

***Characterization of natural antimicrobial peptides adsorbed to
different matrices***

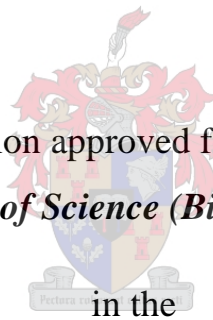
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Summary

Biofouling is the attachment and biofilm formation that leads to negative repercussions such as persistent post-harvest infections, infections obtained from medical implants and continual surface contamination of food processing plants. Much of the problem lies with the resistance that develops against conventional treatments due to the formation of mature biofilms. Thus the focus has shifted from the removal of biofilms to the prevention of initial attachment of organisms. This entails the use of antimicrobial surfaces that either have an inherent antimicrobial activity, e.g. certain metals, or surfaces that are modified by the attachment of antimicrobial agents. The attachment of antimicrobial agents can either be through covalent bonding or adsorption, depending on the intended use of the surface as well as the mode of action of the antimicrobial agent. Antimicrobial peptides (AMPs) are ubiquitous in nature, tend to have a broad spectrum of activity, are very stable and have been shown to maintain activity when covalently bound to solid surfaces. Tyrocidines (Trcs), antimicrobial peptides produced by *Bacillus aneurinolyticus*, are cyclodecapeptides with a broad spectrum of activity against Gram-positive bacteria, fungi, yeasts and the human malaria parasite, *Plasmodium falciparum*. The aim of this study was to determine the antimicrobial activity of surfaces treated with a tyrocidine extract, under which conditions the activity remained stable and to look into possible applications of these peptide-treated surfaces.

The study focussed on different solid surfaces namely mixed cellulose, polyvinylidene fluoride, polycarbonate, cellulose acetate, cellulose (paper)(CL) and high density cellulose packing material (HDC), as a pilot study to assess the antimicrobial activity of Trc and gramicidin S (GS) treated solid surfaces. Peptide desorption and subsequent analysis by mass spectrometry was used to confirm the presence and integrity of the Trcs adsorbed. Scanning electron microscopy was utilised to show that the adsorbed peptides did not affect the structural integrity of the treated filters. However, it was shown that the adsorbed peptides changed the hydrophobic/hydrophilic character by means of a wettability assay.

A cell viability assay and erythrocyte assay were developed from existing methodologies to determine the biological activity of the AMP-functionalised polymeric material. Seven of the AMP treated solid surfaces showed antimicrobial activity when challenged with $>10^5$ *Micrococcus luteus* cells/cm². Although the polycarbonate filter lost antimicrobial activity at the

high cell concentrations, it was shown to have potent antimicrobial activity at lower cell concentrations. Complete inhibition of *M. luteus* growth was observed for both the gramicidin S and tyrocidine extract treated high density cellulose and cellulose filters.

Stability tests showed that the tyrocidines remained adsorbed to cellulose filters and biologically active when exposed multiple water washes, water washes at different temperatures (25°C - 100°C) and pH changes (pH 1-12). The antimicrobial activity was only affected after exposure to the water wash of pH 13 which is possible due to susceptibility of the CL filters to high pH solvents. A preliminary study on the effect of Trcs treated CL filters on the sterilization, germination and effect on tomato seedlings was conducted. It was found that Trcs had no effect on the germination and did not fully sterilise the seeds or environment against fungi. However, it was observed that 5 µg/mL Trcs treated filters promoted root length opposed to the toxic effect seen with filters treated with higher Trc concentrations.

It is hypothesised that Trcs prefer to bind to hydrophilic surfaces exposing the hydrophobic residues and the cationic residue of the peptide to interact with the bacterial membrane to elicit its antimicrobial response. The exposed residues contain some of the hydrophobic residues and the cationic Orn⁹/Lys⁹, which are crucial to the antimicrobial activity of the peptides. Hydrophobic interaction is particularly important for the haemolytic activity which is currently the only viable method of detection of the adsorbed Trcs. Trcs also have a preference for adsorption onto cellulose and cellulose analogues which points to possible application in protective food wrapping and wood surface protection.

Trcs maintains its antimicrobial activity regardless of adsorption to solid surfaces. It can therefore be concluded that Trcs treated solid surfaces hold great potential in preventing the initial bacterial colonization and subsequent biofilm formation. Antimicrobial peptide enriched solid surfaces can thus be developed and tailored to a specific application such as filters, catheters and packaging materials.

Opsomming

Biovervuiling is die aanhegting en vorming van biofilms met negatiewe gevolge soos aanhoudende na-oes infeksies, infeksies op mediese inplantings en voortdurende oppervlak besoedeling van voedselverwerkings fabriek. Die probleem lê grotendeels by die weerstand wat ontwikkel word teen konvensionele behandelings as gevolg van die vorming van volwasse biofilms. Die fokus het gevolglik verskuif vanaf die verwydering van biofilms na die voorkoming van aanvanklike aanhegting van organismes aan oppervlaktes. Dit behels die gebruik van antimikrobiese oppervlaktes wat of 'n inherente antimikrobiese aktiwiteit het, bv. sekere metale óf oppervlaktes wat aangepas is deur die aanhegting van antimikrobiese middels. Die aanhegting van antimikrobiese agente kan of deur kovalente binding óf adsorpsie plaasvind, afhangende van die beoogde gebruik van die oppervlak, sowel as die metode van werking van die antimikrobiese agent. Antimikrobiese peptiede (AMPe) is alomteenwoordig in die natuur, is geneig om 'n breë spektrum van aktiwiteit te hê, is baie stabiel en het getoon dat aktiwiteit in stand gehou word wanneer dit kovalent gebind word op soliede oppervlaktes. Tiroside (Trcs), antimikrobiese peptiede wat deur *Bacillus aneurinolyticus* geproduseer word, is siklo-dekapeptiede met 'n breë spektrum van aktiwiteit teen Gram-positiewe bakterieë, swamme, giste en die menslike malaria parasiet *Plasmodium falciparum*. Die doel van hierdie studie was om die antimikrobiese aktiwiteit te bepaal van oppervlaktes wat met 'n tiroside ekstrak behandel is, te bepaal onder watter omstandighede die aktiwiteit stabiel bly en om te soek na moontlike toepassings van hierdie peptied-behandelde oppervlaktes.

Die studie het gefokus op verskillende soliede oppervlaktes naamlik gemengde sellulose, polyvinylidene fluoried, polikarbonaat, sellulose asetaat, sellulose (papier)(CL) en 'n hoë digtheid sellulose verpakkings materiaal (HDC), as 'n loodsstudie om die antimikrobiese aktiwiteit van die Trcs en gramisidien S (GS) behandelde soliede oppervlaktes te ondersoek. Peptied-desorpsie en daaropvolgende ontleding deur massaspektroskopie is gebruik om die teenwoordigheid en integriteit van die geadsorbeerde Trcs te bevestig. Skandering elektronmikroskopie is gebruik om aan te toon dat die geadsorbeerde peptiede geen invloed op die strukturele integriteit van die behandelde filters het nie. Daar is egter getoon dat die geadsorbeerde peptiede die hidrofobiese / hidrofiliese karakter verander.

'n Lewensvatbaarheid selgebaseerde toets en eritrosiet toets is ontwikkel uit bestaande metodes

om die biologiese aktiwiteit van die AMP-gefunksionaliseerde polimeriese materiaal te bepaal. Sewe van die AMP behandel soliede oppervlaktes het antimikrobiese aktiwiteit getoon wanneer dit met $> 10^5$ *Micrococcus luteus* selle/cm² gedaag is. Hoewel die polikarbonaat filter antimikrobiese aktiwiteit met hoë sel konsentrasies verloor het, is dit getoon dat dit wel uitgeproke antimikrobiese aktiwiteit het teen laer konsentrasies selle. Volledige inhibisie van *M. luteus* groei is waargeneem vir beide die hoë digtheid sellulose en sellulose filters wat met GS en tirosidien ekstrak behandel is.

