitrate (92.7% to 84.5%), while the opposite was the case with the 0.5:0.5 nitrate ratio culture (86.7% to 89.7%).

The baffled cultures outperformed the unbaffled cultures in all kinetic parameters except in μ_{max} and $Y_{p/x}$ (p=antifungals). Antifungal selectivity showed contradictory results between the two nitrogen source configurations when comparing baffled and unbaffled cultures. Considering antifungal production, the presence of sufficient oxygen availability was required to maximise production with regards to antifungal concentration and production kinetics.

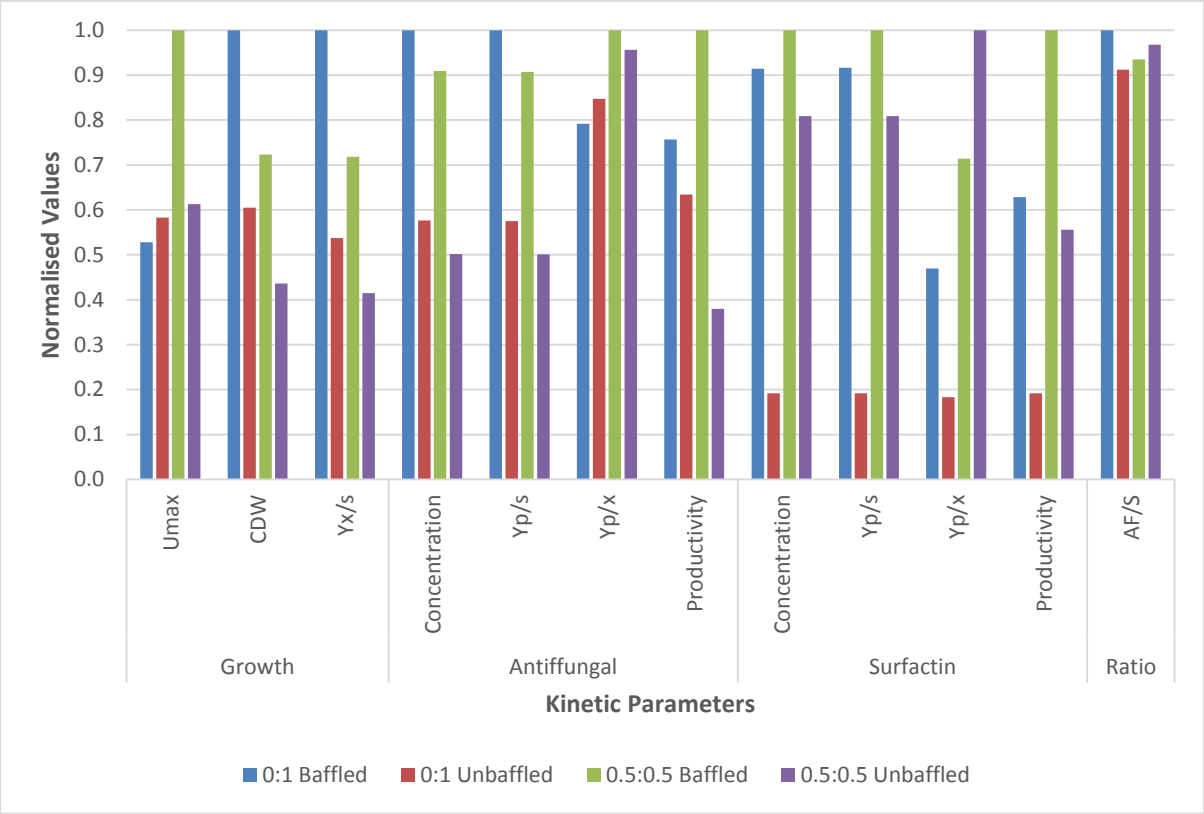

Figure 53: Summary of *B. amyloliquefaciens* growth and lipopeptide related kinetic parameters at maximum antifungal concentration at optimum nitrogen ratios in baffled and unbaffled shake flasks

4.4. Production kinetics under controlled conditions with optimum nitrogen source and dissolved oxygen level

The 0.5:0.5 nitrogen ratio culture was identified as the optimum nitrogen configuration (refer to section 4.2). Similarly, baffled configuration and thus high dissolved oxygen levels were identified as the optimum (refer to section 4.3) for antifungal production. The process performance of these optimum conditions was assessed when cultured in the bioreactor. The bioreactor culture kinetics were compared with the 8 g/l NH_4NO_3 bioreactor culture conducted by Pretorius *et al*. (2015).

The bioreactor experiment was done duplicated. Error bars representing the standard deviation between duplicates are present and used to assess the reproducibility.

4.4.1. Growth, substrate utilisation and product formation

The growth curve, of the bioreactor culture with optimum nitrogen source and dissolved oxygen levels, in Figure 54 had no lag phase. The exponential phase started directly after inoculation from 0 to 12 hours. A short deceleration phase was observed between the exponential and stationary phase, which started around 24 hours. A maximum CDW of 7.8 g/l was reach at 34 hours.

Glucose was utilised until depletion which occurred at 23 hours. The initial absence of glucose utilisation was possibly due to the fact that residual glucose from the inoculum was used and thus the concentration at the time of inoculation was higher than the medium concentration of 40 g/l. Nitrate was utilised at an almost constant rate until utilisation ceased at 23 hours due to glucose depletion. Ammonium started with a concentration of approximately 901 mg/l which was rapidly utilised in the first 10 hours, after which the rate of utilisation decreased until it ceased at 22 hours where approximately 2 mg/l ammoniums remained.

Dissolved oxygen levels decreased rapidly from 100% to 11% in the first 2 hours and became limiting after 4 hours. Nitrate was utilised as the electron acceptor from 4 hours onward since no oxygen was available. Furthermore, the absence of ammonium from 10 hours onward did not influence growth and cell maintenance, suggesting that nitrate was converted to ammonia and utilised for growth and cell maintenance. This was reflected by the continuous rapid utilisation of nitrate from 4 to 23 hours.

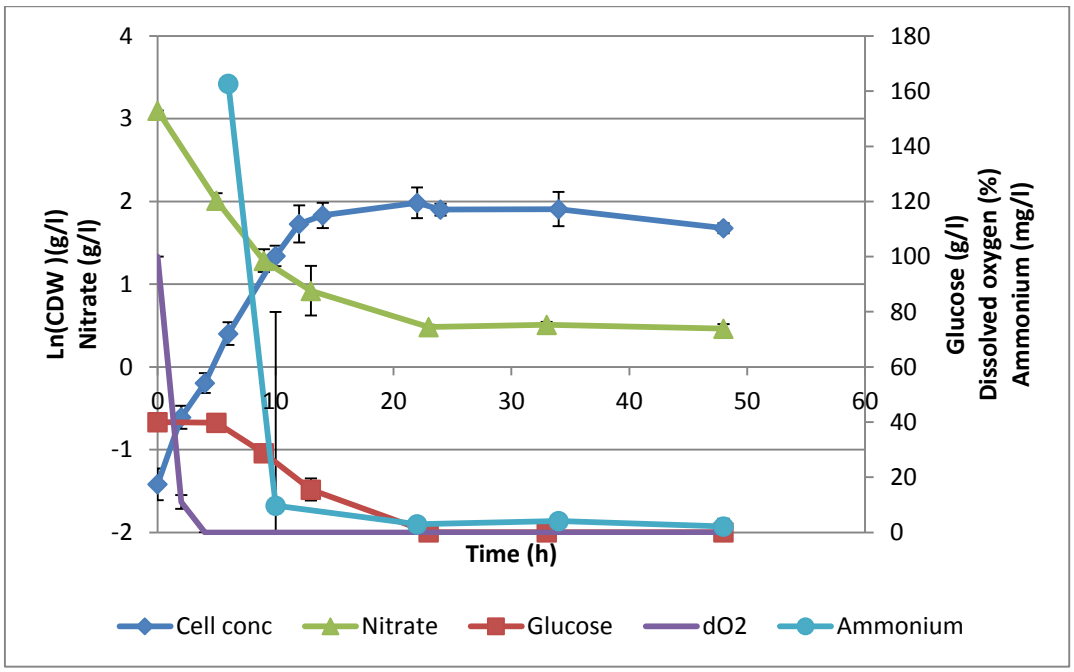

Figure 54: Growth and substrate utilisation of *B. amyloliquefaciens* during controlled batch bioreactor culture with a 0.5:0.5 nitrogen ratio from ammonium and nitrate sources respectively and high oxygen levels by continuous air sparging

Lipopeptide production (Figure 55), surfactin and antifungals, achieved a maximum of 897 mg/l and 290.17 mAU*min at 13 hours. Surfactin production decreased rapidly from 13 to 23 hours, after which it continued to decrease but at a much slower rate. Antifungal production also decreased rapidly after 13 hours until it plateaued at 33 hours.

A slight decrease and/or plateauing was expected with surfactin production, as production is associated with the exponential stage rather than the stationary phase. This has been observed in most shake flask experiments, as well as the 0.5:0.5 nitrogen ratio bioreactor culture (refer to Figure 27). The decrease in antifungals was however not expected, as antifungal production typically continues into the stationary phase where it reaches its maximum, as observed from shake flask cultures (refer to Figure 27). The addition of antifoam could possibly have caused some type of interference or detrimental effect to lipopeptides, or an error could have occurred during analytical analysis of the samples. The exact cause is however currently unknown. The maximum production achieved at 13 hours was used for the discussion, however it should be taken into account that the actual antifungal production could well have been higher (based on trends observed from other experiments).

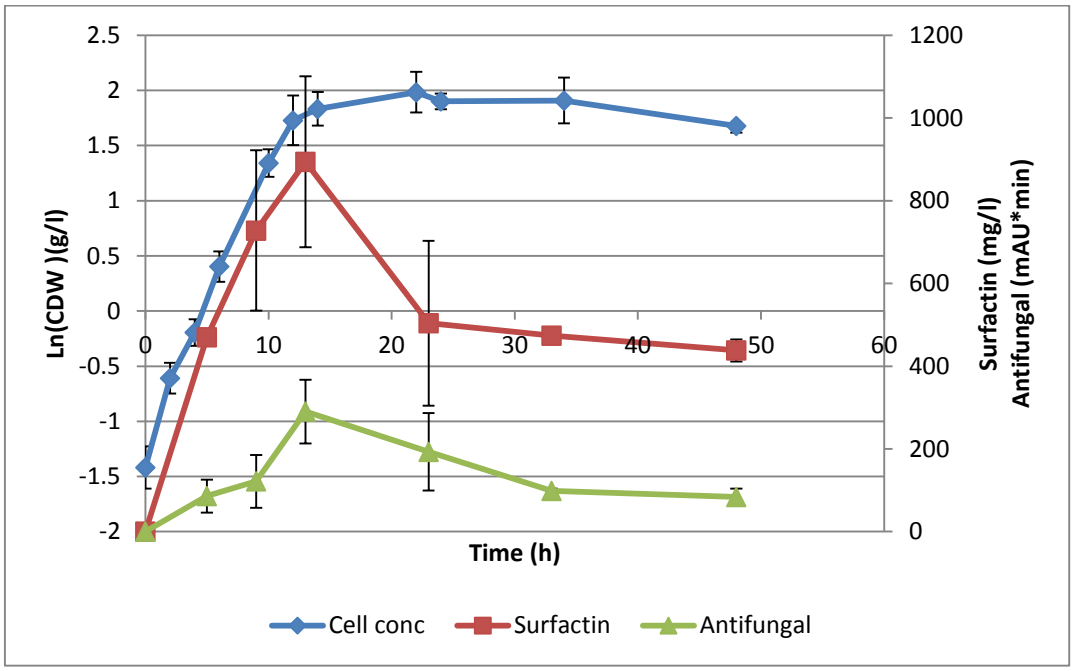

Figure 55: Growth and product formation of *B. amyloliquefaciens* during controlled batch bioreactor culture with a 0.5:0.5 nitrogen ratio from ammonium and nitrate sources respectively and high oxygen levels by continuous air sparging

4.4.2. Comparison of growth and substrate utilisation trends

Growth data between the optimum batch bioreactor cultures are compared in Figure 56 below.

The growth curves followed the same basic trend and exhibited almost identical data points from 24 hours onward. The starting positions differed, however, the 0.5:0.5 nitrogen ratio

bioreactor culture showed no lag phase and started with an optical density of 0.355 and CDW concentration of 0.211 g/l while the 8 g/l NH_4NO_3 culture had a lag phase of 3 hours (thus the low Ln(CDW) starting position), and started with an optical density of 0.092 and CDW concentration of 0.031 g/l. The stationary phase for both experiments started around 24 hours. The 0.5:0.5 nitrogen ratio bioreactor culture showed greater growth values throughout the experiments and achieved a maximum CDW of 7.81 g/l at 34 hours while the 8 g/l NH_4NO_3 culture achieved a maximum CDW of 6.67 g/l at 24 hours.

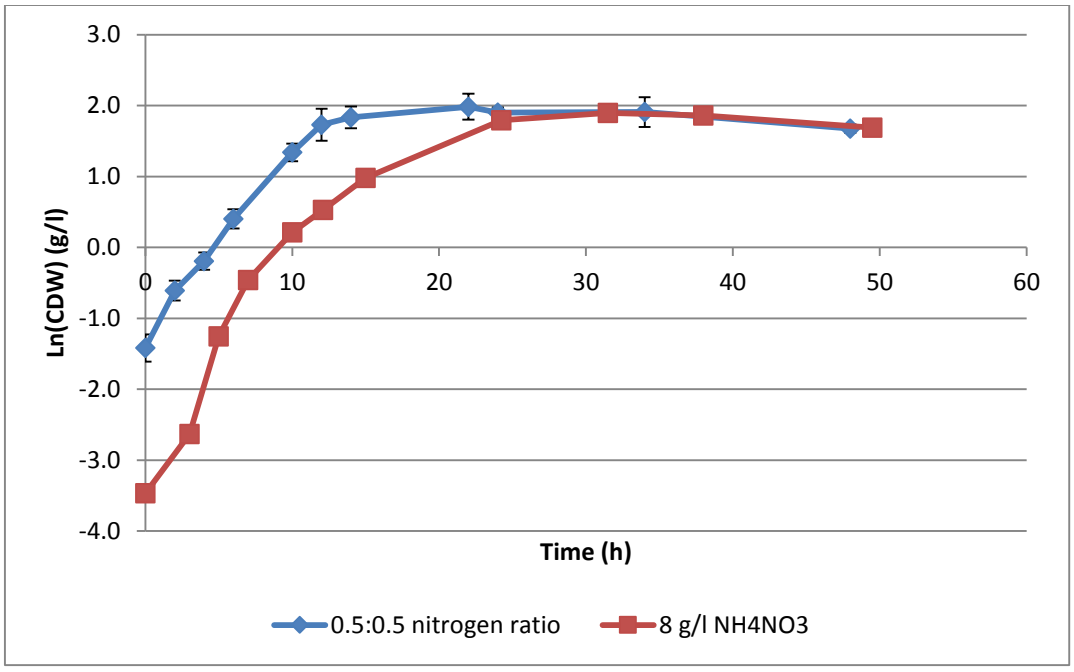

Figure 56: Comparison of *B. amyloliquefaciens* growth with the optimum controlled batch bioreactor cultures. The legends refer to the optimum culture with a 0.5:0.5 nitrogen ratio from ammonium and nitrate sources respectively and the 8 g/l NH_4NO_3 culture from Pretorius *et al*. (2015).

The specific growth rates of the optimum batch bioreactor cultures are given in Figure 57 below.

The 0.5:0.5 nitrogen ratio bioreactor culture had an exponential phase of 12 hours with a maximum specific growth rate of 0.256 h^{-1} while the 8 g/l NH_4NO_3 culture had a much shorter exponential phase of only 4 hours. However, the 8 g/l NH_4NO_3 culture had a maximum specific growth rate double that of the 0.5:0.5 nitrogen ratio bioreactor culture at 0.543 h^{-1}. Both exhibited high R^2 values, indicating good linear fit between the exponential phase data points.

The growth rate of the 0.5:0.5 nitrogen ratio bioreactor culture was higher than both the 4 g/l and 8 g/l NH_4NO_3 bioreactor cultures conducted in this study (refer to section 4.1), which achieved a maximum specific growth rate of 0.237 h^{-1} and 0.190 h^{-1}.

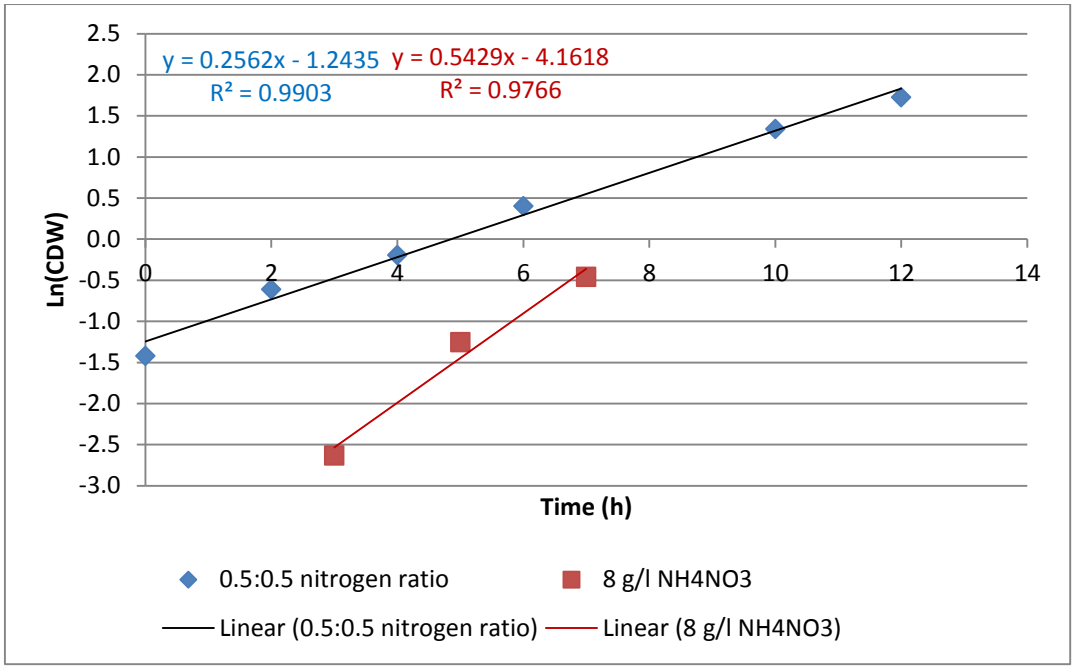

Figure 57: Comparison of *B. amyloliquefaciens* growth rates with the optimum controlled batch bioreactor cultures. The legends refer to the optimum culture with a 0.5:0.5 nitrogen ratio from ammonium and nitrate sources respectively and the 8 g/l NH_4NO_3 culture from Pretorius *et al*. (2015).

Glucose utilisation of the optimum batch bioreactor cultures are compared in Figure 58 below.

The 0.5:0.5 nitrogen ratio bioreactor culture utilised glucose much more rapidly and until depletion at 23 hours. The 8 g/l NH_4NO_3 culture only depleted glucose around 49 hours, regardless of also reaching the stationary phase at 23 hours.