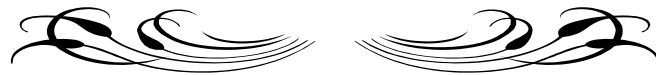
Stabiliteit toetse het getoon dat die tirosidien geadsorbeer en biologies aktief op sellulose filters bly nadat dit blootgestel is aan verskeie water was-stappe, waterwasse by verskillende temperature (25 °C -100 °C) en pH veranderinge (pH 1-12). Die antimikrobiese aktiwiteit was net beïnvloed ná blootstelling aan die water met 'n pH 13, wat moontlik is te danke aan die vatbaarheid van die CL filters by hoë pH oplosmiddels is. 'n Voorlopige studie is gedoen om die uitwerking van Trcs behandelde CL filters op die sterilisasie, ontkieming en tamatiesaailinge te bepaal. Daar is gevind dat Trcs geen effek op die ontkieming het nie, maar dat dit nie volledig die sade en omgewing steriliseer vir fungusiese groei nie. Daar is egter waargeneem dat 5 µg/mL Trcs behandelde filters wortel lengte van die saailinge bevorder teenoor die giftige uitwerking soos waargeneem vir die filters wat met hoër konsentrasies Trcs behandel is.

Dit word gepostuleer dat Trcs verkies om aan hidrofiliese oppervlaktes te bind wat die van die hidrofobiese aminosure en die kationiese residu van die peptied blootstel om aan die bakteriële membraan te bind om gevolglik antimikrobiese reaksie te ontlok. Die blootgestelde deel bevat sommige van die hidrofobiese residue en positiewe Orn⁹/Lys⁹ wat noodsaaklik vir die antimikrobiese aktiwiteit van die peptiede. Die hidrofobiese interaksies is veral belangrik vir die hemolitiese aktiwiteit wat tans die enigste bruikbare metode van opsporing van die geadsorbeerde Trcs is. Trcs het ook 'n tendens vir adsorpsie op sellulose en sellulose analoë wat dui op die moontlike toepassing in beskermende voedselverpakking en die beskerming van houtoppervlaktes. Trcs handhaaf hul antimikrobiese aktiwiteit, ongeag van adsorpsie aan soliede oppervlaktes. Dit kan dus afgelei word dat Trcs-behandelde soliede oppervlaktes die potensiaal het om die aanvanklike kolonisasie van bakterië te voorkom en die daaropvolgende biofilm vorming. Antimikrobiese peptied verrykte soliede oppervlaktes kan dus ontwikkel en aangepas word vir gebruik in spesifieke toepassing soos in filters, kateters en verpakkingsmateriaal.



“Success is not final, failure is not fatal:
it is the courage to continue that counts.”

— Winston S. Churchill



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List of Abbreviations and Acronyms

[M+H] ⁺	singly charged molecular ion
[M+2H] ²⁺	doubly charged molecular ion
ACN	acetonitrile
AMP(s)	antimicrobial peptide(s)
ATCC	American type culture collection
<i>B. aneurinolyticus</i>	<i>Bacillus aneurinolyticus</i>
<i>B. subtilis</i>	<i>Bacillus subtilis</i>
BHI	brain heart infusion
<i>C. albicans</i>	<i>Candida albicans</i>
CFUs	colony forming units
<i>E. coli</i>	<i>Escherichia coli</i>
FTIR	Fourier transform infrared spectroscopy
GS	gramicidin S
HEPES	hydroxyethyl piperazineethanesulfonic acid
HP	High purity
HPLC	high performance liquid chromatography
LB	Luria Bertani
LCMS	liquid chromatography mass spectrometry
<i>L. monocytogenes</i>	<i>Listeria monocytogenes</i>
M	molar
mM	millimolar
<i>M. luteus</i>	<i>Micrococcus luteus</i>
<i>m/z</i>	mass over charge ratio
MALDI	matrix-assisted laser desorption/ionization
MS	electrospray mass spectrometry
NCTC	national collection of type cultures
nm	nanometer
NMR	nuclear magnetic resonance
OD	optical density

Orn	ornithine
O	ornithine
PBS	phosphate buffered saline
<i>P. fluorescence</i>	<i>Pseudomonas fluorescence</i>
Phc A	phenycidine A
Phc(s)	phenycidine(s)
R _f	retention factor
RF-HPLC	reverse phase high performance liquid chromatography
RPMI	Roswell park memorial institute medium
SEM	standard error of the mean
SEM	Scanning electron microscopy
TFA	trifluoroacetic acid
TGS	tryptone glucose and salts culture medium
TOF	time of flight
TpcA	tryptocidine A
TpcB	tryptocidine B
TpcC	tryptocidine C
Tpc(s)	tryptocidines(s)
TrcA	tyrocidine A
TrcA ₁	tyrocidine A ₁
TrcB	tyrocidine B
TrcB ₁	tyrocidine B ₁
TrcC	tyrocidine C
TrcC ₁	tyrocidine C ₁
Trc(s)	tyrocidine(s)
TSB	tryptone soy broth
UPLC	ultra performance liquid chromatography
µg	microgram
µL	microliter

Preface

Agriculture and many industries experience great losses due to persistent bacterial and fungal infections. Various antimicrobial agents, ranging from antibiotics to detergents, are used in an attempt to control and remove these organisms. However, treatment and containment are becoming a problem due to the rising resistance of pathogens in the industrial, agricultural and medical settings to known antimicrobial agents. Studies have indicated that the resistance against antimicrobial agents can be due to an inherent ability of the organism, modification of the bacterial cell target of the antimicrobial agent or the formation of biofilms. Furthermore, removal and eradication of biofilms is accompanied with a range of problems including loss of productivity, increasing costs involved and that it can be time consuming. As a result much effort has gone into developing antimicrobial surfaces that would prevent the initial colonization and subsequent biofilm formation.

Antimicrobial peptides are ideal candidates since it is ubiquitous in nature, has a broad spectrum of activity and are generally stable. The tyrocidines and analogues in the tyrothricin peptide complex, antimicrobial peptides produced by *Bacillus aneurinolyticus*, holds great promise since it is active against a broad spectrum of Gram-positive bacteria, various plant-associated filamentous fungi, the human malaria parasite *Plasmodium falciparum* and as well as the human pathogen *Candida albicans*. Due to its broad spectrum of activity and membranolytic mode of action, that limits the possibility of resistance, tyrocidines are ideal to use in the development of antimicrobial surfaces in various industries. Moreover, if retained on a solid surface it could prevent the colonization of organisms and formation of biofilms. Consequently a peptide enriched solid phase can be developed and tailored to a specific application to not only lower the levels of product loss occurring but also the amount of money lost due to biofilm formation and bacterial/fungal spoilage.

The main goal for this project is to determine the antimicrobial activity of solid surfaces treated with antimicrobial peptides and to develop these surfaces for commercial applications. In order to reach this goal the following objectives need to be met:

1. Purification of tyrocidines from the commercially obtained tyrothricin complex and from amino acid supplemented *Bacillus aneurinolyticus* culture media and the subsequent characterisation of tyrocidines and analogues purified regarding identity, purity and activity (Chapter 2).
2. Characterisation of peptide treated materials regarding the quantification of the amount of peptide bound antimicrobial activity and treated material characteristics (Chapter 3).
3. Treatment of different polymeric surfaces with tyrocidine extract and characterising the retained antimicrobial and haemolytic activity of the surfaces (Chapter 4).
4. Determination of the stability of tyrocidine and tyrocidine analogues adsorbed to cellulose filters and selected materials when exposed to temperature ranges, pH changes and various solvents (Chapter 4).
5. Ascertaining the possible disinfection effect of treated cellulose filters in the application of plant seed germination for plant tissue culture (Chapter 4).

This thesis consists of a literature review, given in Chapter 1, followed by experimental chapters 2-4. All conclusions and summary of future work of this study are presented in Chapter 5. The chapters within this thesis were written as independent units to ease future publication and inevitable repetitions were kept to a minimum.

Outputs of MSc study

- Van Rensburg, W. (2013) Characterisation of natural antimicrobial peptides adsorbed to different matrixes, Biochemistry Forum, University of Stellenbosch, Oral presentation
- Van Rensburg, W. (2015) Characterisation of natural antimicrobial peptides adsorbed to different matrices, Biochemistry Forum, University of Stellenbosch, Oral presentation, MSc defence
- Van Rensburg, W., Rautenbach, M. (2014) Characterization of natural antimicrobial peptides adsorbed to different matrixes. Poster, AMP 2014, Lorient, France
- Van Rensburg, W., Rautenbach, M. (2014) Characterization of tyrocidine, produced by *Bacillus aneurinolyticus*, adsorbed onto different matrixes. Poster, SASBMB conference, Goudini, Western Cape, SA
- Van Rensburg, W., Rautenbach, M. (2015) Modification of polymeric materials with antimicrobial cyclodecapeptides to create sterilising materials. Poster, UNESCO/IUPAC Conference 2015, Port Elizabeth, Eastern Cape, SA
- Rautenbach, M., van Rensburg, W. (2014) Cyclopeptide-Derived antimicrobial compositions and uses thereof. Patent Application No: 2014/04023, Filled: 3 June 2014
- Rautenbach, M., van Rensburg, W. (2015) Method for preventing or treating microbial growth on a manufactured product. PCT patent application PCT/IB2015/054166, Filed 2 June 2015
- Van Rensburg, W., Rautenbach, M. (2015) Stable solid-phase antimicrobial activity of cellulose treated with tyrocidines. Manuscript in preparation for *Acta Biomaterialia*

Chapter 1

Literature review

1.1 Introduction

Colonisation and adhesion of microbial pathogens to solid surfaces (microbial biofouling) is an ever present problem in agriculture, the food industry, the medical field and various other industries. Microbial infections and/or biofilm formation have a negative effect on the system/material it inhabits. This negative effect can be seen in various industries such as post-harvest food spoilage in agriculture, patient infection due to medical instrument contamination such as catheters, persistent contamination of food processing plants etc. However, it is the transfer of pathogens during treatment/utilisation/processing, combined with biofilm associated infections that are of great concern since the formation of biofilms effects the susceptibility of organisms to known antimicrobial treatments.

1.2 Microbial contamination and biofouling

1.2.1 Biofilms

Costerton *et al.* [1] define biofilms as bacterial populations, enclosed in an exopolysaccharide (EPS) matrix, which are adhered to each other and/or a solid surface. The definition presented also includes microbial aggregates and floccules, but also populations attached within porous media. However, biofilm formation is not limited to Gram-positive and -negative bacteria, but can include yeast, fungi and filamentous fungi to form a mono- or mixed culture biofilm [2]. Biofilm formation on any solid surface occurs in three stages: the initial attachment of the cells to the solid surface followed by the formation of microcolonies after which the colonies are enched in an EPS matrix to form mature biofilms [1-3].

The initial adhesion of the cells and subsequent biofilm formation can be influenced by the properties of the solid phase, the growth or bulk media and properties of the cell. Factors that

play a role in the adhesion of the cells regarding properties of the solid phase include the surface texture, hydrophobicity and the conditioning film created by the growth/bulk media [4]. The probability of a cell adhering or colonizing to a specific surface is partially dependent on the surface texture of the solid phase [5] as a rough surface solid phase would offer increased binding surface opposed to a smoother surface. A contributing factor is the hydrophobicity of the solid media. This can be seen with the preference of biofilms to form on hydrophobic, non-polar surfaces like teflon and plastics opposed to polar hydrophilic materials such as glass or metals [6-8]. Increased hydrophobicity of either the solid media or the cell increases the hydrophobic interactions that in turn aid the adhesion of the cell to the solid surface. On a cellular level this is achieved through the presence of fimbriae on the cell. Fimbriae are thought to aid adhesion by overcoming the initial electrostatic repulsion that is between the cell and the solid phase [9] due to the large amounts of hydrophobic amino-acids present in the fimbriae [10]. It has also been found that growth media forms a nutrient film on the solid phase surface that would encourage the initial colonisation of cells. Moreover it has been found that in cases where the solid surface is a nutrient itself, as in the case of cellulose and analogues, microorganisms would be more likely to attach and form biofilms in an aquatic industrial and natural environment [11]. In contrast certain surfaces can have an inherent ability to prevent the adhesion of microorganisms and biofilm formation, as in the case of endothelial cells that contain tissue-associated antibodies [4] and Paneth cell defensins [12, 13] that kill planktonic bacteria on contact.

The properties of the growth media or bulk fluid that influences cell adhesion include the velocity at which the medium flows, pH, temperature, cations, and the presence of antimicrobial agents [4]. In an industrial environment, the forces used during cleaning and disinfecting steps are sufficient to remove biofilms, yet in slower flowing linear systems biofilms can slow down the flow rate [10]. The characteristics of the medium can influence

the scope of microorganism attachment to a solid phase. Factors include pH, nutrient levels, ionic strength and temperature [4]. High levels of cations (sodium, calcium, lanthanum, ferric iron) were found to increase the amount of *Pseudomonas fluorescence* attached to glass surfaces [14, 15]. It was proposed that this was possibly due to the lowered repulsive forces between the glass surfaces and the negatively charged bacterial cell wall caused by the cations present in the medium [14, 15]. It was also found that antimicrobial agents, at lower levels than what is needed to kill planktonic cells, within the growth media affects the adhesion of cells in three possible ways: lowering the expression of fimbriae, effecting swimming and twitching motility and down regulating key quorum sensing systems [16, 17]. Decreased swimming affects the formation of microcolonies, because the cell do not move towards each other whereas increased twitching leads to the cells moving over the solid surface without forming microcolonies [18, 19].

The rate and extent of attachment of the antimicrobial cell is also influenced by the presence of fimbriae, flagella, EPS production and cell surface hydrophobicity [4]. Fimbriae consists of large amounts of hydrophobic amino acids [10] which in turn increases hydrophobicity of the cell surface and will aid the adhesion of the cell to the solid phase by overcoming the initial electrostatic repulsion between them [10]. Apart from the hydrophobicity, cell motility due to the presence of flagella; have been shown to have a significant effect on the formation of biofilms, attachment and even colonisation [20]. Lopez *et al.* [21] conducted a study to determine whether any membrane structures, flagella or pilli, could influence the formation of biofilms and found that *Pseudomonas aeruginosa* contains type IV pilli. Type IV pilli are used for a type of surface-motility called twitching that could possibly be important for the formation of microcolonies [22]. Pilli and fimbriae are important to attach cells to each other and to attach the cell to the solid surface [21]. Once microcolonies are formed the genes transcribing for the production of the extracellular matrix are activated [23] which will result

in the microcolonies developing into mature exopolysaccharide enmeshed biofilms. Once formed, the biofilms are difficult to remove due to a lack of accessibility to the biofilm and rising resistance of biofilms against cleaning agents.