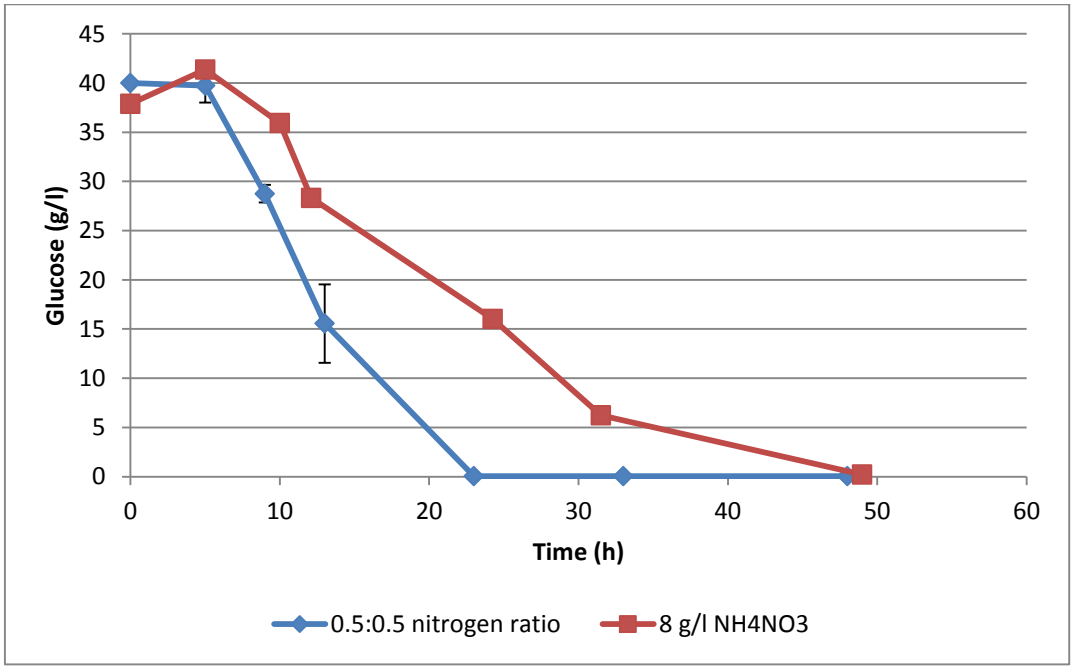

Figure 58: Comparison of *B. amyloliquefaciens* glucose utilisation with the optimum controlled batch bioreactor cultures. The legends refer to the optimum culture with a 0.5:0.5 nitrogen ratio from ammonium and nitrate sources respectively and the 8 g/l NH_4NO_3 culture from Pretorius *et al*. (2015).

Nitrate utilisation of the optimum batch bioreactor cultures are compared in Figure 59 below.

Nitrate utilisation for the 0.5:0.5 nitrogen ratio bioreactor culture ranged from the start to 23 hours, where utilisation ceased due to glucose being depleted and the stationary phase started. The same was not the case with the 8 g/l NH_4NO_3 culture, utilisation ranged from 10 to 49 hours where it became depleted.

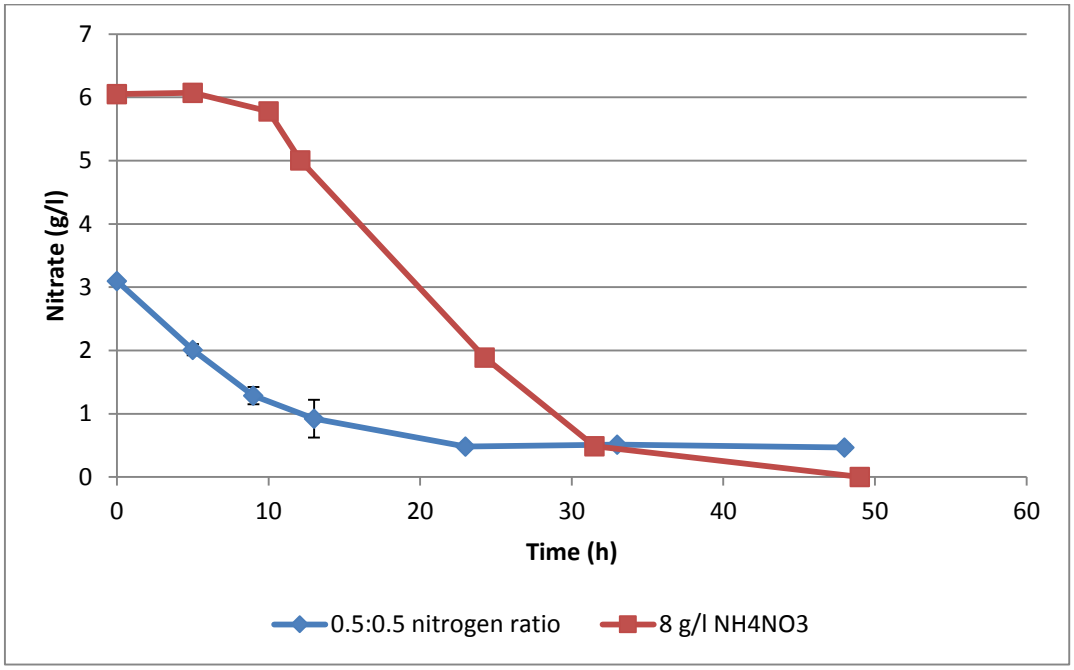

Figure 59: Comparison of *B. amyloliquefaciens* nitrate utilisation with the optimum controlled batch bioreactor cultures. The legends refer to the optimum culture with a 0.5:0.5 nitrogen ratio from ammonium and nitrate sources respectively and the 8 g/l NH_4NO_3 culture from Pretorius *et al*. (2015).

4.4.3. Comparison of lipopeptide production trends

Surfactin production profiles of the optimum batch bioreactor cultures are given in Figure 60 below.

The 0.5:0.5 nitrogen ratio bioreactor culture outperformed the 8 g/l NH_4NO_3 culture, achieving a maximum surfactin concentration of 894 mg/l compared to 302 mg/l. The production trends were also very different. The 0.5:0.5 nitrogen ratio bioreactor culture showed rapid production during the exponential phase until the maximum at 13 hours, after which production decreased. This trend differs somewhat from what has been observed in other experiments, a slight decrease and/or plateauing was expected but not a decrease to the extent observed. The 8 g/l NH_4NO_3 culture on the other hand showed production at an almost constant rate until its maximum at 49 hours. The reason for these distinctly different production trends is unknown.

Similarly, the 0.5:0.5 nitrogen ratio bioreactor culture also outperformed both the 4 g/l and 8 g/l NH_4NO_3 batch bioreactor cultures, conducted in this study, where a maximum of 626 mg/l and 450 mg/l surfactin was achieved. This showed that, as with shake flasks, the use of NH_4Cl and $NaNO_3$ outperforms NH_4NO_3 at the same nitrogen ratio (refer to Appendix 2).

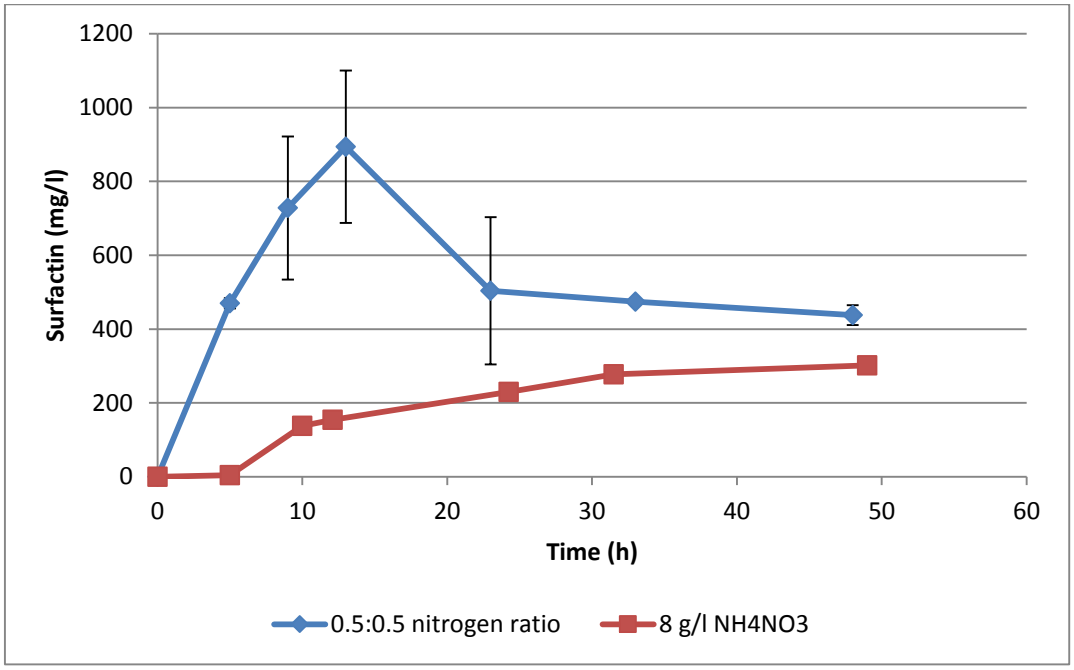

Figure 60: Comparison of surfactin production by *B. amyloliquefaciens* with the optimum controlled batch bioreactor cultures. The legends refer to the optimum culture with a 0.5:0.5 nitrogen ratio from ammonium and nitrate sources respectively and the 8 g/l NH_4NO_3 culture from Pretorius *et al*. (2015).

The antifungal production trends of Figure 61 were quite different. The 0.5:0.5 nitrogen ratio bioreactor culture increased in concentration until a maximum of 290.17 mAU*min at 13 hours, while the 8 g/l NH_4NO_3 culture increased in concentration throughout the experiment until its maximum of 285.66 mAU*min at 49 hours.

The maximum antifungal concentration achieved with the 0.5:0.5 nitrogen ratio bioreactor culture was slightly higher than both the 4 g/l and 8 g/l NH_4NO_3 batch bioreactor cultures of this study, where a maximum of 247 mAU*min and 285.7 mAU*min was achieved. This slight increase in antifungal concentration is not significant, however, it should be noted that the maximum concentration could well be greater if production followed the expected trend observed from other experiments). As with surfactin, the use of NH_4Cl and $NaNO_3$ as opposed to NH_4NO_3 results in greater production (refer to Appendix 2).

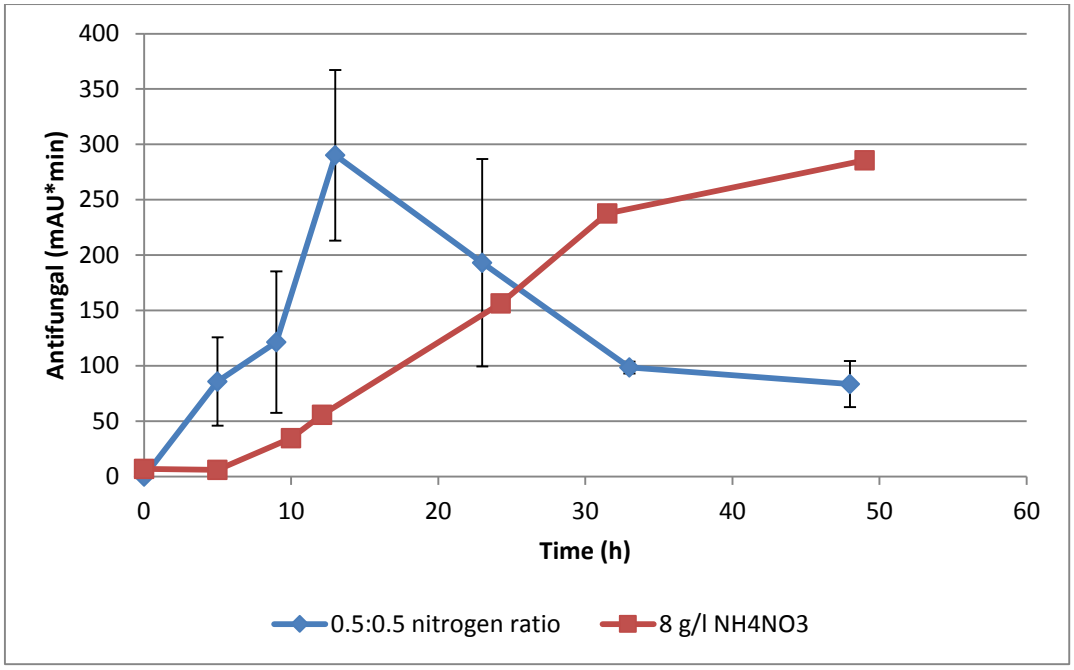

Figure 61: Comparison of antifungal production by *B. amyloliquefaciens* with the optimum controlled batch bioreactor cultures. The legends refer to the optimum culture with a 0.5:0.5 nitrogen ratio from ammonium and nitrate sources respectively and the 8 g/l NH_4NO_3 culture from Pretorius *et al*. (2015).

4.4.4. Comparison of normalised cell and lipopeptide concentrations and associated kinetic parameters

The range of kinetic parameters used throughout this study was once again used to assess the process performance of the optimum batch bioreactor cultures. These kinetic parameters were calculated at the point of maximum antifungal production and are given in Figure 62 below.

The 0.5:0.5 nitrogen ratio bioreactor culture outperformed the 8 g/l NH_4NO_3 culture of Pretorius *et al*. (2015) in almost all parameters, with the exception of the growth kinetics, maximum specific growth rate and CDW. Cell and lipopeptide yield was higher in the 0.5:0.5 nitrate ratio bioreactor culture than in the 8 g/l NH_4NO_3 culture.

The optimum bioreactor cultures achieved similar maximum antifungal concentrations, the 0.5:0.5 nitrogen ratio bioreactor culture leading slightly (290.171 vs 285.66 mAU*min). The 0.5:0.5 nitrogen ratio bioreactor culture outperformed the 8 g/l NH_4NO_3 culture with regards to maximum surfactin concentration. The specific antifungal production ($Y_{p/x}$) achieved was 58.08 and 51.85 mAU*min/g/l for the 0.5:0.5 nitrogen ratio bioreactor culture and the 8 g/l NH_4NO_3 culture. The specific surfactin production ($Y_{p/x}$) was much greater with the 0.5:0.5 nitrogen ratio bioreactor culture than with the 8 g/l NH_4NO_3 culture (0.179 vs. 0.056).

Antifungal productivity was much greater with the 0.5:0.5 nitrogen ratio bioreactor culture (22.32 mAU*min/h) compared to the 8 g/l NH_4NO_3 culture (5.69 mAU*min/h). Similarly, surfactin productivity was greater with the 0.5:0.5 nitrogen ratio bioreactor culture (68.77 g/l/h) compared to the 8 g/l NH_4NO_3 culture (6.13 g/l/h).

These kinetic parameters conclude that the 0.5:0.5 nitrogen ratio bioreactor culture outperformed the previously identified optimum bioreactor culture (8 g/l NH_4NO_3) over all lipopeptide production related criteria.

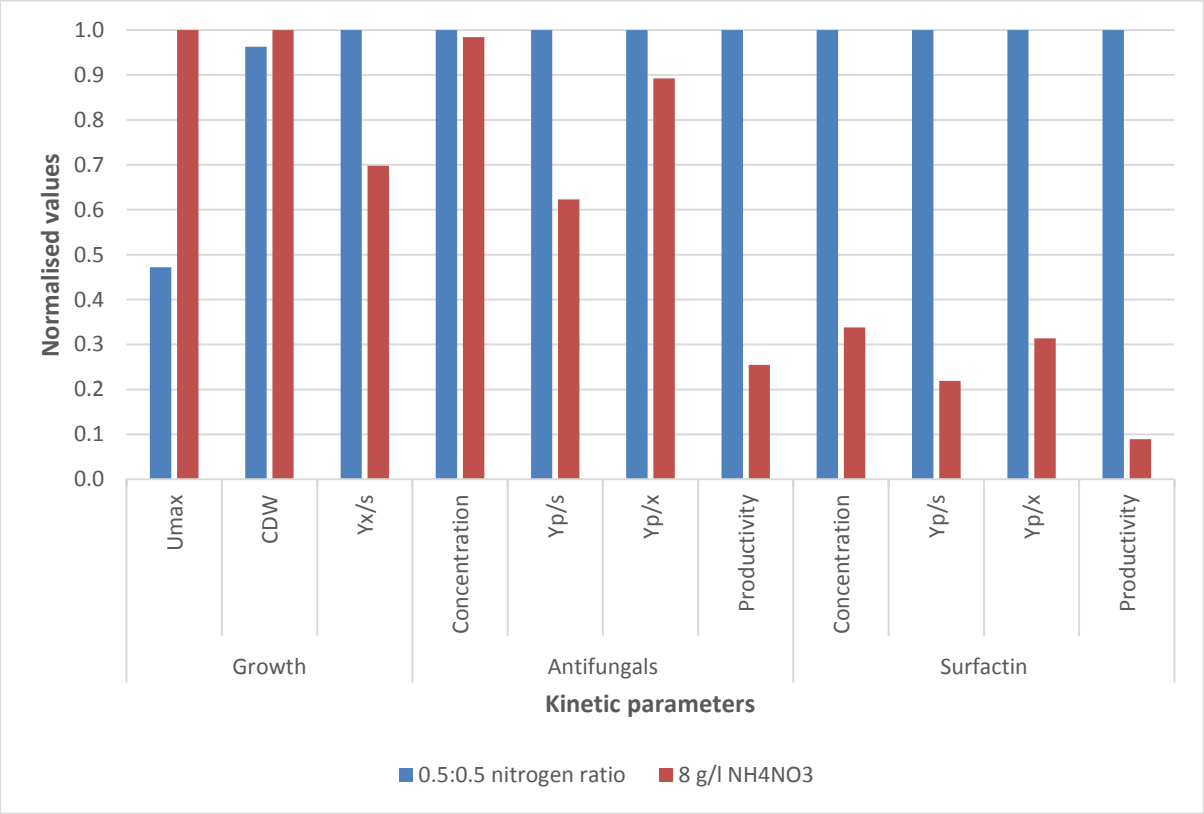

Figure 62: Normalised *B. amyloliquefaciens* growth and lipopeptide related kinetic parameters at maximum antifungal concentration with the optimum controlled batch bioreactor cultures. The legends refer to the optimum culture with a 0.5:0.5 nitrogen ratio from ammonium and nitrate sources respectively and the 8 g/l NH_4NO_3 culture from Pretorius *et al*. (2015).

4.5. Antifungal lipopeptide efficacy against phytopathogens

Antifungal efficacy was tested against six known phytopathogens with cultured supernatant taken from the 4 g/l NH_4NO_3 culture and the culture containing only nitrate ($NaNO_3$ with sufficient oxygen). The culture containing only nitrate was used instead of the 0.5:0.5 nitrogen ratio (optimum) culture since it achieved a slightly higher maximum antifungal concentration. Based on the antifungal lipopeptide production trends, the 0.5:0.5 nitrogen ratio culture is expected to achieve similar efficacy results as the culture containing only nitrate.

Total antifungal concentration was the basis for the antifungal efficacy tests as homologue production and their ratios were not investigated. It is hypothesised that different homologues would have different efficacy against phytopathogens. However, the large number of homologues that have been reported, around 100 (Pretorius et al. 2015), would warrant a substantial amount of research and several more studies. Nevertheless, the effect of different homologues on antifungal efficacy is an important concept that could potentially lead to a bespoke future product.

Supernatant samples were taken from shake flask cultures and underwent acid precipitation for concentration and partial purification. Shake flasks were used instead of bioreactor cultures as no precipitation was achieved with bioreactor cultures, possibly due to the interference of antifoam. The partially purified supernatant was added to the three wells labelled “1”, “2” and “3” while water was added to the forth well labelled “C” to act as the control.