1.2.2 Other types of microbial contamination of surfaces

Microbial contamination of surfaces is not limited to the adhesion of bacteria to surfaces and the subsequent biofilm formation, but can also present itself in the form of fungal spoilage as in the case of wood, fruits and vegetables. Wood biodeterioration entails the breakdown of lignin, cellulose and hemicellulose within the wood by soft-, brown- and white-rot fungi.

Brown rot fungi predominantly break down cellulose and hemicellulose [24]. The breakdown of hemicellulose produces H_2O_2 that breaks down cellulose, but also is small enough to diffuse through the wood spreading the damage farther than the hyphae [24-26]. Brown rot fungi include *Postia placenta* [27], *Neolentinus lepideus* [27], *Gloephyllum trabeum* [28] and *Laetoporeus sulphureus* [28]. White rot fungi break down lignin and cellulose by producing enzymes such as lignin peroxidase [29], Mn-peroxidase [30] and laccase [31, 32]. The combination of enzymes produced differs between fungal species. Examples of white rot fungi are *Pharerochaete chrysosporium* [29, 33], *Lentinula edodes* [34] and *Rigidosporus lignosus* [31, 32]. Soft rot fungi produce cellulase from the hyphae for the breakdown of cellulose. Soft rot fungi include *Chaetomium globosum* [35], *Aspergillus niger* [36] and *Penicillium chrysogenum* [36]. The general concern regarding the biodeterioration of wood is the loss of structural integrity which has negative effects in its application as is the case of wood used in the structural support of roofs, docks and crates which not only causes financial losses but can also present safety issues.

Fruits generally have a low pH around pH 2-3, whereas vegetables can be around pH 4-7, which makes vegetables susceptible to both fungal and bacterial infection whereas fruits are

mostly infected by fungi [37]. Bacterial species connected to postharvest spoilage are *Erwinia* spp., *Pseudomonas* spp. and *Xanthomonas* spp., all of which are Gram negative organisms. An example of bacterial postharvest spoilage can be seen with endives that are susceptible to *Pseudomonas marginalis*, isolated the most of the *Pseudomonas* spp. and *Erwinia herbicola* infection [38]. *Pseudomonas* spp also infects celery and cabbage [39]. *Xanthomonas* spp have been shown to infect bell peppers, papaya, tomato and cucumber to name a few [40].

Fungal species involved in postharvest infection and subsequent spoilage are as vast as the list of possible targets. As examples: apples can be infected with *P. expansum*, *Botrytis cinerea*, *Rhizopus stolonifer*; strawberries by *Botrytis cinerea*, *Rhizopus* spp. [41., 42]; papayas by *Colletotrichum gloeosporioides* [43, 44]; citrus fruits by *P. digitatum*, *P. italicum* and sweet cherries by *P. expansum*, *Rhizopus* spp., *Monilinia* spp., *Cladosporium herbarum* and *Alternaria* spp. [45]. It is thus evident that fruit and vegetables are vulnerable to post harvest infections which would ultimately lead to crop losses. However, the negative result of infection is not limited to postharvest crop losses but includes the harmful effects of mycotoxins, a secondary metabolite produced by some fungi. *P. expansum* produces a mycotoxin named patulin that is mutagenic, genotoxic, neurotoxic and cause immune suppression should the contaminated fruit/vegetable be ingested [46].

1.3 Antimicrobial agents in biofouling

1.3.1 General antimicrobial agents

Microbial biofouling within agriculture, the medical field and other industries is becoming a great problem and have repercussions ranging from loss of product and subsequent increased cost to company within agriculture to persistent infection and sometimes death within medicine. The biggest problem with microbial biofouling, regardless on the industry, is that

once the biofilm has formed and matured it is very difficult to remove. Alternatively infections in natural material such as wood and paper destroy the integrity of the material. As a result much emphasis is placed on treating surfaces and developing surfaces that have an antimicrobial activity to prevent or postpone the initial adhesion of pathogens. Non-fouling surfaces can include surfaces that have an inherent antimicrobial activity such as copper [47] and silver surfaces [48] or antimicrobials attached to the surface such as poly(quaternary ammonium) compounds [49], polyurethanes [50], vancomycin or gentamycin [51].

Post-harvest infections are responsible for 24% crop loss within the USA [52] and 50% crop loss in underdeveloped tropical countries [53]. Preventative measures against post-harvest spoilage include fumigation, dips or washes, chemical sprays or the use of antimicrobial treated box liners, shredded paper or wrappers. Although many treatments exist it is matched by the same problems of antimicrobial spectrum, efficacy, resistance, retained residue above legal limits and produce damage. One of these is the use of biphenyl in wrappers and box liners that only inhibits the spore formation and vegetative growth of citrus pathogens and stimulates the growth of other fruit and vegetable pathogens [54]. Another example is orthophenylphenol in wrappers that, although effective against fungal infection, cause injury and scalding of the fruit [55]. The most prominent drawback of all the preventative measures used is the damage of fruit and vegetables by the active compound used in the treatment. There is therefore a great need for an antifungal treatment or treated wrappers that do not have a damaging effect on the produce.

Microbial biofouling in medicine exists in the form of biofilm formation on surgical implants to catheters [56, 57]. In both cases the removal of the biofilm is difficult since it forms in hard to reach places and the amount of treatment used is determined by the cytotoxicity to the patient. Thus it is crucial to prevent the adhesion of the pathogens altogether. Catheters are

commonly made out of either silicone or latex and it has been found that the hydrophobicity of both the material and organisms determine the type of organisms found in the biofilm [58]. Organisms known to be found in catheter biofilms include *Staphylococcus epidermis*, *Enterococcus faecalis*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*. In a recent study researchers have managed to prevent the adhesion of three pathogens, *Pr. mirabilis*, *S. aureus* and *E. coli*, by impregnating the catheter material with a combination of rifampicin, sparfloxacin and triclosan, opening the door for the use of antimicrobial agent impregnated materials in medicine [59].

Food processing plants easily fall victim to the formation of biofilms of which *Listeria monocytogenes* is commonly associated. Cleaning steps within the plant is generally enough to remove the formed biofilm, however, it is not enough to prevent the formation of biofilms within hard to reach places such as gaskets, valves, joints etc. Furthermore *Listeria spp.* can be reintroduced into the cleaned plant from environmental surfaces (walls, floors etc.) if it is not kept clean. Moreover, studies have shown *Listeria spp.* to become resistant to cleaning agents used within the food processing industry which is a concern since it is responsible for 28% deaths in the USA caused by the intake of contaminated food [60].

1.3.2 Antimicrobial peptides

Antimicrobial peptides (AMPs) have the potential to be the next generation antibiotics and biocides (sterilisation agents). A number of extensive reviews have been written on AMPs over the last two decades [61, 62], therefore only a brief overview will be given on these antimicrobial compounds. Antimicrobial peptides (AMPs) can originate from various sources since it is ubiquitous in nature. It can be found in lactic acid bacteria producing Class I (e.g. nisin) [63-65] and Class II (e. g. pediocin) lantibiotics [66], in fungi producing fungal defensins (e. g. plectasin) [67], in plants producing plant defensins (e.g. thionins and plant

defensins) [68, 69], in insects (ie cecropins) [70, 71], amphibians (e.g. magainins) [72-74] and in higher vertebrates as the cathelicidins (e.g. Human LL-37) [75, 76] and defensins [12, 77, 78]. General characteristics include a broad spectrum of activity usually in the micromolar range or lower, amphipathic structure and a net positive charge to aid in the association to negatively charged microbial cell walls membranes. AMPs differ in mode of action and are not limited to one method of eliciting its antimicrobial activity. Mode of action can be against outer surface lipids (e.g. human beta-defensin 3 against lipid II)[79], outer-membrane proteins associated with Gram negative organisms (e.g. sheep myeloid antimicrobial peptide 29 against outer-membrane protein 1)[80], the inner membrane forming pores (e.g. almethicin)[81] or having a detergent micellization effect (e.g. dermaseptin B)[82], the integral membrane proteins (e.g. clavanin A against proton translocation related proteins)[83], DNA (e.g. lindolicidin)[84], RNA (e.g. Bufarin 2)[85] and intracellular proteins (e.g. pyrrocorricin that inhibits the ATPase actions of DnaK)[86].

In general, after more than three decades of research, the reports of resistance of pathogens against AMPs has been limited and it has been found that AMPs hold promise as a potent anti-biofilm treatment. Human LL-37 has activity against both Gram-negative and Gram-positive organisms, showing an inhibitory effect against planktonic cells and biofilm formation of *Francisella novicida* (tularemia disease) [87]. Moreover it has been observed to inhibit the biofilm formation of *L. monocytogenes*, *P. aeruginosa*, and *Burkholderia cenocepacia* and cause a 50% decrease in biomass of mature biofilms compared to controls [88]. Lactoferrin was observed to prevent the formation of *P. aeruginosa* biofilms at lower concentrations than needed to kill planktonic cells [89]. OSIP108, a decapeptide of plant origin, was shown to inhibit the formation of *Candida albicans* biofilms, with no effect on the growth or cell viability of cells or cytotoxicity to human cells [90]. It was suggested by the study to be implemented in combination with other antifungal peptides that would kill the

planktonic *Candida albicans* cells. The use of a combination strategy against *C. albicans* biofilms was reported with tyrocidines, antimicrobial peptides discussed later, and amphotericin B and caspofungin. It was observed that tyrocidines enhanced the antimicrobial activity of both amphotericin B and caspofungin resulting in lower concentrations of the antimicrobial agents needed to elicit a similar response without tyrocidines present [91].

The attachment of AMPs to solid surfaces could present a possible solution based on its broad spectrum of activity and in most cases a membranolytic mode of action which makes the development of resistance less likely. Antimicrobial surfaces can be developed for a specific application provided that the attached AMPs maintain stability and activity regardless of environmental changes found in the specific industry. AMPs can be covalently attached or adsorbed to solid surfaces depending on the intended purpose of the antimicrobial surface.

Covalently bound antimicrobial peptides would be beneficial for example medical implants since the surface need to maintain activity without antimicrobial leaching that could have a negative effect on the host. A medical implant surface coupled with a synthetic peptide, Tet-20 (KRWRIRVRVIRKC) showed a broad spectrum of activity *in vitro* and *in vivo*, prevented biofilm formation and was not cytotoxic to the host cells [92]. A study done by Bagheri *et. al.* [18] showed that prior knowledge of the mode action of the AMPs was crucial since it would determine the position of attachment on the surface, but also whether attachment to a solid surface would hinder the peptide's activity. The authors suggested that mainly membranolytic peptides be selected for covalent binding since peptides with other modes of action might not reach its intended cell target that would consequently lower the known antimicrobial activity. The other form of attachment, namely adsorption, can be used in cases where tethering disrupts the antimicrobial activity, where peptides have a cell target other than the membrane

and need to diffuse from the solid surface to be active, where leaching is not a concern and the solid surface is not intended to remain active.

1.3.3 *The tyrocidines and analogues*

A substantial number of antimicrobial peptides are produced by microorganisms [61, 62]. Dubos and Hotchkiss [93] isolated the first group of antimicrobial peptides, an antibiotic complex called tyrothricin, in the early 1940's from a *Bacillus aneurinolyticus* culture (also known as *Brevibacillus parabrevis*, originally denoted *B. brevis* [94-98]). Tyrothricin was discovered a decade after penicillin, but was the first antibiotic (or antimicrobial peptide complex) to be implemented for clinical use as a topical antibiotic. The tyrothricin complex comprises of tyrocidines, a group of cationic cyclic decapeptides ($\pm 60\%$), and the neutral linear gramicidins ($\pm 40\%$) [99]. Although tyrothricin has both haemolytic activity and is leukocytolytic, it was shown that it has very little toxicity when applied topically [100, 101]. The interest in using tyrocidines for the development of solid surfaces lies therein that studies have shown tyrocidines to have a broad spectrum of antimicrobial activity against the human malaria parasite *Plasmodium falciparum* [102], Gram positive bacteria [103], yeasts such as *Candida albicans* [91] and filamentous fungi [104]. Furthermore, our group has observed that the tyrocidines (Trcs) adsorb to various surfaces during purification and preparation for activity assays resulting in peptide loss and lowered activity, respectively. The group of cyclic decapeptides, the Trcs and Trc analogues, is therefore the focus of the research reported in this dissertation and will be discussed in more detail in the following sections.

1.3.3.1 Tyrocidine and analogue structure

The general structure of tyrocidines (Trcs) are $cyclo-[f^1P^2X^3x^4N^5Q^6X^7V^8O^9L^{10}]$ and is conserved with variances occurring at the aromatic dipeptide moiety ($X^3x^4 = Trp^{3,4}/Phe^{3,4}$; lower case denotes D-amino acid residue) which is part of the variable pentapeptide moiety

(X³X⁴N⁵Q⁶X⁷), at the cationic residue in position 9 (Lys⁹/Orn⁹) and at the aromatic residue in position 7 (Tyr⁷/Phe⁷/Trp⁷) [105, 106] (Figure 1.1, Table 1.1). The Trp/Phe^{3, 4} and Tyr⁷ substitutions denotes Trc A-C, the Lys/Orn⁹ substitution denoting Trc A₁-C₁ and the Tyr⁷/Phe⁷/Trp⁷ substitution either peptides from Trc, phenycidine or tryptocidine group. Possible Trcs and analogues found within the produced peptide profile are summarised in Table 1.1.

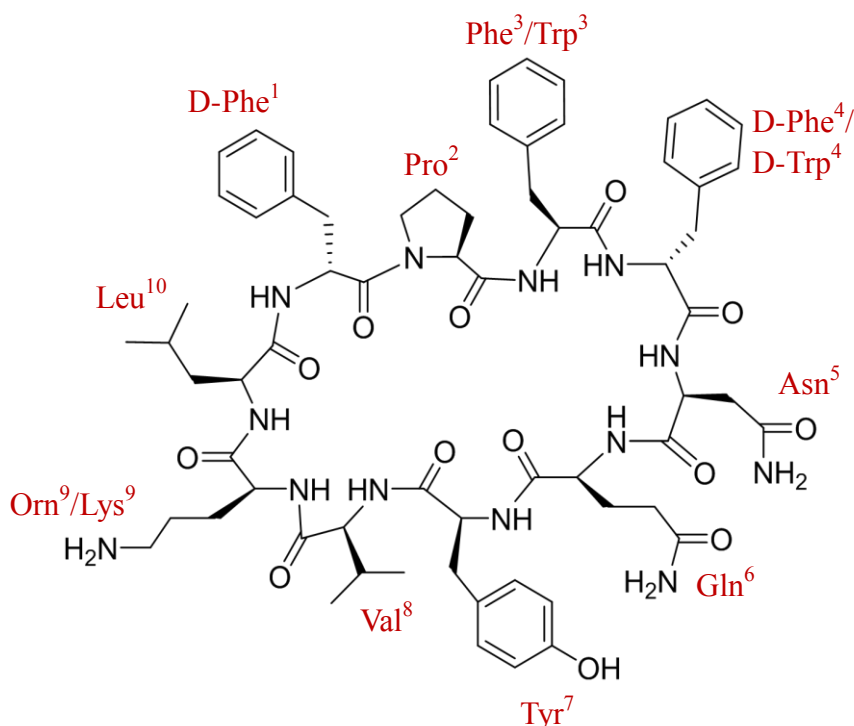


Figure 1.1: Structure of tyrocidine A with variable residues Trp³ and Trp³-DTrp⁴ in TrcB and TrcC respectively and Lys⁹ in TrcA₁, B₁ and C₁