The six phytopathogens considered were *Botrytis cinerea, Alternaria brassicicola, Aspergillus sclerotiorum, Monilinia fructigena, Penicillium expansum* and *Rhizopus stolonifer*

4.5.1. Efficacy against *Botrytis cinerea*

The antifungal efficacy test in Figure 63 showed growth inhibition around the wells containing supernatant for both cases. The fungus *B. cinerea* grew away from these wells toward and over the control well. Biological production of antifungal lipopeptides was effective in inhibiting the growth of *B. cinerea.*

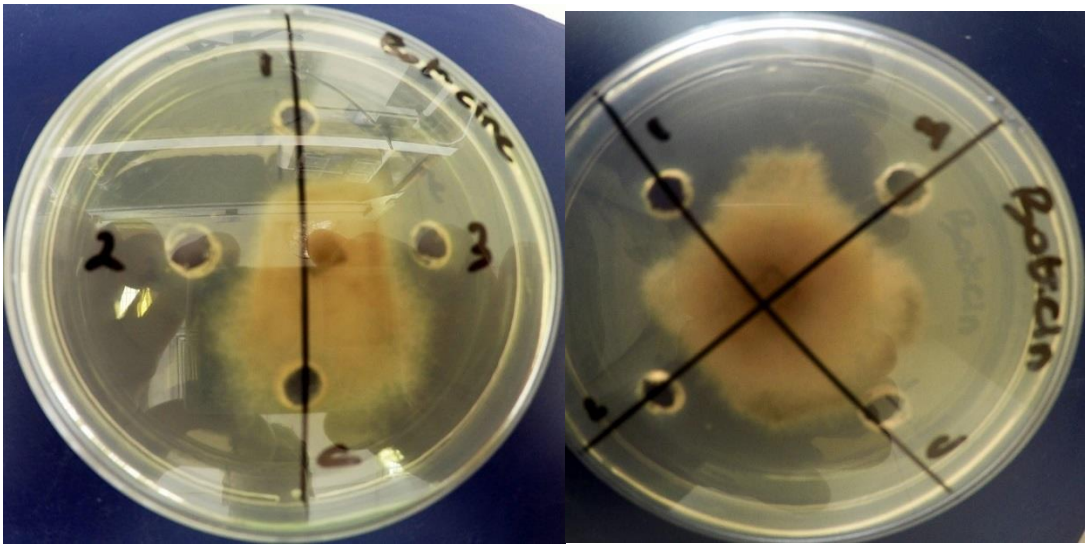

Figure 63: Antifungal efficacy against *B. cinerea* on PDA plates from a) 4 g/l NH_4NO_3 b) culture containing only nitrate. Well “C” filled with water to act as control, all other wells (1 to 3) filled with supernatant

4.5.2. Efficacy against *Alternaria brassicicola*

The antifungal efficacy test in Figure 64 showed growth inhibition in the sense that *A. brassicicola* grew toward the control well (C) instead of towards the wells containing supernatant.

The growth rate of *A. brassicicola* was much slower than that of *B. cinerea* and thus took longer to reach the wells. The efficacy test showed that antifungal lipopeptides were effective against A. *brassicicola.*

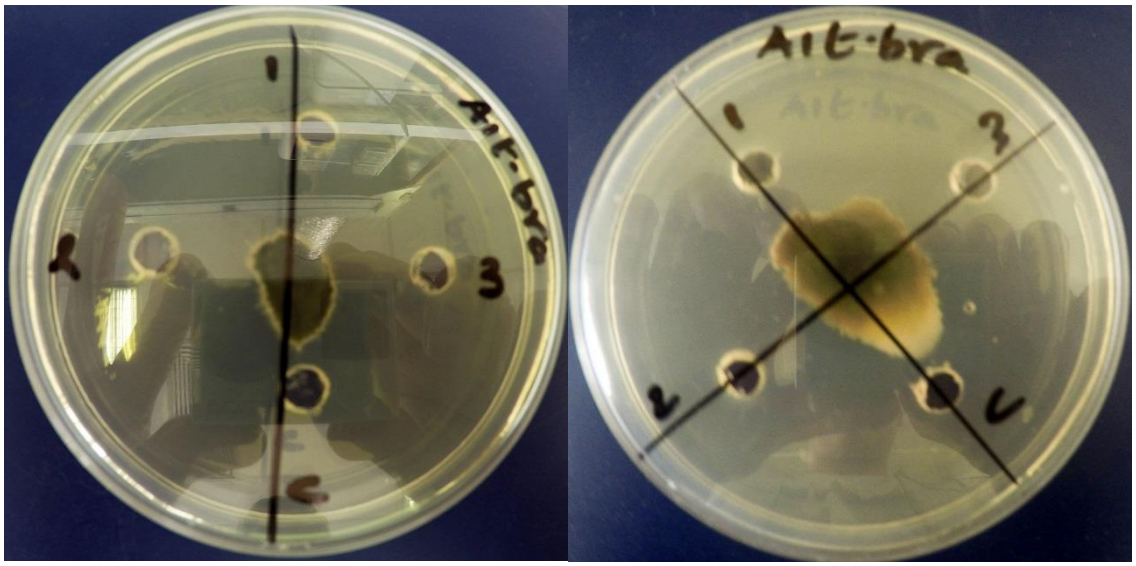

Figure 64: Antifungal efficacy against *A. brassicicola* on PDA plates from a) 4 g/l NH_4NO_3 b) culture containing only nitrate. Well "C" filled with water to act as control, all other wells (1 to 3) filled with supernatant

4.5.3. Efficacy against *Aspergillus sclerotiorum*

The antifungal efficacy test in Figure 65 did not show growth inhibition around or close to the wells containing supernatant. The efficacy test with 4 g/l NH_4NO_3 showed that the fungus grew more towards the control well (C), hinting at potential inhibition, but also came in contact with the third well. The efficacy test from the culture containing only nitrate showed radial fungus grew, encountering well two and three and the control well.

The antifungal lipopeptides did not seem to be effective in inhibiting *A. sclerotiorum* growth. This could indicate that the required antifungal homologues that are effective against the fungus were present in inadequate concentrations, or not at all. It could also be that the lipopeptides are simply not effective against *A. sclerotiorum.*

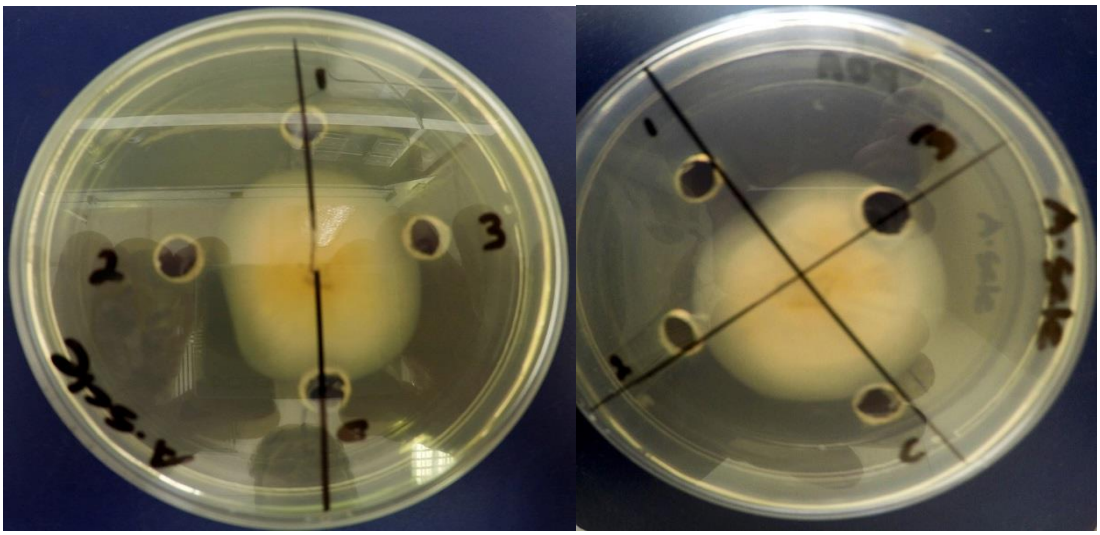

Figure 65: Antifungal efficacy against *A. sclerotiorum* on PDA plates from a) 4 g/l NH_4NO_3 b) culture containing only nitrate. Well "C" filled with water to act as control, all other wells (1 to 3) filled with supernatant

4.5.4. Efficacy against R*hizopus stolonifer*

The antifungal efficacy test of Figure 66 showed fungal inhibition around the wells containing supernatant. *R. stolonifer* grew in a filamentous manner, spreading over the entire plate.

A small inhibition zone was present around each of the three supernatant wells. This showed that antifungal lipopeptides are effective in inhibiting the growth of *R. stolonifer.*

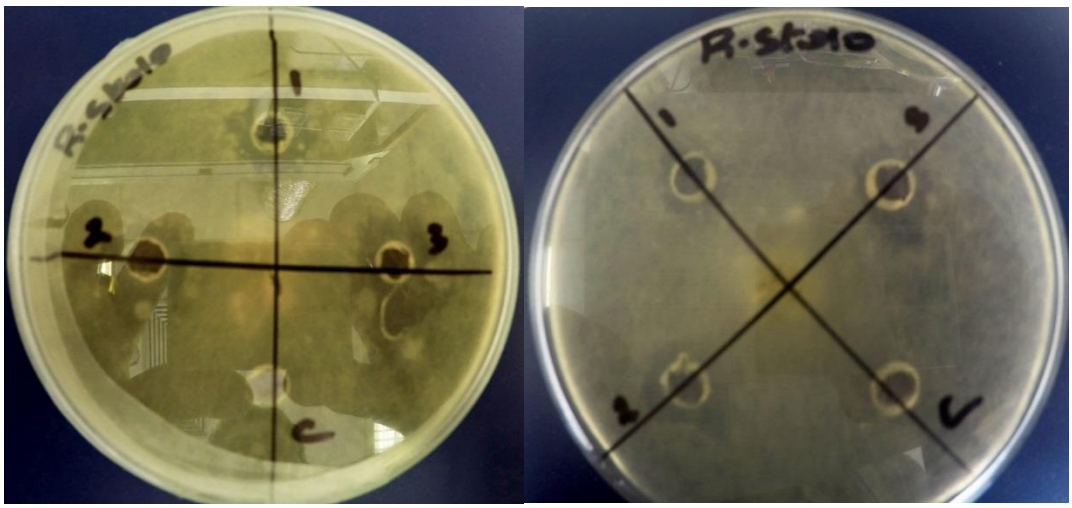

Figure 66: Antifungal efficacy against *R. stolonifer* on PDA plates from a) 4 g/l NH_4NO_3 b) culture containing only nitrate. Well "C" filled with water to act as control, all other wells (1 to 3) filled with supernatant

4.5.5. Efficacy against *Monilinia fructigena*

Inhibition was shown on the antifungal efficacy test in Figure 67. In the 4 g/l NH_4NO_3 plate, the greatest distribution of *M. fructigena* growth was close to and around the control well. This was

not the case with the culture containing only nitrate as growth is shown toward the well containing supernatant.

It is possible that different antifungal homologues and homologue ratios were present in the supernatant used for the efficacy tests, possibly attributed to the different media used. The culture containing 4 g/l NH_4NO_3 may have contained the homologue(s) that influenced *M. fructigena* growth, while the culture containing only nitrate did not, or in inadequate concentrations. Furthermore, the 4 g/l NH_4NO_3 efficacy test may have contained the required homologues at the minimum concentration required to interfere with growth, but did not inhibit it completely as some growth was observed around wells one and two.

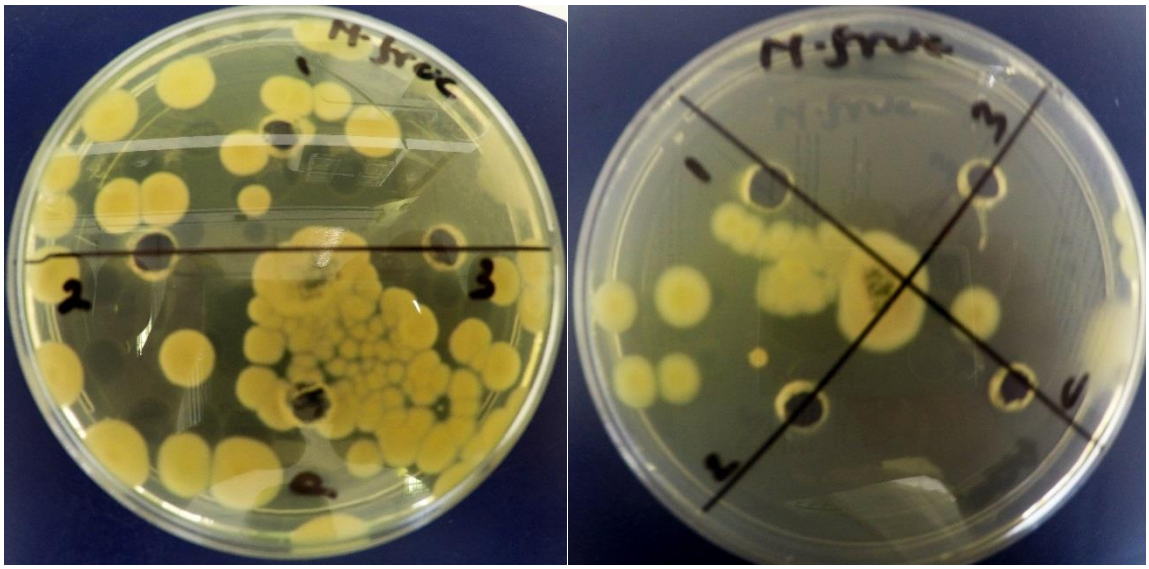

Figure 67: Antifungal efficacy against *M. fructigena* on PDA plates from a) 4 g/l NH_4NO_3 b) culture containing only nitrate. Well "C" filled with water to act as control, all other wells (1 to 3) filled with supernatant

4.5.6. Efficacy against *Penicillium expansum*

Inhibition was similar in Figure 68 as in Figure 67. The greatest distribution of *P. expansum* growth was shown close to and around the control well. In the 4 g/l NH_4NO_3 efficacy test, zones of no growth were present around the wells containing supernatant. A similar growth distribution is expected in the efficacy test conducted with the culture containing only nitrate if it was incubated longer.

The absence of growth around the wells containing supernatant, regardless of growth over the entire plate, showed that the fungus grew where no or low concentrations of antifungal lipopeptides were present. Antifungal lipopeptides were thus shown to be effective against *P. expansum*.

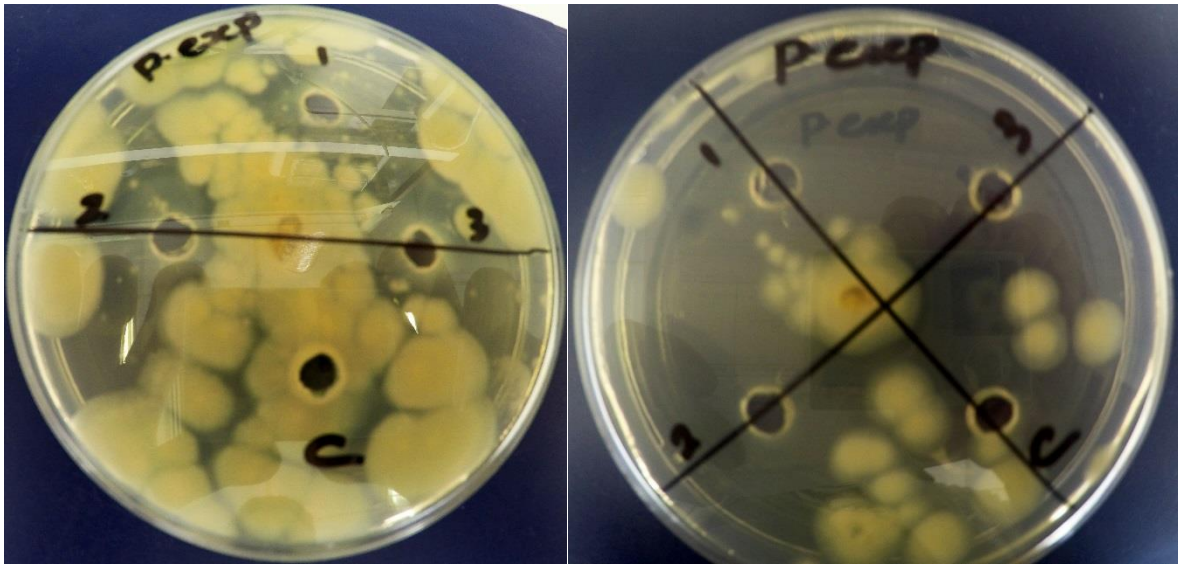

Figure 68: Antifungal efficacy against *P. expansum* on PDA plates from a) 4 g/l NH_4NO_3 b) culture containing only nitrate. Well "C" filled with water to act as control, all other wells (1 to 3) filled with supernatant

4.6. Preliminary investigation into the use of continuous cultures as a tool for rigorous kinetic evaluation

The process performance of continuous cultures, with 4 g/l NH_4NO_3, was investigated to evaluate the method as the potential next step in kinetic optimisation for antifungal lipopeptide production. No duplications were conducted, as such no error bars are present.

4.6.1. Identifying steady state by growth and glucose concentration

The 4 g/l NH_4NO_3 growth curve of Figure 69 is comprised of a batch growth curve for the first 10 hours, after which the configuration was switched to continuous culture by turning on the feed and waste pumps. The batch curve portion showed no lag phase and the exponential phase started directly after inoculation, ranging from 0 to 6 hours. During the continuous configuration of the growth curve the culture reached steady state, confirmed by constant optical density and DNS glucose concentrations, between 170 hours and 198 hours. The average CDW reached at steady state was 3.99 g/l with a standard deviation of 0.0027.

Glucose concentration, as determined by DNS, decreased rapidly during the exponential and deceleration phases (0 to 30 hours) which was the time period where rapid growth was shown. As the bacteria continued towards steady state, glucose utilisation also became more constant. The average residual DNS glucose concentration during steady state was 1.48 g/l with a standard deviation of 0.023.

HPLC, glucose and nitrate, samples were taken during steady state, however no nitrate analysis was done prior to steady state. Nitrate utilisation was expected to be most rapid

during the exponential phase, since the dissolved oxygen became depleted after 2 hours, after which the rate of utilisation would decrease as time progressed until a constant rate was reached during steady state as shown by the constant nitrate concentration in Figure 69.