The peptides have a β -turn/sheet structure that contributes to dimerization and the consequent amphipathic character of the peptide [107, 108]. This in turn aids the peptide's association with bacterial membranes, followed by the proposed destabilization of the membrane that then leads to cell death [109]. Its β -sheet structure with intra-molecular hydrogen bonds lends structural rigidity to the peptide that it is unaffected by temperature and solvent composition [110, 111]. Studies have indicated that the loss of biological activity is dependent on the

Table 1.1: Summary of expected Trcs and Trc analogues within the peptide profile

Identity	Abbreviation	Sequence ^a	Theoretical Monoisotopic M _r ^b
Major Trcs			
Tyrocidine C ₁	TrcC ₁	<i>Cyclo</i> -(fPWwNQYVKL)	1361.692
Tyrocidine C	TrcC	<i>Cyclo</i> -(fPWwNQYVOL)	1347.676
Tyrocidine B ₁	TrcB ₁	<i>Cyclo</i> -(fPWfNQYVKL)	1322.681
Tyrocidine B ₁ '	TrcB ₁	<i>Cyclo</i> -(fPFwNQYVKL)	1322.681
Tyrocidine B	TrcB	<i>Cyclo</i> -(fPWfNQYVOL)	1308.655
Tyrocidine B'	TrcB	<i>Cyclo</i> -(fPFwNQYVOL)	1308.655
Tyrocidine A ₁	TrcA ₁	<i>Cyclo</i> -(fPFfNQYVKL)	1283.670
Tyrocidine A	TrcA	<i>Cyclo</i> -(fPFfNQYVOL)	1269.653
Tyrocidine Analogues			
Tryptocidine C	TpcC	<i>Cyclo</i> -(fPWwNQWVOL)	1370.692
Tryptocidine C ₁	TpcC ₁	<i>Cyclo</i> -(fPWwNQWVKL)	1384.708
Tryptocidine B	TpcB	<i>Cyclo</i> -(fPWfNQWVOL)	1331.681
Tryptocidine B ₁	TpcB ₁	<i>Cyclo</i> -(fPWfNQWVKL)	1345.697
Tryptocidine A	TpcA	<i>Cyclo</i> -(fPFfNQWVOL)	1292.647
Tryptocidine A ₁	TpcA ₁	<i>Cyclo</i> -(fPFfNQWVKL)	1306.686
Phenycidine A	PheA	<i>Cyclo</i> -(fPFfNQFVOL)	1253.660
Phenycidine A ₁	PheA ₁	<i>Cyclo</i> -(fPFfNQFVKL)	1267.675
Linear Gramicidins			
Val – Gramicidin A	VGA	VGAIAvVvW-IWIWIW	1881.078
Val – Gramicidin B	VGB	VGAIAvVvW-IFIWIW	1842.067
Val – Gramicidin C	VGC		
Gramicidin S	GS	<i>Cyclo</i> -(fPVOLfPVOL)	

^a Peptide sequences (Tang *et al.*, [106]) represented with conventional one letter abbreviations with the exception of Orn which is denoted with O. D-form amino acids and in Uppercase, L-Form is in lower case.

^b Theoretical monoisotopic M_r calculated from the molecular weights of all the constituent amino acids.

concentration of Trcs due to aggregation [111] caused by the amphipathicity of the molecule [110]. The formation of micelles can be strengthened by the addition of an organic acid to the solvent and the use of and polar solvent [110]. Trcs form supermicelle structures through non-covalent bonds between the different peptides [112]. TrcA, TrcB and TrcC forms homo-polymers and hetero-polymers were as TrcB readily associates with TrcC [113]. The cyclic

structure of the peptide has been shown to play a key role in this self-association since no association and subsequent aggregation was observed for the linear form of the Trcs [113].

Gramicidins, produced as part of the tyrothricin complex, are neutral linear pentadecapeptides that can be divided in three groups (Table 1.1): gramicidins A, B and C. The general structure (gramicidin A) is HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-NHCH₂CH₂OH. The structure of each gramicidin differs on position 11 with Phe for gramicidin B and Tyr for gramicidin C. In each group of gramicidins a difference occurs on position 1 with either incorporating Ile or Val [95].

Gramicidins A, B and C should not be confused with gramicidin S (GS). GS is produced by *Aneurinibacillus migulanus* (previously known as *Bacillus brevis*) and it consists of two identical pentapeptides to form one cyclic decapeptide. The pentapeptide sequence is D-Phe-L-Pro-L-Val-L-Orn-L-Leu and one of these pentapeptide moieties also occurs in the tyrocidines and analogues (Table 1.1). It is important to note the occurrence of D-Phe and L-Orn which are not commonly found in proteins [95]. GS shares the broad spectrum of activity with the Trcs, but also has activity against Gram-negative bacteria [114].

1.3.3.2 Tyrocidine and analogue synthesis

Trcs and analogues are non-ribosomally produced [115] by *B. aneurinolyticus* during the late logarithmic growth phase under oxygen limiting conditions [116]. Production is achieved by the sequential addition of amino acids available within the growth medium into the Trc structure by a polyenzyme complex [117, 118]. Substitution of amino acids at the four variable sites occurring within the Trc structure (refer to Figure 1.1 and Table 1.1), giving rise to the various Trc analogues, can be attributed to the low specificity of the enzymes involved with the recognition and incorporation of the specific amino acids, in particular the

aromatic residues in the variable pentapeptide sequence (xXNQX; x, X denotes D- and L-aromatic amino acid residues, respectively) [118].

As a result the peptide production profile can be shifted with the addition of selected amino acids to the culture medium of the producer organism. Studies have shown that phenylalanine (Phe) and tryptophan (Trp) are the only amino acids that have an effect on the peptide profile but also negatively influence the initial growth of the organism. [119]. Supplementation of the growth medium with Phe leads to the production of mainly TrcA and analogues (TrcA, TrcA₁, PheA) suppressing the production of TrcB, TrcC and VGA. This is beneficial since TrcA is difficult to purify in the presence of VGA. Supplementation with Trp leads to the predominant production of TpcC and analogues (TrcC, TrcC₁, TpcC₁) with an increase in the production of VGA [98, 119]. Even though the full biological function of Trcs to the producing organism is still unknown, the consensus is that it is a key player in sporulation and defence [120-123].

Stokes and Woodward [124] did a study on the effect different nitrogen sources have on the production of tyrothricin and how stationary cultures and submerged (fermenter based) cultures differ in production. They found that stationary cultures produced tyrothricin whereas the fermenter-based counterpart produced ample cells but very little tyrothricin. The development of synthetic media showed that the addition of asparagine gave the best growth of *B. aneurionolyticus* in stationary cultures. The synthetic medium contained, other than the nitrogen source: K₂HPO₄, MgSO₄·7H₂O, NaCl, MnSO₄·4H₂O, FeSO₄·7H₂O, CaH₄(PO₄)₂·H₂O with separately autoclaved glucose to prevent the toxic effect seen with caramelization of glucose. It was observed that the pH of the cultures are commonly pH 8.4, rising from the initial pH of 7.