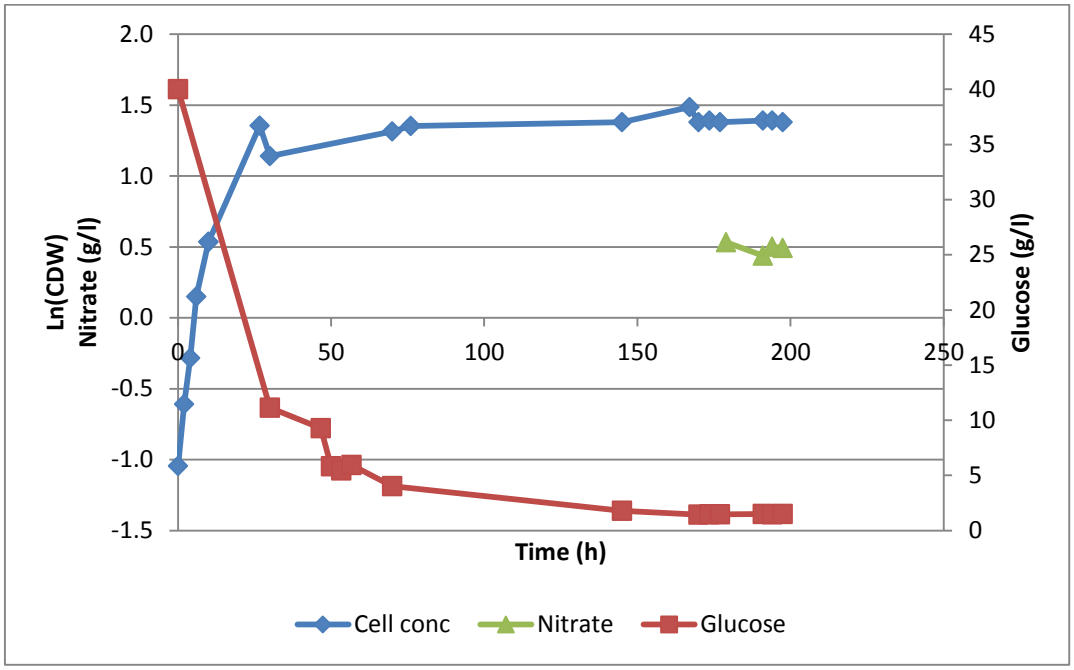

Figure 69: Growth and substrate utilisation of *B. amyloliquefaciens* in 4 g/l controlled continuous bioreactor cultures

4.6.2. Steady state substrate and product concentrations

The substrate and product concentrations during steady state are given in Table 11 below.

Steady state at a dilution rate of 0.1 was reached from 170 hours. Samples for glucose, nitrate and lipopeptides were taken from 179 hours onward, after steady state was confirmed by optical density and DNS glucose concentrations.

The average steady state glucose concentrations were 0.1 g/l with a standard deviation of 0.0363, while the average DNS concentrations were around 1.49 g/l with a standard deviation of 0.013. This difference was expected as different methods were used, with DNS being less sensitive as it relies on colorimetric analysis. The average steady state surfactin concentration was 115 mg/l with a standard deviation of 18.51. Similarly, the average steady state antifungal concentration was 66.191 mAU*min with a standard deviation of 18.825.

Table 11: Substrate and product concentrations during steady state of the 4 g/l controlled continuous bioreactor culture at a dilution rate of 0.1

Time	Glucose (g/l)	Nitrate (g/l)	Surfactin (mg/l)	Antifungals (mAU*min)
0	40	4	0	0
179	0.050	0.533	102	67.23
191	0.130	0.439	100	41.03
194	0.091	0.502	140	69.94
197.5	0.122	0.492	118	86.56

These results show that continuous cultures can be used for rigorous kinetic evaluation and optimisation, however, continuous cultures have their own set of challenges that need to be addressed and overcome. The risk and likelihood of contamination is greater than with other production strategies as more equipment and higher volumes of nutrients need to be sterilised and kept sterile. Furthermore, it was observed that the cultured bacteria (*B. amyloliquefaciens*) would spread through the feed tube and, if left unchecked, would contaminate the feed reservoir, thereby decreasing the concentration of nutrients being supplied to the bioreactor. Lipopeptide production was also much lower than with shake flaks or batch bioreactor cultures, which makes continuous cultures as a production strategy unadvised.

4.7. Experimental repeatability

Error bars were used to indicate the level of reproducibility between duplicate and triplicate experiments. The size of these error bars reflected the standard deviation of the experiments from the mean. The experimental repeatability of the 4 g/l NH_4NO_3 shake flask and 8 g/l batch bioreactor experiments were assessed at the time where maximum antifungal production was achieved, and given in Figure 70. The repeatability was calculated by Equation 7.

Equation 7: Experimental repeatability

$$repeatability = \left(1 - \frac{standard\ deviation}{average}\right) \times 100\%$$

The specific growth rate and CDW showed repeatability above 90% for both shake flask and batch bioreactor cultures, indicating that these two parameters were statistically repeatable. The cell yield parameter ($Y_{x/s}$) showed high repeatability with the shake flask culture and significantly lower repeatability with the batch bioreactor culture. This was due to the fact that the parameter is dependent on both CDW and the glucose concentration at the specific point. The large difference in glucose concentrations at similar CDW concentrations was responsible for the poor repeatability of $Y_{x/s}$ in the batch bioreactor culture.

The maximum antifungal concentration repeatability for the batch bioreactor culture was very high, above 90%, while the shake flask cultures was acceptable at 65%. The large difference in repeatability between the two was due to the fact that one of the triplicate shake flasks achieved a maximum antifungal concentration of 560 mAU*min while the other two were closer to one another (370 and 285 mAU*min). The different maximum antifungal concentration could possibly be due to differences in nitrate utilisation. More nitrate was utilised in the culture that achieved 560 mAU*min than in the other two, thus more nitrate would have been available for lipopeptide production.

The lipopeptide results exhibited higher variance than CDW, glucose and nitrate throughout most of the experiments. This may be due to a difference in area/concentration ratios of the different lipopeptide homologues. The assumption that these ratios are constant for all homologues may not be correct. The large number of homologues that exist, more than 100, along with the absence of standards that contain all the homologues, makes it difficult to know which homologues are present in samples and in what ratios. Thus, different homologues with different area/concentration ratios may be produced which would result in the variance in lipopeptide results being greater than that in other parameters.

The repeatability of specific lipopeptide production ($Y_{p/x}$) was acceptable for surfactin in both shake flask (73%) and batch bioreactor (66%) cultures. The specific antifungal production was extremely high for the batch bioreactor culture (99%) while it was a little more than half that for the shake flask culture (54%). This was due to the higher antifungal concentration in the one shake flask repeat than in the other, while the CDW concentrations for all triplicates were very similar. The repeatability of lipopeptide production per unit substrate consumed ($Y_{p/s}$) was acceptable (65%) for antifungal and good (84%) for surfactin with the shake flask culture. In the case of batch bioreactor cultures lipopeptide production per unit substrate consumed was slightly below acceptable levels (51%) for antifungal production and poor for surfactin (24%). The reason for the low repeatability was, as mentioned earlier, the difference in glucose concentration between duplicates at the specific time (19.1 and 30.72 g/l).

Productivity repeatability is dependent on lipopeptide production and time. The repeatability was acceptable for antifungals (65%) and good for surfactin (84%) in shake flasks. Both antifungal (93%) and surfactin (73%) productivity was good in batch bioreactor cultures.

The results from Figure 70 indicating that both shake flask and bath bioreactor cultures are repeatable and statistically reliable. The biological nature of the study adds to the difficulty and unpredictability that influences the repeatability.

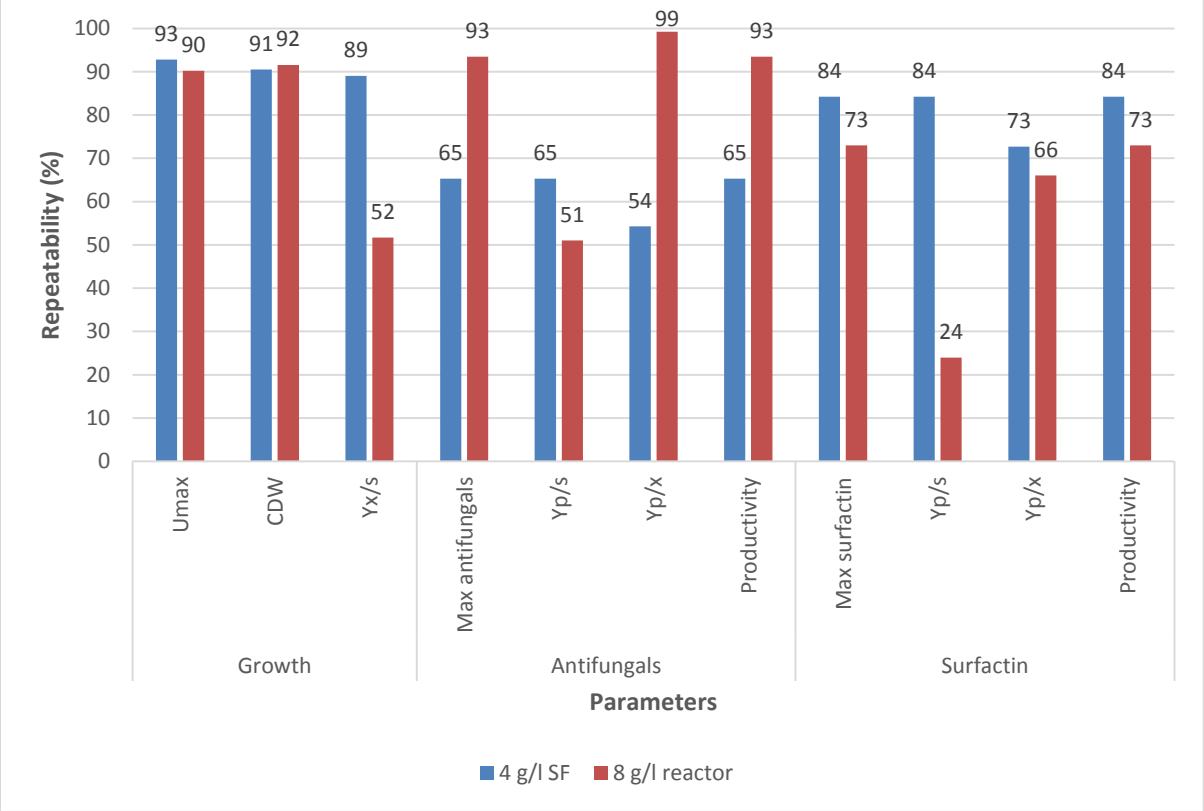

Figure 70: Experimental repeatability of growth and production related kinetic parameters based on shake flask and batch bioreactor cultures with 4 g/l NH_4NO_3

Chapter 5: Conclusions and recommendations

5.1. Conclusions

The project focussed on improving upstream production of antifungal lipopeptides by *B. amyloliquefaciens* through kinetic evaluation for use as biocontrol agents against fungal phytopathogens affecting the fruit industry.

The rigorous kinetic evaluation of identified process conditions, namely nitrogen concentration, nitrogen source and dissolved oxygen, led to improvements in antifungal lipopeptide associated kinetic parameters. An increase from 4 g/l to 8 g/l NH_4NO_3 improved antifungal lipopeptide production and selectivity. The use of two distinct nitrogen sources (NH_4Cl and $NaNO_3$) improved antifungal lipopeptide production and selectivity with the optimum 0.5:0.5 nitrogen ratio. Comparison studies carried out in shake flasks with and without baffles revealed that high oxygen levels favoured increased lipopeptides. Finally, the efficacy studies carried out using partially purified lipopeptide, obtained as acid precipitate of the cell free supernatant, showed growth inhibition against all the test phytopathogens, except *A. sclerotiorum.*

The nitrogen concentration in the medium significantly influenced the process kinetics in terms of growth and antifungal lipopeptide (iturin and fengycin) production, and the selectivity for antifungal lipopeptides relative to the co-produced antibacterial lipopeptides (surfactin).

The increased nitrogen concentration facilitated by 4 g/l to 8 g/l NH_4NO_3 decreased μ_{max}, while CDW and biomass formed per unit substrate consumed ($Y_{x/s}$) increased in shake flask studies and decreased in bioreactor studies. Antifungal production increased at higher NH_4NO_3 concentrations, the antifungal concentration improved from 404.90 to 604.67 mAU*min in shake flasks and from 247.26 to 340.69 mAU*min in bioreactor cultures. Antifungal production decreased significantly after reaching its maximum concentration in the bioreactor cultures, possibly due to some interference/interaction caused by antifoam. The use of 8 g/l in the bioreactor led to greater yields ($Y_{p/s}$, $Y_{p/x}$) and greater antifungal productivity. The selectivity towards antifungals increased slightly between shake flasks (89.8% to 92.0%) and greatly between bioreactor cultures (73.8% to 92.0%).

The nitrogen source in the medium significantly influenced the process kinetics in terms of growth and antifungal lipopeptide production, and the selectivity for antifungal lipopeptides relative to the co-produced antibacterial lipopeptides.

The varying NH_4-N and NO_3-N concentrations showed that ammonium was utilised before nitrate, if both were present. Rapid ammonium utilisation was observed during the first six hours, during which between 85% and 97% ammonium was used. Rapid nitrate utilisation started after the period of rapid ammonium utilisation even though ammonium was not completely utilised.

Ammonium was rapidly utilised during the exponential phase of growth. Furthermore, increasing the ratio of ammonium-to-nitrate from 0.25 to 0.75 increased the μ_{max}. The medium containing only nitrate or ammonium as nitrogen source showed slower growth rates as compared to the medium containing both nitrogen sources, as evident from their very low calculated μ_{max} values of 0.124 h^{-1} and 0.166 h^{-1} respectively. This implied that presence of both nitrogen sources is important to improve the yield of biomass with respect to substrate, $Y_{x/s.}$

In the absence of nitrate, surfactin and antifungal concentration decreased rapidly, after reaching their maximum values, at the end of the exponential phase. This could be attributed to the assimilation of lipopeptide by the bacteria for cell maintenance and survival due to a lack of readily available nitrate source. No change in production trends was observed when ammonium was absent.

Ammonium was thus required for growth and surfactin production, while the presence of nitrate was essential to maintain surfactin concentration, which otherwise would have been consumed by the bacteria, and also to ensure continued antifungal production. The presence of nitrate was more desirable than ammonium during the later stage of production, since it supported the antifungal production despite low biomass growth rates.

The optimum nitrogen source ratio for antifungal production was with the 0.5:0.5 nitrogen ratio as it achieved the second highest maximum antifungal concentration (888.3 mAU*min), highest antifungal productivity (26.92 mAU*min/h), the highest specific antifungal production ($Y_{p/x}$) and competitively high antifungal selectivity (87%). The second best antifungal performance was with the culture containing only nitrate, which achieved the highest maximum antifungal concentration (977.3 mAU*min) and second highest antifungal selectivity (93%), which was very close to the highest antifungal selectivity (0.937) obtained with the 0.75:0.25 nitrogen ratio.

The oxygen availability significantly influenced the process kinetics in terms of growth and antifungal lipopeptide production, and the selectivity for antifungal lipopeptides relative to the co-produced antibacterial lipopeptides.

Under oxygen limiting conditions, such as with unbaffled shake flasks, both ammonium and nitrate were used rapidly from the beginning of the culture. While ammonium was utilised for biomass growth and surfactin production, nitrate supported both the maintenance of surfactin concentration, by preventing assimilation of it by the cells, and antifungal production. Under oxygen depleted conditions, nitrate would have served as an alternative electron acceptor to facilitate anaerobic growth. Surfactin production was low under oxygen limiting conditions when ammonium was absent, further indicating that ammonium was used for surfactin production.

The decrease in dissolved oxygen availability in the cultures, decreased the process performance by reducing the biomass growth, antifungal and surfactin production associated kinetics. Lipopeptide production was enhanced in the presence of oxygen, which is contradictory to what literature (Davis et al. 1999) suggested. Decreasing the dissolved oxygen concentration, decreased the amount of nitrate available for lipopeptide production as it was used as an electron acceptor to facilitate anaerobic growth.

High antifungal selectivity was achieved with both high and low dissolved oxygen levels. The decrease in dissolved oxygen slightly increased antifungal selectivity with the 0.5:0.5 nitrogen ratio culture (87% increased to 90%). The opposite was the case with the culture containing only nitrate (93% to 85%).

Kinetics under controlled conditions with optimum nitrogen source and dissolved oxygen levels

The optimum nitrogen source (0.5:0.5 ratio) and dissolved oxygen levels (high) under controlled bioreactor cultures were compared with the optimum identified by Pretorius *et al*. (2015), namely 8 g/l NH_4NO_3.

The newly optimised bioreactor culture outperformed that of Pretorius *et al*. (2015) in every kinetic parameter except μ_{max} and CDW. Antifungal productivity improved from 5.69 to 22.32 mAU*min/h while the specific antifungal production improved from 51.85 to 58.08 mAU*min/g/l. Only a slight increase in maximum antifungal concentration (285.66 to 290.17 mAU*min) was achieved, however, it is possible that a higher concertation could have been achieved if the suggested interference of antifoam was

addressed as antifungal (and surfactin) production decreased extensively after the exponential phase.

***B. amyloliquefaciens* produces a culture supernatant containing antifungal lipopeptides effective against phytopathogens prevalent in postharvest fruit.**

Antifungal efficacy tests showed that lipopeptides, in the form of partially purified cell free supernatant, from *B. amyloliquefaciens* were effective in inhibiting fungal growth against almost all tested phytopathogens. The antifungals produced with the culture containing 4 g/l NH_4NO_3 showed inhibition against all fungus species except *A. sclerotiorum.* Similarly, the culture containing only nitrate from $NaNO_3$ showed inhibition against all fungus species except *A. sclerotiorum* and *M. fructigena.* Inhibition against *M. fructigena* with the 4 g/l NH_4NO_3 culture, but not the culture containing only nitrate, suggest different homologues or homologue ratios were present in the supernatant.

Continuous cultures as the potential next step in rigorous kinetic evaluation

A continuous culture, with 4 g/l NH_4NO_3 achieved steady state, as confirmed by optical density and DNS glucose concentrations, after approximately 170 hours. Continuous cultures can thus be utilised as the next step in antifungal kinetic evaluation and optimisation where one parameter can be varied while all others are kept constant.

The use of metabolic products (cell free supernatant), produced by *B. amyloliquefaciens* would allow standardisation and quantification of antifungal lipopeptide products, which could be applied to postharvest fruit in order to control diseases affecting fruit. Furthermore, these products would be exempt from quarantine restrictions as they contain no living organisms, unlike other products that rely on cells and spores for fungal inhibition. The partially purified lipopeptides were highly effective against *B. cinerea, A. brassicicola, M. fructigena, P. expansum* and *R. stolonifer.* The high effectiveness of the antifungal lipopeptides in combination with the kinetic data from this study could be used to produce tailored antifungal products.

5.2. Recommendations

The effect of antifoam on lipopeptide production and downstream purification should be investigated in order to prevent and account for detrimental interaction. An alternative method for handling foaming should also be investigated.

During the colorimetric analysis, used to quantify ammonium concentration, it was observed that the reaction used in the method is temperature sensitive. In order to ensure that the reaction stops and the optical density thus ceases to increase after the required 15-minute waiting time, the test tubes should be quenched in ice cold water. This will also deliver more accurate results between duplicates and triplicates.

In order to improve lipopeptide production even further, the use of fed-batch reactor systems should be investigated. Different exponential feed rates and thus growth rates would need to be evaluated in order to obtain the optimum.

The use of continuous bioreactor cultures for rigorous kinetic evaluation and investigation should be considered. The use of a chemostat, where all parameters are kept constant while varying a single variable, could prove invaluable for further improvements to lipopeptide production.

The effect of different nitrogen sources on homologue production should be investigated as different homologues may have different efficacies against different phytopathogens. This information could be used to more precisely standardise the lipopeptide product to ensure effectiveness against a certain phytopathogen(s).

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Appendices

Appendix 1: Optimisation of the two-stage inoculum

The lag phase occurs when bacteria adapt to a new environment, during which nutrients are used and energy expended to produce enzymes and intermediates necessary for growth and survival. The lag phase is undesirable as it decreases productivity and requires the use of limited nutrients for maintenance, which decreases the concentration of nutrients available for product formation.

The lag phase can be decreased or potentially eliminated by inoculating a culture with an optimised inoculum. An inoculum is optimised by investigating the growth and incubation times to determine the ideal incubation period that would result in an inoculum of highly active cells in the exponential phase. This would allow the culture being inoculated to reach the exponential phase quicker and improve product formation (Shuler and Kargi, 2013). A non-optimised inoculum is one where incubation times that have not been confirmed to be ideal for the specific bacteria is used often resulting in cells that are past the exponential phase.

Inoculum data is based on the average between triplicate experiments, with error bars indicating the standard deviation between them.

- **First stage inoculum**

The first stage (Figure 71) was inoculated from a nutrient agar plate that had been stored at 4°C. A lag phase of approximately 8 hours was observed, which is to be expected as the bacteria on the plate would be less active at the low temperature environment of the fridge than at room temperature.

The exponential phase takes place from 8 to 18 hours. The exponential phase is followed by the deceleration phase and then the stationary phase, which ranges from 24 to 48 hours. The maximum CDW over the triplicates is 8.22 g/l at 48 hours with a standard deviation of 1.3.

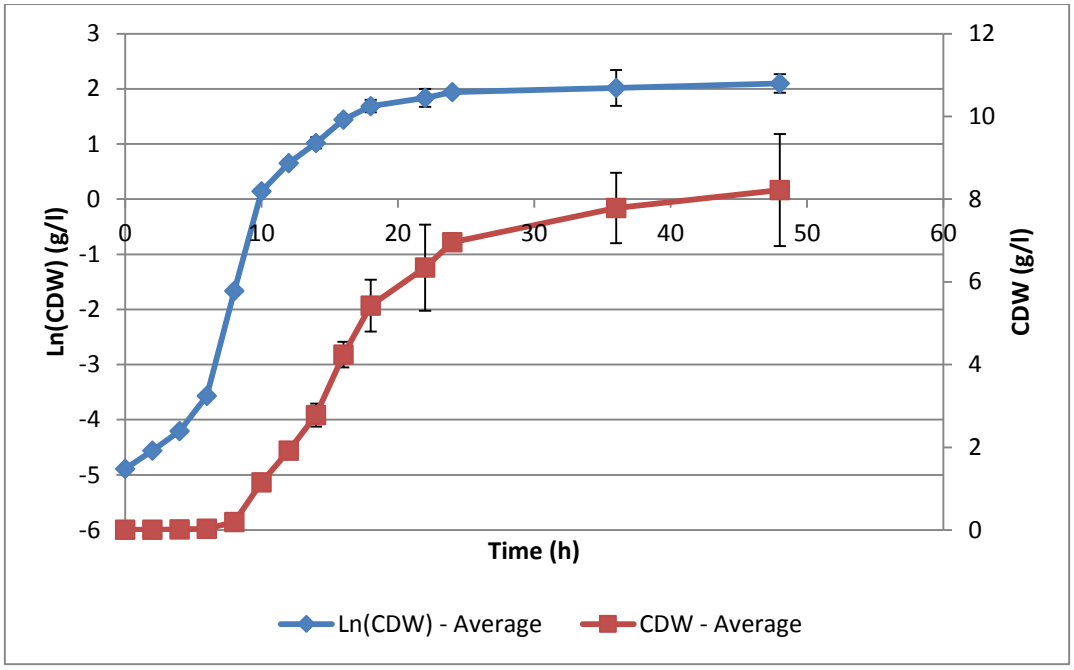

Figure 71: First stage inoculum growth data of *B. amyloliquefaciens*

The maximum specific growth rates (Figure 72) of the triplicates are close to one another with high R^2 values, indicating a good linear fit between the points selected as the exponential phase.

The average maximum specific growth rate between the triplicates is 0.194 h^{-1} with a standard deviation of 1.28 and a R^2 value of 0.988.

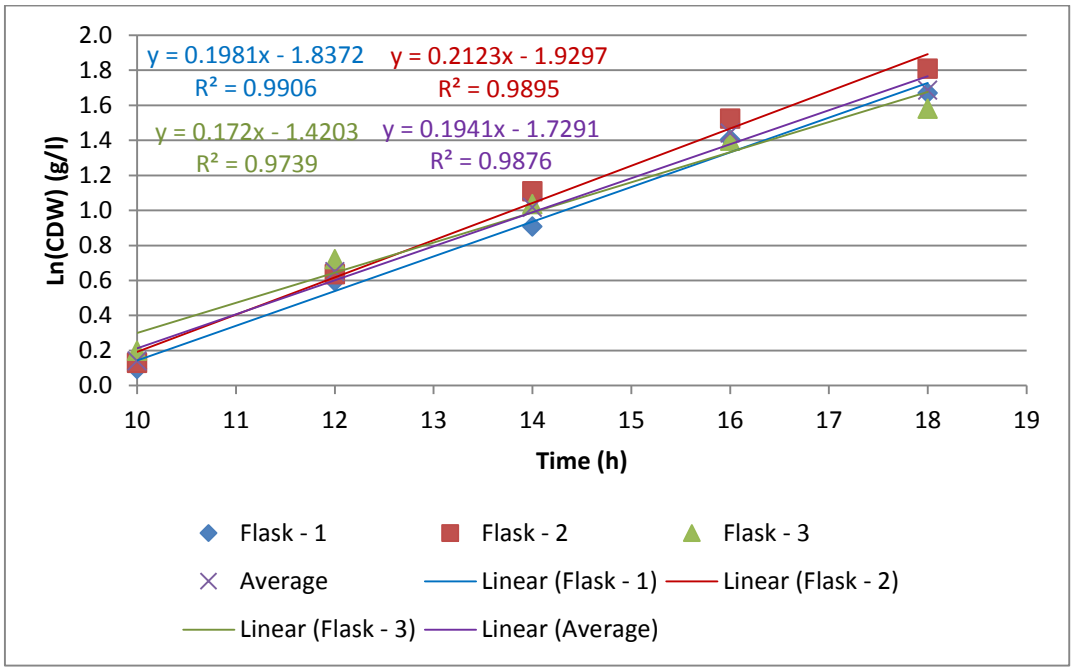

Figure 72: Maximum specific growth rate during 1st stage inoculum *B. amyloliquefaciens inoculum*

The incubation time of the first stage with regard to optical density is evaluated in Figure 73. The first stage should be incubated until the bacteria is in the exponential phase, in order to ensure active cells are used for the inoculation of the second stage. The first stage should be incubated between 10 and 18 hours, or until an optical density between 2.3 and 10 or above is reached, before incubating the second stage.

Error bars were small or insignificant, showing low standard deviation between triplicates and thus displaying good reproducibility. No contamination was present in the inoculum, which was confirmed by checking a sample under the microscope (Zeiss®) with 100x magnification before inoculating the second stage.

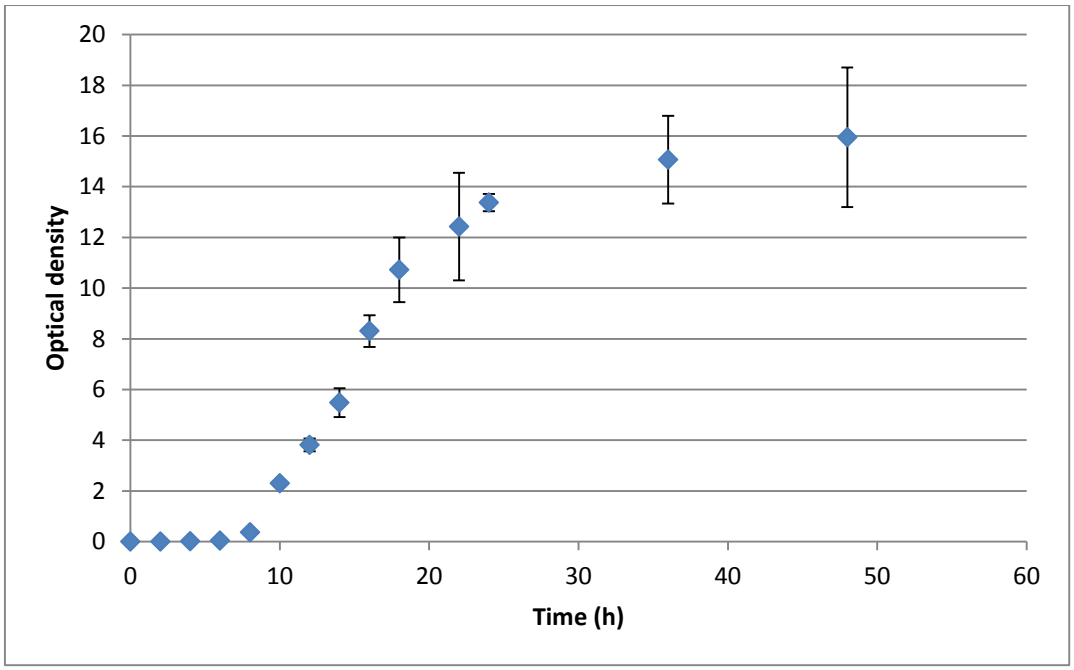

Figure 73: First stage incubation time evaluation for second stage inoculation of *B. amyloliquefaciens*

- **Second stage inoculum**

The second stage inoculum (Figure 74) was inoculated from a first stage inoculum with an optical density of 10, obtained after incubation for 14 hours. The expected optical density after 14 hours of incubation was approximately 5.5. The increase by almost double in optical density is probably due to the fact that the first stage was inoculated from a newly created nutrient agar plate, resulting in the bacteria still being somewhat active and needing less time to adapt to the new environment and thus achieving greater growth.

No notable lag phase was observed, the exponential phase occurs from 0 to 12 hours. Foaming started directly after placing the inoculated second stage in the incubator, which further supports the no lag phase data. The average maximum CDW was found to be 7.24 g/l at 26 hours with a low standard deviation of 0.9 over the triplicates.

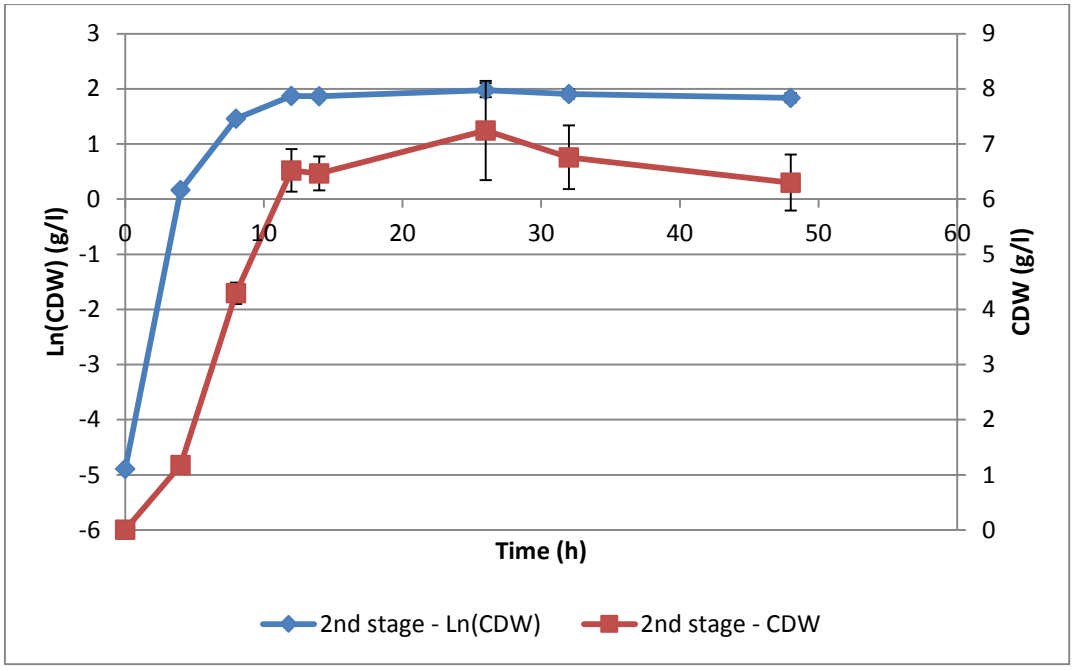

Figure 74: Second stage inoculum growth data of *B. amyloliquefaciens*

The maximum specific growth rates (Figure 75) of the triplicates are close to one another, all with similar R^2 values. The average maximum specific growth rate of the second stage was found to be 0.214 h^{-1} with a R^2 value of 0.9196, showing an acceptable linear fit.

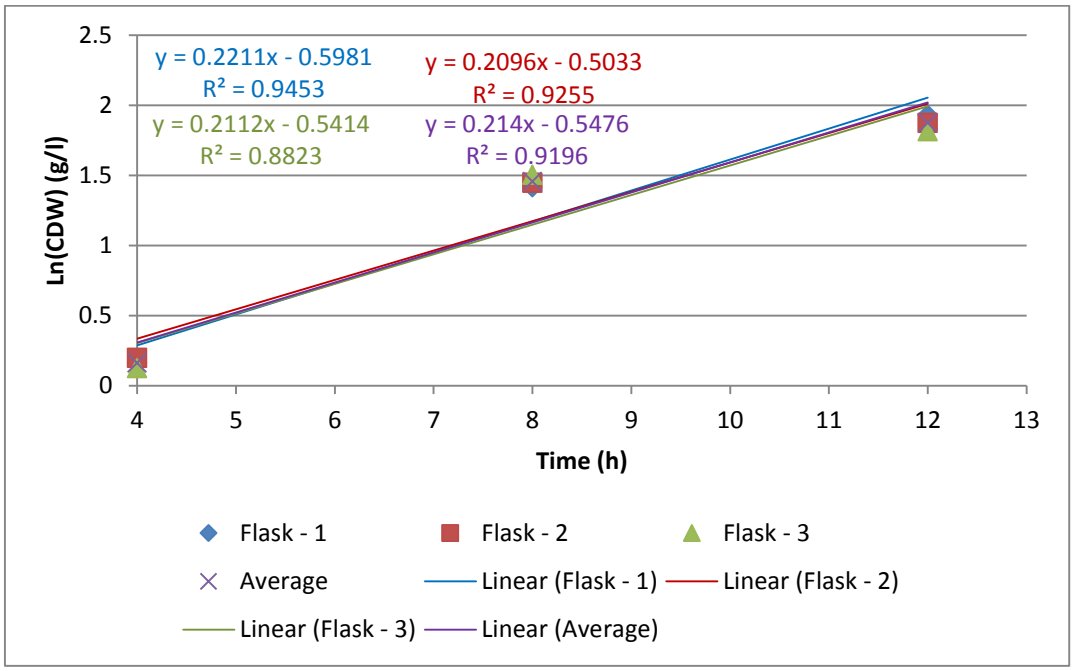

Figure 75: Second stage inoculum exponential phase and μmax of *B. amyloliquefaciens*

The incubation time of the second stage with regard to optical density is evaluated in Figure 76. The second stage should be incubated between 4 and 12 hours, or until an optical density between 2.3 and 12 or above is reached.

The small or unnoticeable error bars show low standard deviation between triplicates and thus indicates good reproducibility. No contamination was present in the inoculum, confirmed by checking a sample from 48 hours under the microscope (Zeiss®) at 100x magnification.

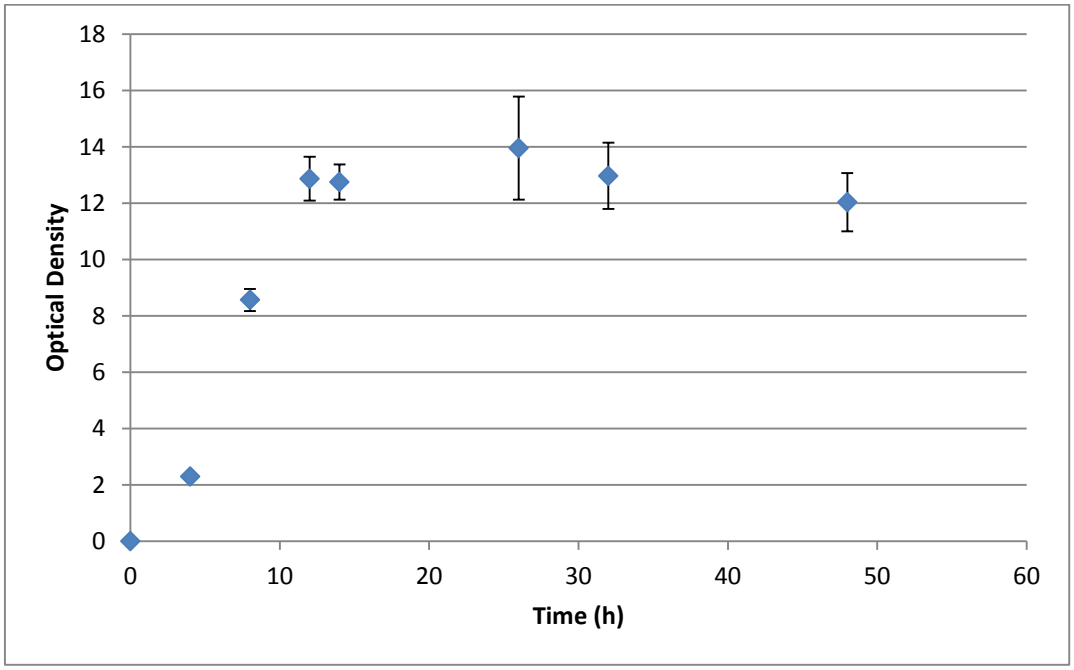

Figure 76: Second stage incubation time evaluation for second stage inoculation of *B. amyloliquefaciens*

- **Growth comparison with optimum and non-optimum inoculums**

The growth curves obtained with both the optimised and non-optimised inocula (Figure 77) follow the same trend expected from Pretorius et al. (2015). The growth curve following from the optimised inoculum started out with a higher optical density value than the non-optimised inoculum (1.22 *vs*. 0.86 respectively), thus higher CDW concentrations and a lag/acceleration phase of two hours, half that of the non-optimised inoculum. The maximum CDW concentration with the optimised inoculum was 7.18 g/l compared to 3.42 g/l with the non-optimised inoculum.

The use of the optimised inoculum increased the starting optical density and decreased the duration of the lag/acceleration phase, which would indirectly benefit lipopeptide production as the exponential phase is reached quicker and less nutrients and energy need to be exhausted to get there.