1.3.3.3 Biological activity of the tyrocidines

Tyrocidines have a very broad spectrum of antimicrobial activity ranging from Gram positive bacteria to fungi and yeast. In 2007 Hoppe *et al.* [102] isolated six Trcs (A, A₁, B, B₁, C, and C₁, see Table 1.1 and Fig. 1.1 for details on structures) from a commercially available tyrothricin complex which all showed antimicrobial activity against the human malaria parasite *Plasmodium falciparum*. The authors also noted that an increase in hydrophobicity was related to the anti-plasmodial activity concluding that hydrophobicity plays a major role in the anti-plasmodial activity of the Trcs. It has been found that Trcs are active against *L. monocytogenes* in the micromolar range, also that the antimicrobial activity was strain specific and dependent on the peptide structure [103, 125]. It was found that the more polar TrcB and TrcC showed more activity against *L. monocytogenes* compared to TrcA [103, 125]. Trc also readily inhibits the metabolic processes in two Gram-positive organisms, *S. aureus* and *Streptococcus haemolytic* [105]. To combat streptogramin resistance, a streptogramin-Trc antibiotic was synthesised that possesses potent antibacterial activity, but remains unaffected by resistance mechanisms developed against streptogramin [126].

Studies have shown Trcs to have an eight fold better activity against *Fusarium solani* and four fold better activities against *B. cinerea* when compared to a known anti-fungal bifonazole and are known to affect the morphology of the fungi causing hyperbranching upon treatment [104]. Trcs have also been shown to cause cell leakage from *Neurospora* hyphae [127]. The antifungal activity have also been shown to be unaffected in the presence of Mg²⁺, Na⁺ and K⁺, but is negatively affected by the presence of Ca²⁺ [104]. With *C. albicans* as target, Trcs have exhibited activity against planktonic cells (in the micromolar range), were able to prevent the formation of *C. albicans* biofilms and cause the disruption of *C. albicans* cells found in mature biofilms [91]. Trcs have also been shown to have synergism with

caspofungin and amphotericin, commonly used antimycotics, against *C. albicans* decreasing the amount needed to kill 50% of the biofilm 12-fold and 9-fold respectively [91].

Trcs are toxic when injected in to the bloodstream since it is both haemolytic and leukocytolytic [101], but show very little toxicity when applied topically [100]. Moreover, it is most effective when in direct contact with the infecting bacteria since Trcs lose their activity in the presence of blood and serum [128]. Early studies on the antibacterial activity of tyrothricin against staphylococci, streptococcus and pneumococcus have shown that the activity observed *in vitro* does little to predict the amount of peptide needed at a local infection level. This change in concentration Trcs needed to elicit a response was attributed to either resistant organisms or the occurrence of substances found at the wound site that inhibits the activity of tyrothricin against the organism [129].

Trcs also play an integral part in the sporulation of the producing organism, *B. aneurinolyticus*. When the condition under which the organism has been cultured inhibits the production of peptide, no sporulation is observed [130, 131]. Also with the addition of tyrothricin to the culture media the growth of the organism is stopped (spore formation), but it has no effect on the culture if it is in stationary growth phase [121].

In a study where the producer organism was transferred (exponentially growing cells) to a nitrogen-free medium, it was shown that the addition of Trc (concentration dependent at 5 μ M) to the growth medium induced the sporulation of the organism and production of linear gramicidins [120, 122, 132]. This correlates with the finding that the production of Trcs is followed 1-2 hours later by the linear gramicidins in a nutrient rich medium [133] since both linear gramicidin and Trcs are involved in spore formation [123]. The addition of Trc had three observed biological consequences. The first was the observed complete loss of intracellular ATP possibly linked to the antibiotic properties of Trcs causing cell leakage.

Other studies have suggested that this depletion of ATP is not linked to the observed Trc induced sporulation for various reasons. The addition of GS, having a similar surfactant mode of action, causes ATP and cell leakage but not sporulation [120, 122]. The addition of Trc to the cells, when sporulation can no longer be induced, caused depletion in ATP but no subsequent sporulation and the synthesis of Trc by the producer organism is not accompanied by a lowering of ATP levels within the cell [120, 122]. The second effect the addition of Trcs has is the increased use of exogenous nucleotide precursors possibly due to the depletion of intracellular ATP and nucleotide levels. A sharp decrease in RNA synthesis proposed to be responsible for the repression of vegetative genes [120, 122]

1.3.3.4 Tyrocidine mode of action

Upon its isolation in 1939, Trcs were thought to be primarily membranolytic in action and shown to disrupt membrane permeability [134-136]. Studies on the effect of Trcs against *Neurospora* hyphae indicated damage to the membrane resulting in the loss of cell contents, such as intercellular K⁺, various small metabolites, as well as non-dialyzable molecules and the destruction of the membrane potential [127]. Trcs are also considered specific surface active haemolysins binding to a component in the erythrocytes membrane and leading to subsequent haemolysis [137]. VGA are also found to antagonise the membrane disruption activity of Trcs [135].

Even though Trcs were thought to be mainly membranolytic, studies have indicated that there might be other modes of action to which membrane lysis is just a result. Trcs inhibit RNA synthesis both *in vitro* and *in vivo*, but have no effect on DNA synthesis [120, 131]. The peptides do this by forming complexes with DNA resulting in hypochromicity, which in turn inhibits the transcription of RNA [120, 138]. One hypothesis is that Trcs binds to GC rich regions within the DNA but that this is also dependent on the conformation of the DNA

[139]. The effect can be reversed by the addition of linear gramicidins, even though gramicidins on their own inhibit transcription of RNA, which weakens the association of Trcs to the DNA [121, 138, 139]. This antagonising effect of linear gramicidins can also be seen with gramicidin A that inhibits the disruption of model membranes by Trcs [135]. It is suggested that linear gramicidins and Trcs form complexes within themselves with a higher possibility than to bind to DNA. This interplay between Trcs and linear gramicidins on the effect of DNA/transcription possibly clarify the peptides effect on sporulation in that some genes need to be switched on and others not [138]. The cyclic structure of the peptide has been shown to play a key role in this association since when the ring was broken no association occurred [110, 139].

Kuo and Gibbons [140] suggested the activity of Trcs be centred on the idea of a hydrophobic (Val⁸, Leu¹⁰, D-Phe¹, Pro², Phe³ and D-Phe⁴) and hydrophilic region (Asn⁵, Gln⁶ and Tyr⁷). The biological value is unknown, but the disruption thereof leads to almost complete loss on activity. An acetylation on the positive residue (Orn⁹/Lys⁹) leads to a decrease in binding to the enzyme suggesting the importance of the positive charge for the initial association of Trcs to membranes [141]. Structure-activity studies have shown that Trc A with a mutation in sequence at position 3, with Val, showed a twofold decrease in activity (MIC in µg/mL) against *B. subtilis* 168 [96] and a substitution with glycine, at the same position, showed a fivefold decrease compared to the Val substitution [142]. The Val substitution also showed lower inhibition of active membrane transport and *in vitro* transcription. A substitution at position 4, with ornithine, showed an almost complete loss in activity against *B. subtilis* and eliciting no effect against active membrane transport and *in vitro* transcription [96]. Thus highlighting the need to conserve the dipeptide unit (Phe³/Trp³, Phe⁴/Trp⁴), in order for Trcs to elicit antimicrobial activity. There is, however, some preference of the analogues to elicit an antimicrobial response. TrcB and TrcC are more active against Gram positive organisms

[103] whereas TrcA has a greater activity against *Plasmodium falciparum* [102]. Orn analogues have a greater activity opposed to Lys containing analogues [102, 103, 125] since Lys amino group remains associated with the head groups in the aqueous phase while the longer butylene chain interacts with the acyl chains [143] which is detrimental to its activity. Tyr and Phe containing analogues (Tres and Phcs) is more active than Trp containing analogues (Tpcs) which can be possibly attributed to the Trp causing steric hindrance that interferes with target interaction [125]. Trp has a shallow insertion into the target cell membrane and has a tendency to form hydrogen bonds (NH group) with fatty acyl carbonyl groups [144] whereas Phe that inserts deeper [145, 146].