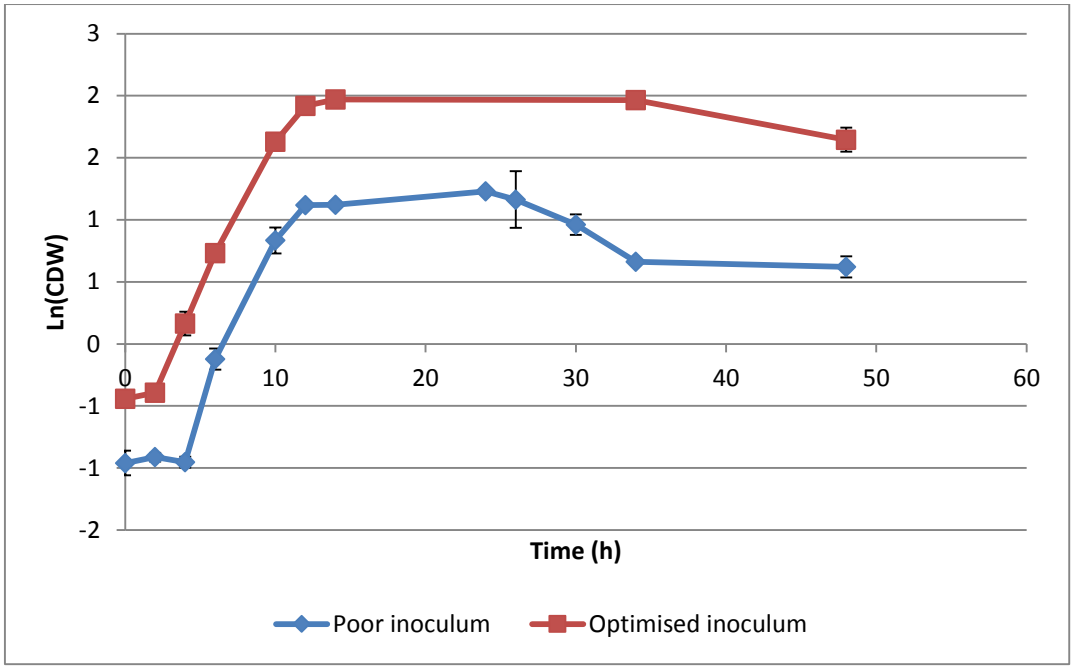

Figure 77: Growth curve of *B. amyloliquefaciens* from optimum and non-optimum 4 g/l inoculum

Appendix 2: Equations and calculations

- **Kinetic parameter equations**

The equations utilised to determine the different kinetic parameters are given below:

The maximum specific growth rate (μ_{max}) was determined by graphical means. A plot of ln(x) *vs*. t was made for the exponential phase, with the slope representing the maximum specific growth rate.

Equation 8: Maximum specific growth rate

$$\ln(x) = \ln(x_0) + \mu t$$

The yield parameters ($Y_{x/s}$, $Y_{p/s}$, $Y_{p/x}$ and productivity) were all calculated in a similar manner. CDW, glucose and lipopeptides concentrations at the start of the culture (time zero) and at a specific sample time after that (time i) were used.

Equation 9: Cell yield per gram substrate

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

Equation 10: Lipopeptide yield per gram substrate

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

Equation 11: Specific lipopeptide production

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

Equation 12: Productivity

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

- **Nitrogen source ratio calculation**

The ratio of nitrogen supplied by ammonium to nitrogen supplied by nitrate was determined to ensure the total nitrogen concentration matches that present in 4 g/l NH_4NO_3.

Determine nitrogen concentration present in 4 g/l NH_4NO_3.

$$[N] = 4\frac{g}{l} NH_4NO_3 \times \left(\frac{Mw_N}{Mw_{NH_4NO_3}}\right)$$

$$[N] = 4\frac{g}{l}NH_4NO_3 \times \left(\frac{14.0067\frac{g}{mol}}{80.052\frac{g}{mol}}\right)$$

$$[N] = 1.4\ g/l$$

Determine ratio of nitrogen supplied by ammonium to nitrogen supplied by nitrate for 4 g/l and 8 g/l NH_4NO_3.

Ammonium:

$$[NH_4] = 4\frac{g}{l}NH_4NO_3 \times \left(\frac{Mw_{NH_4}}{Mw_{NH_4NO_3}}\right)$$

$$[NH_4] = 4\frac{g}{l}NH_4NO_3 \times \left(\frac{18.04\frac{g}{mol}}{80.052\frac{g}{mol}}\right)$$

$$[NH_4] = 0.901\ g/l$$

$$[N] = [NH_4] \times \left(\frac{Mw_N}{Mw_{NH_4}}\right)$$

$$[N] = 0.901\ g\frac{NH_4}{l} \times \left(\frac{14.0067\frac{g}{mol}}{18.04\frac{g}{mol}}\right)$$

$$[N] = 0.7\ g\frac{N}{l}$$

Nitrate:

$$[NO_3] = 4\frac{g}{l}NH_4NO_3 \times \left(\frac{Mw_{NO_3}}{Mw_{NH_4NO_3}}\right)$$

$$[NO_3] = 4\frac{g}{l}NH_4NO_3 \times \left(\frac{62.005\frac{g}{mol}}{80.052\frac{g}{mol}}\right)$$

$$[NO_3] = 3.099\ g/l$$

$$[N] = [NO_3] \times \left(\frac{Mw_N}{Mw_{NO_3}}\right)$$

$$[N] = 3.099\ g\frac{NO_3}{l} \times \left(\frac{14.0067\frac{g}{mol}}{62.005\frac{g}{mol}}\right)$$

$$[N] = 0.7\ g\frac{N}{l}$$

Thus 4 g/l NH_4NO_3 contains nitrogen in an 0.5:0.5 ratio supplied by ammonium and nitrate. Increasing the concentration to 8 g/l NH_4NO_3, does not change the ratio, the amount of both ammonium and nitrate simply doubles.

Determining concentration of ammonium and nitrate required to make desired ratio

Using the 0.5:0.5 nitrogen ratio for illustration purposes

$$[NH_4] = (Fraction\ \times [N]) \times \left(\frac{Mw_{NH_4}}{Mw_N}\right)$$

$$[NH_4] = \left(0.5\ \times 1.4\ g\frac{N}{l}\right) \times \left(\frac{18.04\frac{g}{mol}}{14.0067\frac{g}{mol}}\right)$$

$$[NH4] = 0.901\ g/l$$

$$[NO_3] = (Fraction\ \times [N]) \times \left(\frac{Mw_{NO_3}}{Mw_N}\right)$$

$$[NO_3] = \left(0.5\ \times 1.4\ g\frac{N}{l}\right) \times \left(\frac{62.005\frac{g}{mol}}{14.0067\frac{g}{mol}}\right)$$

$$[NO_3] = 3.099\ g/l$$

Appendix 3: Standard curves

The standard curves for CDW, glucose, nitrate ammonium and surfactin are given below.

The CDW standard curve is given in Figure 78 below.

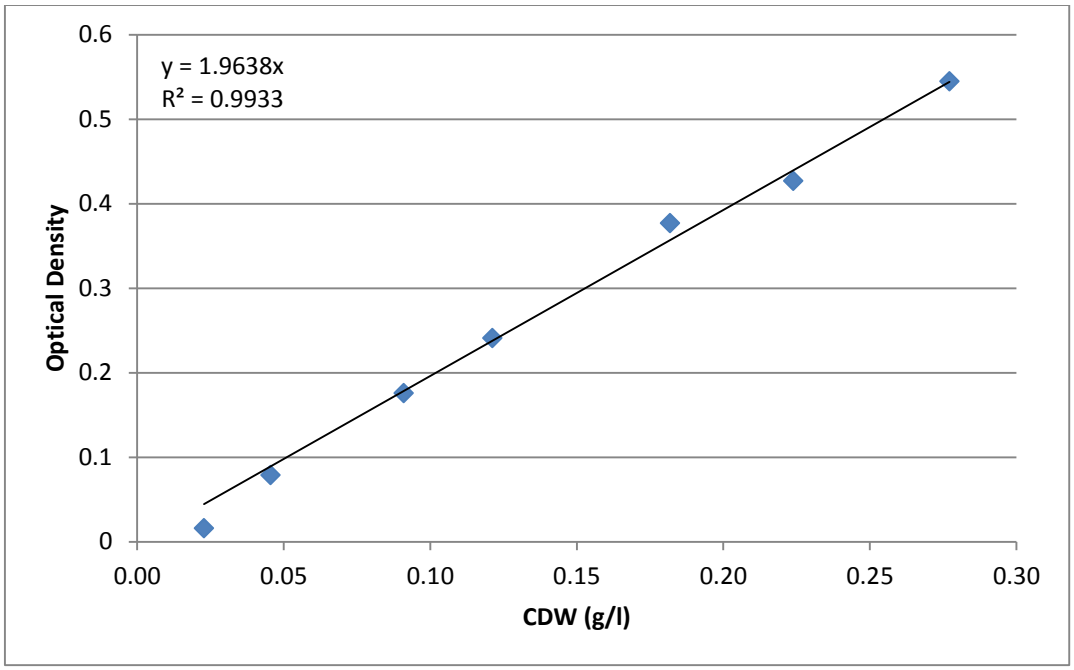

Figure 78: Standard curve for cell concentration analysis

The DNS glucose standard curve is given below in Figure 79 below. The error bars represent the standard deviation from the mean obtained from two replicates.

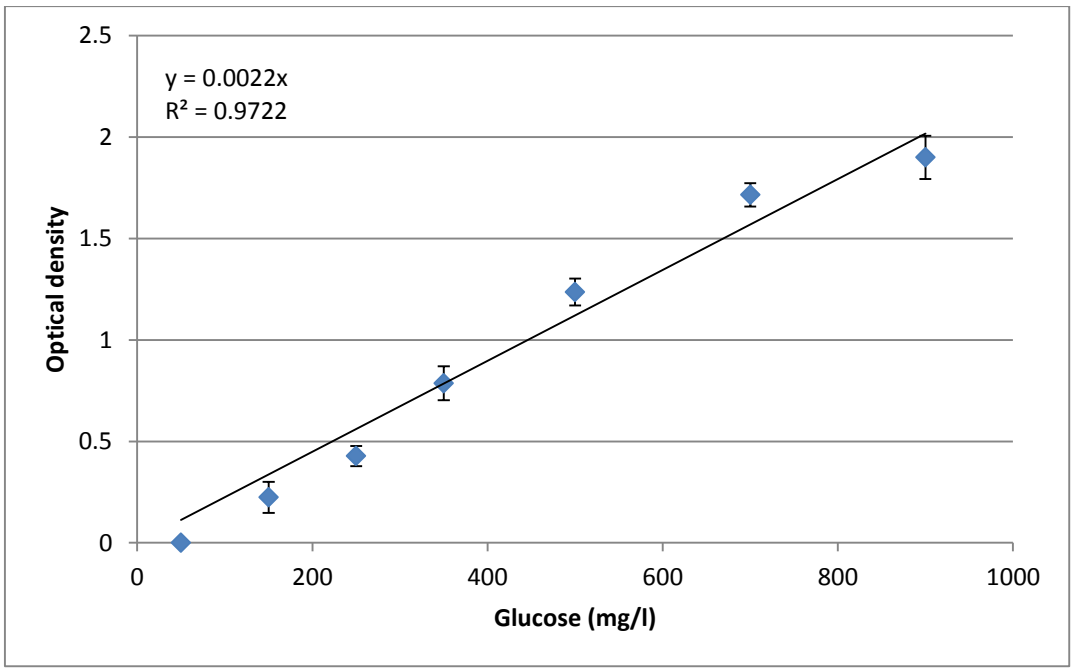

Figure 79: Standard curve for glucose concentration by colorimetric analysis

The HPLC glucose standard curve is given below in Figure 80 below.

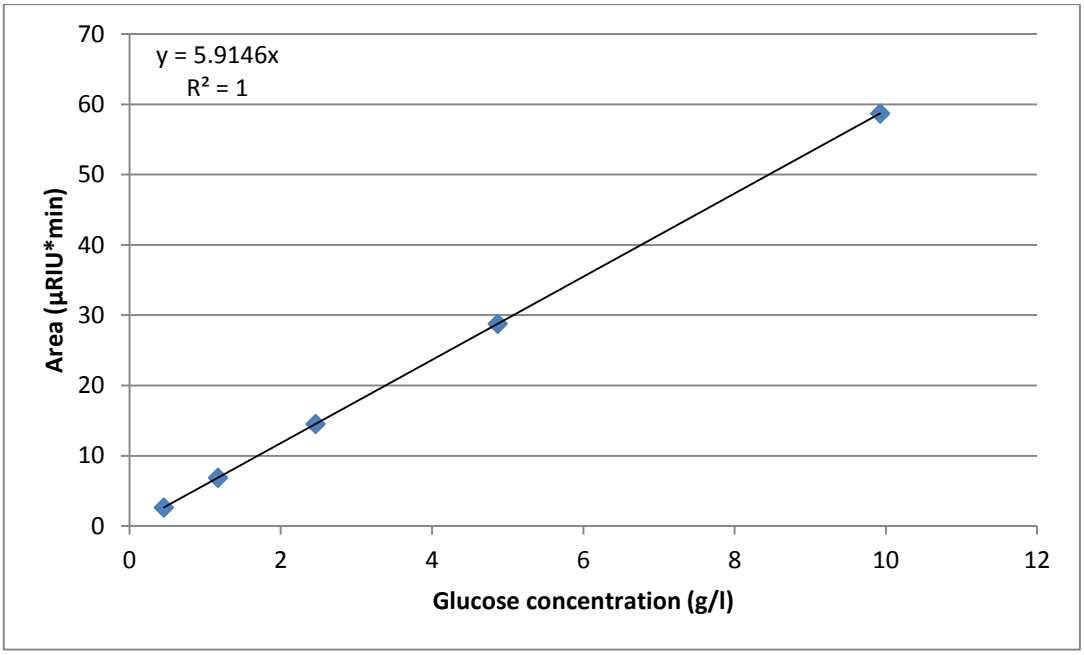

Figure 80: Standard curve for glucose concentration by HPLC analysis

The nitrate standard curve is given in Figure 81 below.

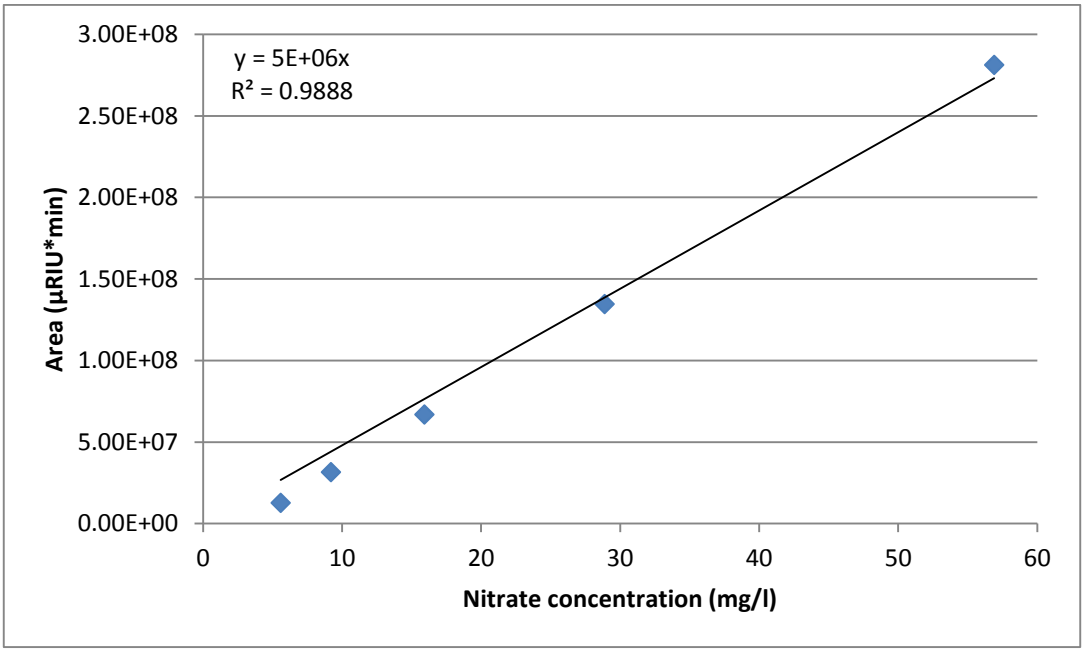

Figure 81: Standard curve for nitrate concentration by ion chromatography analysis

The ammonium standard curve is given in Figure 82 below.

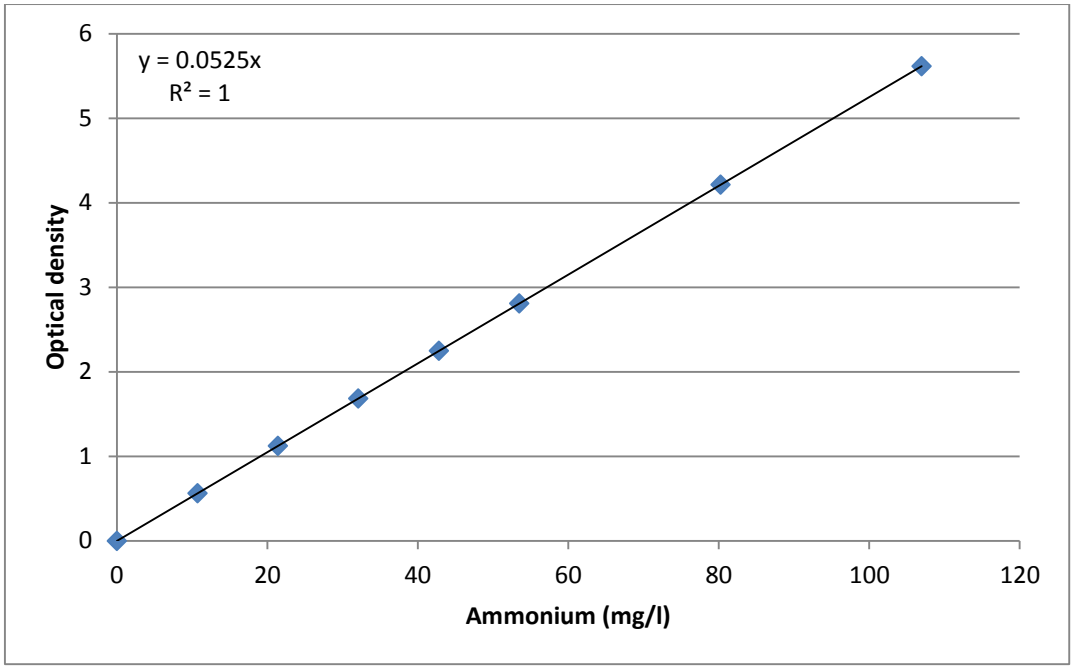

Figure 82: Standard curve for ammonium concentration by colorimetric analysis

The surfactin standard curve is given in Figure 83 below.

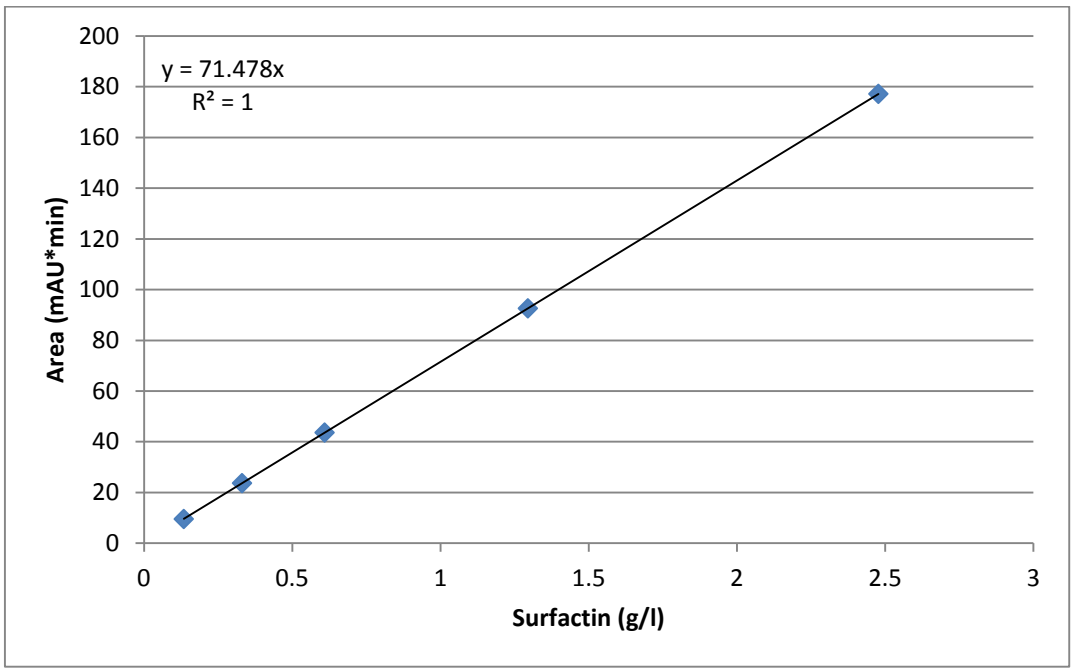

Figure 83: Standard curve for surfactin concentration by HPLC analysis

Appendix 4: Acid precipitation methodology

Antifungal efficacy tests made use of crude supernatant that underwent partial purification and concentration by means of acid precipitation. Samples (approximately 15 ml) were taken from the nitrate only nitrogen ratio culture and centrifuged to obtain the culture supernatant, which was combined in a flask and placed on a magnetic stirrer. 4 M HCl was added drop by drop to the uniformly stirred culture supernatant, until the pH of the solution reach a value of 2. The precipitate obtained by the acidification process was centrifuged and the resulting supernatant was discarded. The centrifuged crude lipopeptide product was resuspended in an alkaline solution of pH 11, using 1 M NaOH. The solution was then filtered through 0.22 µm filters before used as the crude lipopeptides in the antifungal efficacy tests.

Appendix 5: Chemicals and nutrients information

Name	Formula	Manufacturer	Supplier	Country
Glucose		Saarchem	Merck chemicals	South Africa
Ammonium nitrate	NH_4NO_3	Saarchem	Merck chemicals	South Africa
Disodium hydrogen orthophosphate	Na_2HPO_4	Saarchem	Merck chemicals	South Africa
Potassium dihydrogen orthophosphate	KH_2PO_4	Saarchem	Merck chemicals	South Africa
Magnesium sulphate monohydrate	$MgSO_4H_2O$	Saarchem	Merck chemicals	South Africa
Manganese sulphate heptahydrate	$MnSO_4.7H_2O$	Saarchem	Merck chemicals	South Africa
Calcium chloride tetrahydrate	$CaCl_2.4H2O$	Saarchem	Merck chemicals	South Africa
Ammonium chloride	NH_4Cl	Saarchem	Merck chemicals	South Africa
Sodium nitrate	$NaNO_3$	Saarchem	Merck chemicals	South Africa
Nutrient agar	N/A	Biolab	Merck chemicals	South Africa
Yeast extract	N/A	Biolab	Merck chemicals	South Africa

Appendix 6: Experimental data

The experimental and analysed data used in the graphs and tables of this study is given in the tables below.

- **Standard curve data**

Table 12: CDW standard curve data

Dilution	Optical density	Weight Before (g)	Weight After (g)	CDW (g/l)
1	2.316	0.124	0.153	5.820
2	2.147			2.910
4	1.815			1.455
8	1.312			0.728
16	0.684			0.364
21	0.545			0.277
26	0.427			0.224
32	0.377			0.182
48	0.241			0.121
64	0.176			0.091
128	0.079			0.045
256	0.016			0.023

Table 13: HPLC glucose and nitrate standard curve data

Glucose		Nitrate	
Concentration (g/l)	Area (µRIU*min)	Concentration (mg/l)	Area (µRIU*min)
9.927	58.713	56.908	2.81E+08
4.866	28.780	28.884	1.35E+08
2.459	14.544	15.921	6.68E+07
1.169	6.915	9.182	3.15E+07
0.453	2.681	5.585	1.27E+07

Table 14: DNS glucose standard curve data

DNS Glucose	
Concentration (mg/l)	**Optical density**
50	0.000
150	0.224
250	0.428
350	0.787
500	1.237

Table 15: Ammonium standard curve data

Ammonium	
Concentration (mg/l)	**Optical density**
0	0.000
10.70	0.562
21.40	1.123
32.09	1.685
42.79	2.247
53.49	2.808
80.24	4.212
106.98	5.617

- **Effect of nitrogen concentration experimental data**

Table 16: Nitrogen concentration biomass data

Shake flasks culture											
4 g/l NH_4NO_3						8 g/l NH_4NO_3					
		CDW		Ln(CDW)				CDW		Ln(CDW)	
t	time	Stdv	Average	Stdv	Average	t	time	Stdv	Average	Stdv	Average
0	10:00	0.007	0.643	0.012	-0.442	0	10:00	0.047	0.454	0.104	-0.793
2	12:00	0.037	0.675	0.054	-0.394	2	12:00	0.067	0.564	0.117	-0.577
4	14:00	0.116	1.183	0.096	0.165	4	14:00	0.009	0.769	0.012	-0.263
6	16:00	0.037	2.078	0.018	0.731	6	16:00	0.188	1.793	0.106	0.580
10	20:00	0.352	5.104	0.070	1.628	10	20:00	0.415	4.210	0.100	1.434
12	22:00	0.251	6.807	0.037	1.918	12	22:00	0.609	5.350	0.114	1.673
14	00:00	0.130	7.180	0.018	1.971	14	00:00	0.492	7.299	0.069	1.986
34	20:00	0.343	7.136	0.048	1.964	22	08:00	0.505	8.044	0.063	2.084
48	10:00	0.494	5.200	0.097	1.646	24	10:00	1.036	8.151	0.129	2.093
						34	20:00	1.654	6.790	0.251	1.895
						48	10:00	1.757	5.627	0.311	1.695
Bioreactor culture											
4 g/l NH_4NO_3						8 g/l NH_4NO_3					
		CDW		Ln(CDW)				CDW		Ln(CDW)	
t	time	Stdv	Average	Stdv	Average	t	time	Stdv	Average	Stdv	Average
0	10:00	N/A	0.438	N/A	-0.825	0	10:00	0.053	0.246	0.217	-1.414

2	12:00	N/A	0.474	N/A	-0.747	2	12:00	0.079	0.347	0.229	-1.070
4	14:00	N/A	0.882	N/A	-0.126	4	14:00	0.000	0.572	0.000	-0.558
6	16:00	N/A	1.299	N/A	0.261	6	16:00	0.000	0.847	0.000	-0.167
10	20:00	N/A	3.255	N/A	1.180	8	18:00	0.006	1.203	0.005	0.185
12	22:00	N/A	5.420	N/A	1.690	10	20:00	0.000	1.578	0.000	0.456
14	00:00	N/A	6.774	N/A	1.913	12	22:00	0.193	2.434	0.080	0.888
22	08:00	N/A	7.545	N/A	2.021	14	00:00	0.311	3.540	0.088	1.262
24	10:00	N/A	6.696	N/A	1.901	22	08:00	1.286	5.980	0.217	1.777
						24	10:00	1.359	5.846	0.235	1.752
						33	20:00	0.015	4.772	0.003	1.563

Table 17: Nitrogen concentration substrate data

t	time	Glucose (g/l)							
		4 g/l SF		8 g/l SF		4 g/l Bioreactor		8 g/l Bioreactor	
		Stdv	Concentration	Stdv	Concentration	Stdv	Concentration	Stdv	Concentration
0	10:00	0	40	0	40	N/A	40	0	40
5	15:00	5.967	32.879	0.741	40.298	N/A	35.636	0.791	36.977
9	19:00	2.008	19.719	3.192	31.111	N/A	28.573	4.446	33.371
13	23:00	1.590	6.060	2.089	18.147	N/A	14.665	8.241	24.902
23	09:00	0	0	0.000	0.000	N/A	0	3.961	4.860
33	19:00	0	0	0.000	0.000	N/A	0	0	0
48	10:00	0	0	0.000	0.000	N/A	0	0	0

t	time	Nitrate (g/l)							
		4 g/l SF		8 g/l SF		4 g/l Bioreactor		8 g/l Bioreactor	
		Stdv	Concentration	Stdv	Concentration	Stdv	Concentration	Stdv	Concentration
0	10:00	0	3.098	0	6.196	N/A	3.099	0.000	6.196
5	15:00	0.035	2.296	0.187	4.185	N/A	2.992	0.987	3.845
9	19:00	0.269	1.937	0.092	4.087	N/A	1.719	0.671	2.837
13	23:00	0.617	1.776	0.325	3.448	N/A	1.813	1.072	2.292
23	09:00	0.657	1.526	0.582	3.262	N/A	0.412	0.658	0.899
33	19:00	0.544	1.339	0.729	3.188	N/A	0.415	0.684	0.912
48	10:00	0.641	1.529	0.576	3.196	N/A	0.421	0.031	0.434

Table 18: Nitrogen concentration product data

t	time	Surfactin (mg/l)							
		4 g/l SF		8 g/l SF		4 g/l Bioreactor		8 g/l Bioreactor	
		Stdv	Concentration	Stdv	Concentration	Stdv	Concentration	Stdv	Concentration
0	10:00	0	0	0	0	N/A	0	0	0
5	15:00	11.37	388.67	2.83	358	N/A	408	125.865	377
9	19:00	107.19	539.33	5.66	444	N/A	380	130.108	428
13	23:00	154.97	460.00	46.67	518	N/A	626	121.622	450
23	09:00	99.85	443.33	0.00	422.00	N/A	322	141.421	412
33	19:00	73.82	491.33	82.02	432.00	N/A	170	132.936	406
48	10:00	71.19	452.00	67.88	482	N/A	40	125.865	401

t	time	Antifungals (mAU*min)							
		4 g/l SF		8 g/l SF		4 g/l Bioreactor		8 g/l Bioreactor	
		Stdv	Concentration	Stdv	Concentration	Stdv	Concentration	Stdv	Concentration
0	10:00	0	0	0	0	N/A	0	0	0
5	15:00	2.008	91.996	4.691	74.888	N/A	85.788	68.686	113.176
9	19:00	14.558	209.414	4.141	146.266	N/A	115.538	57.922	223.387
13	23:00	137.289	329.875	14.292	264.614	N/A	247.258	22.209	340.688
23	09:00	112.552	320.369	97.173	475.778	N/A	131.566	102.044	267.594
33	19:00	125.616	399.887	152.898	583.532	N/A	73.584	12.304	245.118
48	10:00	140.583	404.899	181.657	604.668	N/A	48.888	91.183	189.342

Table 19: Nitrogen concentration average kinetic parameters

Average kinetic parameters												
Exp	Growth			Antifungal				Surfactin				Ratio
	μ_{max} (h^{-1})	CDW (g/l)	$Y_{x/s}$ (g/g)	Concentration (mAU*min)	$Y_{p/s}$ (area/g/l)	$Y_{p/x}$ (area/g/l)	Productivity (area*min/h)	Concentration (mg/l)	$Y_{p/s}$ (g/g)	$Y_{p/x}$	Productivity (mg/l/h)	AF/S
4 g/l SF	0.252	5.200	0.114	404.899	10.122	91.507	8.435	452	0.011	0.101	9.417	0.898
8 g/l SF	0.175	5.627	0.129	604.668	8.594	77.826	7.161	482	0.007	0.060	6.208	0.920
4 g/l Bioreactor	0.237	6.097	0.223	247.258	9.760	43.699	19.020	626	0.025	0.111	48.154	0.738
8 g/l Bioreactor	0.190	2.987	0.209	340.688	26.043	124.350	26.207	450	0.038	0.166	34.615	0.920

Table 20: Nitrogen concentration normalised average kinetic parameters

Normalised average kinetic parameters												
Exp	Growth			Antifungal				Surfactin				Ratio
	μ_{max} (h^{-1})	CDW (g/l)	$Y_{x/s}$ (g/g)	Concentration (mAU*min)	$Y_{p/s}$ (area/g/l)	$Y_{p/x}$ (area/g/l)	Productivity	Concentration (mg/l)	$Y_{p/s}$ (g/g)	$Y_{p/x}$	Productivity (mg/l/h)	AF/S
4 g/l SF	1.00	0.85	0.51	0.67	0.39	0.74	0.32	0.72	0.30	0.61	0.20	0.98
8 g/l SF	0.69	0.92	0.58	1.00	0.33	0.63	0.27	0.77	0.20	0.36	0.13	1.00
4 g/l Bioreactor	0.94	1.00	1.00	0.41	0.37	0.35	0.73	1.00	0.66	0.67	1.00	0.80
8 g/l Bioreactor	0.75	0.49	0.94	0.56	1.00	1.00	1.00	0.72	1.00	1.00	0.72	1.00

- **Effect of nitrogen source experimental data**

Table 21: Nitrogen source biomass data

Nitrogen ratios											
0:1						0.25:0.75					
		CDW (g/l)		Ln(CDW)				CDW (g/l)		Ln(CDW)	
t	time	Stdv	Average	Stdv	Average	t	time	Stdv	Average	Stdv	Average
0	10:00	0.022	0.361	0.061	-1.020	0	10:00	0.013	0.287	0.045	-1.249
2	12:00	0.004	0.450	0.009	-0.797	2	12:00	0.020	0.505	0.039	-0.685
4	14:00	0.008	0.604	0.012	-0.503	4	14:00	0.210	0.825	0.245	-0.213
6	16:00	0.063	0.774	0.084	-0.258	6	16:00	0.082	1.083	0.077	0.078
10	20:00	0.127	1.176	0.106	0.159	10	20:00	0.264	2.229	0.123	0.797
12	22:00	0.174	1.650	0.107	0.497	12	22:00	0.061	3.352	0.018	1.209
22	08:00	0.269	3.823	0.072	1.339	14	00:00	0.174	3.708	0.047	1.310
24	10:00	0.356	4.903	0.073	1.588	24	10:00	0.326	5.498	0.060	1.703
34	20:00	0.134	6.310	0.021	1.842	34	20:00	0.158	6.502	0.024	1.872
48	10:00	0.278	8.168	0.034	2.100	48	10:00	0.077	6.540	0.012	1.878
0.5:0.5						0.75:0.25					
		CDW (g/l)		Ln(CDW)				CDW (g/l)		Ln(CDW)	
t	time	Stdv	Average	Stdv	Average	t	time	Stdv	Average	Stdv	Average
0	10:00	0.013	0.292	0.046	-1.233	0	10:00	0.004	0.277	0.013	-1.283
2	12:00	0.148	0.711	0.208	-0.356	2	12:00	0.061	0.369	0.161	-1.007
4	14:00	0.094	1.092	0.088	0.085	4	14:00	0.037	0.531	0.068	-0.635

6	16:00	0.189	1.751	0.105	0.556	6	16:00	0.128	0.897	0.141	-0.115
10	20:00	0.645	4.573	0.136	1.514	10	20:00	0.152	2.393	0.062	0.871
14	00:00	1.100	7.376	0.155	1.990	12	22:00	0.049	4.276	0.012	1.453
22	08:00	1.236	6.172	0.212	1.806	14	00:00	0.170	4.192	0.041	1.433
24	10:00	0.322	5.966	0.055	1.785	22	08:00	1.019	6.695	0.158	1.893
34	20:00	0.657	5.902	0.115	1.771	24	10:00	0.670	7.067	0.097	1.952
48	10:00	0.207	5.619	0.037	1.726	34	20:00	1.466	4.345	0.328	1.432
						48	10:00	0.854	4.510	0.188	1.495

1:0					
		CDW (g/l)		**Ln(CDW)**	
t	**time**	**Stdv**	**Average**	**Stdv**	**Average**
0	10:00	0.040	0.454	0.086	-0.793
2	12:00	0.064	0.410	0.152	-0.899
4	14:00	0.074	0.650	0.114	-0.435
6	16:00	0.077	0.947	0.082	-0.057
10	20:00	0.110	1.700	0.066	0.529
12	22:00	0.322	2.274	0.145	0.815
14	00:00	0.396	2.667	0.152	0.973
22	08:00	0.160	5.584	0.029	1.720
24	10:00	0.231	5.766	0.040	1.751
34	20:00	0.358	5.505	0.064	1.704
48	10:00	1.470	5.369	0.027	1.652

Table 22: Nitrogen source substrate data

t	time	Glucose (g/l)									
		0:1		0.25:0.75		0.5:0.5		0.75:0.25		1:0	
		Stdv	Concentration	Stdv	Concentration	Stdv	Concentration	Stdv	Concentration	Stdv	Concentration
0	10:00	0.000	40.000	0.000	40.000	0.000	40.000	0.000	40.000	0.000	40.000
5	15:00	1.496	43.898	1.102	44.175	4.190	36.296	0.483	44.307	2.948	41.167
9	19:00	0.405	40.152	0.525	40.291	0.414	30.331	0.629	38.804	1.776	37.804
13	23:00	2.245	36.566	1.803	38.343	1.095	15.671	1.094	33.155	0.601	36.168
23	09:00	2.579	26.607	1.339	24.973	0.000	0.000	0.000	0.000	2.826	24.720
33	19:00	0.771	16.521	1.941	12.513	0.000	0.000	0.000	0.000	3.536	23.145
48	10:00	0.028	0.083	0.000	0.000	0.000	0.000	0.000	0.000	2.963	22.918
t	time	Nitrate (g/l)									
		0:1		0.25:0.75		0.5:0.5		0.75:0.25		1:0	
		Stdv	Concentration	Stdv	Concentration	Stdv	Concentration	Stdv	Concentration	Stdv	Concentration
0	10:00	0.000	6.196	0.000	4.647	0.000	3.098	0.000	1.549	0.000	0.000
5	15:00	0.509	4.274	0.010	3.122	0.148	2.374	0.027	1.573	0.007	0.701
9	19:00	0.507	3.958	0.020	3.250	0.409	2.161	0.001	1.453	0.023	0.707
13	23:00	0.149	3.756	0.301	3.202	0.351	1.664	0.034	1.392	0.030	0.718
23	09:00	0.061	3.211	0.386	2.421	0.418	1.218	0.104	0.688	0.077	0.655
33	19:00	0.385	2.384	0.269	1.896	0.541	1.541	0.102	0.686	0.061	0.670
48	10:00	0.059	1.963	0.063	1.586	0.439	1.307	0.102	0.727	0.036	0.646

t	time	Ammonium (mg/l									
		0:1		0.25:0.75		0.5:0.5		0.75:0.25		1:0	
		Stdv	Concentration	Stdv	Concentration	Stdv	Concentration	Stdv	Concentration	Stdv	Concentration
0	10:00	0.000	0.000	0.000	450.000	0.000	901.000	0.000	1350.000	0.000	1803.000
6	16:00	1.242	1.124	6.649	25.219	10.230	18.686	5.048	199.937	12.317	166.794
10	20:00	0.000	0.000	2.345	8.324	5.890	16.210	3.166	34.794	2.036	56.381
22	08:00	0.275	0.159	0.038	0.210	0.154	9.587	0.116	0.476	0.725	1.384
34	20:00	0.099	0.057	N/A	N/A	1.138	1.644	0.927	4.413	0.671	0.584
48	10:00	1.486	1.695	0.781	2.463	0.232	0.286	1.389	2.883	0.240	0.330

Table 23: Nitrogen source product data

t	time	Surfactin (mg/l)									
		0:1		0.25:0.75		0.5:0.5		0.75:0.25		1:0	
		Stdv	Concentration	Stdv	Concentration	Stdv	Concentration	Stdv	Concentration	Stdv	Concentration
0	10:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	15:00	16.166	512.667	20.000	504.000	5.292	428.000	4.000	488.000	11.372	485.333
9	19:00	4.619	538.667	20.232	691.333	13.317	664.667	9.018	539.333	19.630	524.667
13	23:00	16.042	551.333	46.130	768.000	15.556	809.000	19.218	533.333	18.148	530.667
23	09:00	9.899	661.000	55.148	715.333	11.314	684.000	32.527	575.000	38.158	306.000
33	19:00	18.148	697.333	22.480	781.333	9.899	773.000	49.497	547.000	12.728	67.000
48	10:00	5.033	706.667	113.072	731.333	1.414	761.000	22.627	598.000	12.055	71.333

t	time	Antifungals (mAU*min)									
		0:1		0.25:0.75		0.5:0.5		0.75:0.25		1:0	
		Stdv	Concentration	Stdv	Concentration	Stdv	Concentration	Stdv	Concentration	Stdv	Concentration
0	10:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	15:00	1.750	86.961	3.107	93.394	2.016	98.439	2.112	71.654	7.051	81.835
9	19:00	11.651	180.391	4.005	212.951	1.684	188.765	3.008	204.881	8.566	235.562
13	23:00	5.763	256.717	9.662	393.358	4.725	426.799	9.327	360.956	20.739	377.226
23	09:00	49.690	666.512	18.561	581.913	3.330	728.745	125.388	618.237	21.601	167.827
33	19:00	19.451	877.482	56.630	724.051	48.102	888.303	129.355	593.860	14.136	155.876
48	10:00	48.488	977.253	253.447	698.887	30.957	847.068	127.876	673.958	6.580	131.698

Table 24: Nitrogen source average kinetic parameters

Average kinetic parameters												
NH4 Ratio	Growth			Antifungal				Surfactin				Ratio
	μ_{max} (h^{-1})	CDW (g/l)	$Y_{x/s}$ (g/g)	Concentration (mAU*min)	$Y_{p/s}$ (area/g/l)	$Y_{p/x}$ (area/g/l)	Productivity	Concentration (mg/l)	$Y_{p/s}$ (g/g)	$Y_{p/x}$	Productivity (mg/l/h)	AF/S
0	0.124	8.168	0.196	977.253	24.482	125.183	20.359	706.667	0.018	0.091	14.722	0.927
0.25	0.196	6.402	0.222	724.051	26.342	118.405	21.941	781.333	0.028	0.128	23.677	0.861
0.5	0.225	5.908	0.140	888.303	22.208	158.154	26.918	773.000	0.019	0.138	23.424	0.867
0.75	0.258	4.510	0.106	673.958	16.849	159.209	14.041	598.000	0.015	0.141	12.458	0.937
1	0.166	2.471	0.526	377.226	98.438	187.029	29.017	530.667	0.138	0.263	40.821	0.929

Table 25: Nitrogen source normalised average kinetic parameters

Normalised average kinetic parameters												
NH4 Ratio	Growth			Antifungal				Surfactin				Ratio
	μ_{max} (h^{-1})	CDW (g/l)	$Y_{x/s}$ (g/g)	Concentration (mAU*min)	$Y_{p/s}$ (area/g/l)	$Y_{p/x}$ (area/g/l)	Productivity	Concentration (mg/l)	$Y_{p/s}$ (g/g)	$Y_{p/x}$	Productivity (mg/l/h)	AF/S
0	0.480	1.000	0.372	1.000	0.249	0.669	0.702	0.904	0.128	0.344	0.361	0.990
0.25	0.761	0.784	0.423	0.741	0.268	0.633	0.756	1.000	0.205	0.486	0.580	0.920
0.5	0.872	0.723	0.267	0.909	0.226	0.846	0.928	0.989	0.140	0.523	0.574	0.926
0.75	1.000	0.552	0.201	0.690	0.171	0.851	0.484	0.765	0.108	0.537	0.305	1.000
1	0.644	0.302	1.000	0.386	1.000	1.000	1.000	0.679	1.000	1.000	1.000	0.992

- **Effect of dissolved oxygen experimental data**

Table 26: Dissolved oxygen biomass data

0.5:0.5 Baffled						0.5:0.5 Unbaffled					
		CDW (g/l)		Ln(CDW)				CDW (g/l)		Ln(CDW)	
t	time	Stdv	Average	Stdv	Average	t	time	Stdv	Average	Stdv	Average
0	10:00	0.013	0.292	0.046	-1.233	0	10:00	0.037	0.319	0.116	-1.148
2	12:00	0.148	0.711	0.208	-0.356	2	12:00	0.012	0.499	0.025	-0.696
4	14:00	0.094	1.092	0.088	0.085	4	14:00	0.041	0.688	0.060	-0.375
6	16:00	0.189	1.751	0.105	0.556	6	16:00	0.029	0.837	0.035	-0.178
10	20:00	0.645	4.573	0.136	1.514	10	20:00	0.080	1.690	0.047	0.524
14	00:00	1.100	7.376	0.155	1.990	12	22:00	0.079	2.028	0.038	0.707
22	08:00	1.236	6.172	0.212	1.806	14	00:00	0.075	2.588	0.029	0.951
24	10:00	0.322	5.966	0.055	1.785	22	08:00	0.294	5.362	0.054	1.678
34	20:00	0.657	5.902	0.115	1.771	24	10:00	0.352	5.250	0.067	1.657
48	10:00	0.207	5.619	0.037	1.726	34	20:00	0.186	4.930	0.038	1.595
						48	10:00	0.128	3.561	0.036	1.270
0:1 Baffled						0:1 Unbaffled					
		CDW (g/l)		Ln(CDW)				CDW (g/l)		Ln(CDW)	
t	time	Stdv	Average	Stdv	Average	t	time	Stdv	Average	Stdv	Average
0	10:00	0.022	0.361	0.061	-1.020	0	10:00	0.023	0.740	0.031	-0.302
2	12:00	0.004	0.450	0.009	-0.797	2	12:00	0.008	0.558	0.015	-0.583
4	14:00	0.008	0.604	0.012	-0.503	6	16:00	0.002	1.020	0.002	0.020

6	16:00	0.063	0.774	0.084	-0.258	14	00:00	0.206	2.916	0.071	1.069
10	20:00	0.127	1.176	0.106	0.159	22	08:00	0.172	5.330	0.032	1.673
12	22:00	0.174	1.650	0.107	0.497	24	10:00	0.445	6.648	0.067	1.893
22	08:00	0.269	3.823	0.072	1.339	34	20:00	0.334	4.752	0.070	1.557
24	10:00	0.356	4.903	0.073	1.588	48	10:00	0.156	3.180	0.049	1.156
34	20:00	0.134	6.310	0.021	1.842						
48	10:00	0.278	8.168	0.034	2.100						
0	10:00	0.022	0.361	0.061	-1.020						

Table 27: Dissolved oxygen substrate data

t	time	Glucose (g/l)							
		0.5:0.5 Baffled		0.5:0.5 Unbaffled		0:1 Baffled		0:1 Unbaffled	
		Stdv	Concentration	Stdv	Concentration	Stdv	Concentration	Stdv	Concentration
0	10:00	0.000	40.000	0.000	40.000	0.000	40.000	0.000	40.000
5	15:00	4.190	36.296	2.864	44.018	1.496	43.898	5.242	37.782
9	19:00	0.414	30.331	1.970	40.430	0.405	40.152	0.377	36.772
13	23:00	1.095	15.671	1.569	32.331	2.245	36.566	1.379	28.621
23	09:00	0.000	0.000	1.303	9.831	2.579	26.607	0.063	5.271
33	19:00	0.000	0.000	0.000	0.000	0.771	16.521	0.000	0.000
48	10:00	0.000	0.000	0.000	0.000	0.028	0.083	0.000	0.000
t	time	Nitrate (g/l)							
		0.5:0.5 Baffled		0.5:0.5 Unbaffled		0:1 Baffled		0:1 Unbaffled	

		Stdv	Concentration	Stdv	Concentration	Stdv	Concentration	Stdv	Concentration
0	10:00	0.000	3.098	0.000	3.098	0.000	6.196	0.000	6.196
5	15:00	0.148	2.374	0.044	2.676	0.509	4.274	0.033	3.909
9	19:00	0.409	2.161	0.219	2.063	0.507	3.958	0.047	3.637
13	23:00	0.351	1.664	0.174	1.555	0.149	3.756	0.011	2.998
23	09:00	0.418	1.218	0.120	0.590	0.061	3.211	0.025	1.117
33	19:00	0.541	1.541	0.003	0.608	0.385	2.384	0.013	0.384
48	10:00	0.439	1.307	0.162	0.610	0.059	1.963	0.057	0.451
t	**time**	**Ammonium (mg/l)**							
		0.5:0.5 Baffled		**0.5:0.5 Unbaffled**		**0:1 Baffled**		**0:1 Unbaffled**	
		Stdv	**Concentration**	**Stdv**	**Concentration**	**Stdv**	**Concentration**	**Stdv**	**Concentration**
0	10:00	0.000	0.000	901.000	0.000	901.000	0.000	0.000	0.000
6	16:00	0.148	10.230	18.686	4.510	12.990	1.242	1.124	3.919
10	20:00	0.409	5.890	16.210	0.429	0.908	0.000	0.000	4.187
22	08:00	0.351	0.154	9.587	0.674	0.997	0.275	0.159	0.119
34	20:00	0.418	1.138	1.644	2.309	3.258	0.099	0.057	1.557
48	10:00	0.541	0.232	0.286	0.000	0.000	1.486	1.695	1.104

Table 28: Dissolved oxygen product data

t	time	Surfactin (mg/l)							
		0.5:0.5 Baffled		0.5:0.5 Unbaffled		0:1 Baffled		0:1 Unbaffled	
		Stdv	Concentration	Stdv	Concentration	Stdv	Concentration	Stdv	Concentration
0	10:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	15:00	5.292	428.000	5.292	512.000	16.166	512.667	1.414	55.000
9	19:00	13.317	664.667	15.556	477.000	4.619	538.667	1.414	61.000
13	23:00	15.556	809.000	9.018	564.667	16.042	551.333	7.071	103.000
23	09:00	11.314	684.000	29.698	657.000	9.899	661.000	32.527	139.000
33	19:00	9.899	773.000	2.828	660.000	18.148	697.333	0.000	148.000
48	10:00	1.414	761.000	24.042	625.000	5.033	706.667	1.414	125.000
t	time	Antifungals (mAU*min)							
		0.5:0.5 Baffled		0.5:0.5 Unbaffled		0:1 Baffled		0:1 Unbaffled	
		Stdv	Concentration	Stdv	Concentration	Stdv	Concentration	Stdv	Concentration
0	10:00	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	15:00	2.016	98.439	10.319	93.141	1.750	86.961	4.158	105.114
9	19:00	1.684	188.765	27.477	62.081	11.651	180.391	0.781	149.524
13	23:00	4.725	426.799	6.645	190.446	5.763	256.717	13.791	205.494
23	09:00	3.330	728.745	53.558	452.895	49.690	666.512	110.282	445.919
33	19:00	48.102	888.303	45.793	489.231	19.451	877.482	2.623	563.283
48	10:00	30.957	847.068	27.753	490.306	48.488	977.253	3.548	501.951

Table 29: Dissolved oxygen average kinetic parameters

Average kinetic parameters												
Exp	Growth			Antifungal				Surfactin				Ratio
	μ_{max} (h^{-1})	CDW (g/l)	$Y_{x/s}$ (g/g)	Concentration (mAU*min)	$Y_{p/s}$ (area/g/l)	$Y_{p/x}$ (area/g/l)	Productivity	Concentration (mg/l)	$Y_{p/s}$ (g/g)	$Y_{p/x}$	Productivity (mg/l/h)	AF/S
0:1 Baffled	0.124	8.168	0.196	977.253	24.482	125.183	20.359	706.667	0.018	0.091	14.722	0.927
0:1 Unbaffled	0.137	4.942	0.105	563.283	14.082	134.041	17.069	148.000	0.004	0.035	4.485	0.845
0.5:0.5 Baffled	0.235	5.908	0.140	888.303	22.208	158.154	26.918	773.000	0.019	0.138	23.424	0.867
0.5:0.5 Unbaffled	0.144	3.561	0.081	490.306	12.258	151.229	10.215	625.000	0.016	0.193	13.021	0.897

Table 30: Dissolved oxygen normalised average kinetic parameters

Normalised average kinetic parameters												
Exp	Growth			Antifungal				Surfactin				Ratio
	μ_{max} (h^{-1})	CDW (g/l)	$Y_{x/s}$ (g/g)	Concentration (mAU*min)	$Y_{p/s}$ (area/g/l)	$Y_{p/x}$ (area/g/l)	Productivity	Concentration (mg/l)	$Y_{p/s}$ (g/g)	$Y_{p/x}$	Productivity (mg/l/h)	AF/S
0:1 Baffled	0.528	1.000	1.000	1.000	1.000	0.792	0.756	0.914	0.916	0.470	0.629	1.000
0:1 Unbaffled	0.583	0.605	0.537	0.576	0.575	0.848	0.634	0.191	0.191	0.183	0.191	0.912
0.5:0.5 Baffled	1.000	0.723	0.718	0.909	0.907	1.000	1.000	1.000	1.000	0.714	1.000	0.935
0.5:0.5 Unbaffled	0.613	0.436	0.414	0.502	0.501	0.956	0.379	0.809	0.809	1.000	0.556	0.968

- **Bioreactor culture with optimum conditions**

Table 31: Optimum conditions biomass data

0.5:0.5 Bioreactor with oxygen (this study)					8 g/l NH_4NO_3 with oxygen (Pretorius et al. (2015)				
	CDW (g/l)		Ln(CDW)			CDW (g/l)		Ln(CDW)	
t	Stdv	Average	Stdv	Average	t	Stdv	Average	Stdv	Average
0	0.047	0.211	0.192	-1.419	0	N/A	0.031	N/A	-3.465
2	0.076	0.600	0.139	-0.610	3	N/A	0.072	N/A	-2.630
4	0.100	0.756	0.121	-0.195	5	N/A	0.286	N/A	-1.253
6	0.206	1.648	0.137	0.403	7	N/A	0.632	N/A	-0.458
10	0.476	3.501	0.124	1.341	10	N/A	1.238	N/A	0.213
12	1.272	4.803	0.225	1.728	12.1	N/A	1.693	N/A	0.527
14	0.955	5.611	0.152	1.833	15	N/A	2.669	N/A	0.982
22	1.345	6.386	0.184	1.984	24.25	N/A	6.018	N/A	1.795
24	0.478	6.365	0.071	1.901	31.5	N/A	6.664	N/A	1.897
34	1.408	7.810	0.208	1.908	38	N/A	6.426	N/A	1.860
48	0.316	5.130	0.059	1.677	49.5	N/A	5.406	N/A	1.688

Table 32: Optimum conditions substrate data

Glucose (g/l)					
0.5:0.5 Bioreactor			8 g/l NH4NO3		
t	Stdv	Concentration	t	Stdv	Concentration
0	0.000	40	0	N/A	37.88
5	1.736	39.7476	5	N/A	41.36
9	0.898	28.7484	10	N/A	35.94
13	3.983	15.5568	12.1	N/A	28.32
23	0.046	0.0652	24.25	N/A	16.02
33	0.016	0.0636	31.5	N/A	6.22
48	0.180	0.0636	49	N/A	0.22
Nitrate (g/l)					
0.5:0.5 Bioreactor			8 g/l NH4NO3		
t	Stdv	Concentration	t	Stdv	Concentration
0	0.000	3.098	0	N/A	6.055
5	0.088	2.010	5	N/A	6.074
9	0.137	1.285	10	N/A	5.777
13	0.300	0.921	12.1	N/A	5.005
23	0.028	0.482	24.25	N/A	1.889
33	0.034	0.510	31.5	N/A	0.487
48	0.054	0.464	49	N/A	0.000

Table 33: Optimum conditions product data

Surfactin (mg/l)					
0.5:0.5 Bioreactor			8 g/l NH4NO3		
t	Stdv	Concentration	t	Stdv	Concentration
0	0.000	0.000	0	N/A	0.000
5	14.142	470.000	5	N/A	4.000
9	193.747	728.000	10	N/A	138.000
13	206.475	894.000	12.1	N/A	154.000
23	199.404	504.000	24.25	N/A	230.000
33	2.828	474.000	31.5	N/A	277.000
48	26.870	438.000	49	N/A	302.000
Antifungals (mAU*min)					
0.5:0.5 Bioreactor			8 g/l NH4NO3		
t	Stdv	Concentration	t	Stdv	Concentration
0	0.000	0.000	0	N/A	6.985
5	39.967	85.864	5	N/A	5.956
9	63.879	121.416	10	N/A	34.667
13	77.012	290.174	12.1	N/A	55.672
23	93.750	193.174	24.25	N/A	156.285
33	5.436	98.482	31.5	N/A	237.512
48	20.806	83.558	49	N/A	285.66

Table 34: Optimum conditions average kinetic parameters

Average kinetic parameters											
Optimum	Growth			Antifungal				Surfactin			
	μ_{max} (h^{-1})	CDW (g/l)	$Y_{x/s}$ (g/g)	Concentration (mAU*min)	$Y_{p/s}$ (area/g/l)	$Y_{p/x}$ (area/g/l)	Productivity	Concentration (mg/l)	$Y_{p/s}$ (g/g)	$Y_{p/x}$	Productivity (mg/l/h)
0.5:0.5 Bioreactor	0.2562	5.207	0.204	290.174	11.871	58.080	22.321	894	0.037	0.179	68.769
8 g/l NH_4NO_3	0.5429	5.406	0.143	285.66	7.400	51.849	5.687	302	0.008	0.056	6.163

Table 35: Optimum conditions normalised average kinetic parameters

Normalised average kinetic parameters											
Optimum	Growth			Antifungal				Surfactin			
	μ_{max} (h^{-1})	CDW (g/l)	$Y_{x/s}$ (g/g)	Concentration (mAU*min)	$Y_{p/s}$ (area/g/l)	$Y_{p/x}$ (area/g/l)	Productivity	Concentration (mg/l)	$Y_{p/s}$ (g/g)	$Y_{p/x}$	Productivity (mg/l/h)
0.5:0.5 Bioreactor	0.472	0.963	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
8 g/l NH_4NO_3	1.000	1.000	0.698	0.984	0.623	0.893	0.255	0.338	0.219	0.314	0.090

- **Continuous culture experimental data**

Table 36: Continuous culture biomass and DNS data

Ln(CDW)			DNS Glucose	
t	Optical density	Value	t	Concentration (g/l)
0	0.703	-1.045	0	40.000
2	1.020	-0.609	30	11.127
4	1.446	-0.284	46.67	9.273
6	2.280	0.149	50	5.836
10	3.094	0.536	53.34	5.455
10	3.094	0.536	56.67	5.927
26.67	7.440	1.354	70	4.018
30	5.921	1.141	145	1.795
70	6.060	1.314	170	1.436
76	6.363	1.353	173.5	1.482
145	6.565	1.379	177	1.477
167	7.474	1.485	191	1.500
170	6.565	1.379	194	1.477
173.5	6.666	1.391	197.5	1.500
177	6.565	1.379		
191	6.666	1.391		
194	6.666	1.391		
197.5	6.565	1.379		

Table 37: Steady state HPLC data

Steady state values - HPLC				
Total t	**Glucose (g/l)**	**Nitrate (g/l)**	**Surfactin (mg/l)**	**Antifungals (mAU*min)**
0	40.000	4.000	0.000	0.000
179	0.050	0.533	102.000	67.234
191	0.130	0.439	100.000	41.030
194	0.091	0.502	140.000	69.938
197.5	0.122	0.492	118.000	86.560