1.5 Antimicrobial Resistance

As previously discussed, various antimicrobial agents are isolated, created or developed to counter surface contamination, infection, biofilm formation or eradication of mature biofilms. However, the success and continuous application of each antimicrobial agent or combination solution hinges on the possibility of resistance developing against the antimicrobial strategy. Though the ideal would be that pathogens do not have any resistance to any form of antimicrobial agents, it is unrealistic. Resistance can occur in either a passive method, entailing an inherent mechanism that is present even in the absence of AMPs, or in an induced method, entailing a response to the AMPs or the cell stress it causes [147]. The mode of action of the AMPs will determine the ease at which the target organism will develop resistance. It can develop through the hydrolytic inactivation of the AMPs which can be seen with human pathogens *P. aeruginosa*, *E. faecalis*, *P. mirabilis* and *S. pyogenes* that produces proteinases that hydrolyses LL-37 [148]. Resistance can occur by the extracellular trapping of the AMPs preventing access to the cell which can be seen with *S. aureus* against defensins, protegrins and lantibiotics [149] where *S. aureus* produces staphylokinase that inhibits the action of α -defensins through complex formation [150]. This was also observed for *B. subtilis*

strains that produce surfactin, an anionic lipopeptide that forms complexes with cationic GS rendering this potent AMP inactive [151]. Exogenous surfactin was also observed to protect non-surfactin-producing *B. subtilis* strains against the membranolytic activity of GS [151]. This resulted in surfactin and gramicidin S producers being able to be cultured together. Resistance can also develop through the use of a drug exporter as can be seen with *Yersinia spp.* that have an efflux pump and potassium anti-porter system that consists of RosA and RosB proteins. These proteins are activated by a temperature shift to 37°C but also the presence of cationic antimicrobial peptides such as polymyxin B, norobiocin, rifampicin and tetracycline. The efflux pump has a dual action of pumping the antimicrobial peptides out of the cell and acidifying the cytoplasm which would inactivate the antimicrobial peptides preventing its antimicrobial action [152]. The cell envelope can also be modified as is the case of *Salmonella spp.* that elicits its resistance through the use of PhoP-PhoQ virulence regulators that increases the acylation of lipid A, that is a major component of the outer membrane which in turn prevents the membranolytic activity of antimicrobial agents against the organism [153]. Pathogens can also use a combination strategy to become resistant to AMPs such as the human pathogen *Neisseria meningitidis* that achieved resistance against polymyxin B with a combination of lipid A modification and MtrC-MtrD-MtrE (*mtr*) efflux pump [154].

Resistance can also occur due to the formation of biofilms. This resistance against treatment with detergents, biocides, antibiotic, bacteriophage and amoebae [1, 21], as seen in industrial, medical and agricultural environments, can be due to various reasons which include slow growth rate of the microbes in the biofilm, slow penetration of treatment due to the extracellular matrix and possible changes brought about in the organisms' physiology by binding to a solid surface [2].

Studies have found that the growth rate of organisms found in the biofilms have a much slower growth rate than planktonic cells, but also that biofilms found in the lower part of the biofilm had slower metabolism than cells found on the outside of the biofilm. This is possibly due to oxygen limitation within the biofilm. Another factor contributing to difference in metabolism between cells is nutrient limitation/starvation, since the cells on the outside would be in direct contact with the growth media, in an industrial setting, that would lead to a higher metabolism compared to the inside cells [21, 155]. The slower metabolism of the inner cells also causes a slower uptake of the antimicrobial agents and in effect decreasing the activity of the antimicrobial agent [156]. This explains why biofilms are not susceptible to minimum inhibitory concentration (MIC) levels of antimicrobial agents as determined in their planktonic cultures. MIC values are determined with planktonic cells in the exponential growth phase, in effect testing the activity of an antimicrobial agent against rapidly dividing cells [156], whereas biofilms are slow growing and less susceptible to the antimicrobial agent. Planktonic cells, grown from cell isolated from a biofilm, regain antimicrobial agent sensitivity once diluted in fresh media [16]. The hypothesis is that the fresh media do not only supply the cells with fresh nutrients but also removes protective cell-cell signalling [16] concluding that the predominant resistance is due to the cells being in a biofilm.

Cells in biofilms produce an EPS matrix that covers the biofilm, but also attach the cells to each other [2, 21]. Furthermore this matrix limits the diffusion of antimicrobials in to the biofilm by creating a diffusion barrier and limiting the activity only to the cells on the surface [2, 21, 156]. Negatively charged matrices also bind to positively charged antibacterial compounds and can prevent its activity as in the case of extracellular alginate from *P. aeruginosa* [17]. In general the diffusion or penetration of the antimicrobial agent into the polysaccharide matrix is greatly dependent on the biofilm and the antimicrobial used [157].

Once cells are associated to a solid surface the cells undergo a change in phenotype to a protected mode or growth, biologically programmed to exist once adhered/growth on a solid surface [2, 156]. Resistance can also possibly be due to the presence of persister cells. These cells, although they are genetically identical to the rest of the colony are protected against antimicrobial agents due to the ability to secrete an anti-toxin that block the antimicrobial agents [21]. Some cells can produce secondary metabolites during the stationary phase: antibiotics, pigments, other small molecules which serve as a signalling molecule to initiate biofilm formation or biofilm formation of other organisms in the same area [21].

Trcs have a broad spectrum of antimicrobial activity, has shown activity against mature *Candida* biofilms [91], as well as *L. monocytogenes* biofilms (personal communication A. Leussa and M. Rautenbach). Although tyrothricin, containing the Trcs, has been in medical use for more than 70 years it is interesting to note that no resistance has been reported against this peptide complex or the individual peptides in the complex. Resistance is less likely to develop due to these peptides' mode of action being both membranolytic and directed at intra-cellular targets. This makes Trcs ideal candidates for the development of antimicrobial surfaces that can be used to combat surface contamination or be used directly as a protective treatment for example wood or leather.

1.7 References

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Chapter 2

Production, purification and characterisation of tyrocidines and analogues produced by *Bacillus aneurinolyticus*

2.1 Introduction

The rising resistance to currently used antimicrobials have led to the dire need for the discovery and development of novel antimicrobials. Tyrocidines (Trcs) are small cyclic decapeptides with a broad spectrum antimicrobial activity against which resistance is less likely to develop due to its membranolytic mode of action [1-3]. Current research has been limited to discovering the mode of production of Trcs [4-6] and investigating its antimicrobial activity against Gram-positive bacteria [1, 7, 8], the malaria parasite [9] and fungi [10-12]. To date very little research was done on the activity of Trcs and Trc analogues absorbed or attached to surfaces. Based on its broad spectrum of activity and subsequent broad spectrum of application, the aim of this study was to determine the activity and stability of Trc impregnated solid surfaces with the end goal of developing a green application in either agriculture or industrial setting.

Commercial tyrothricin contains both Trcs and linear gramicidins. The latter could be removed with an established organic extraction method [13] resulting in a pure Trc extract that could be used as a control in antimicrobial assays. The tyrothricin peptide complex produced by *B. aneurinolyticus* consists of neutral pentadecapeptides, linear gramicidins, and the basic/cationic cyclic decapeptides, Trcs. The Trc sequence (TrcA) consist of *cyclo*-[f¹P²F³f⁴N⁵Q⁶Y⁷V⁸O⁹L¹⁰] and is conserved with variances occurring at Trp^{3,4}/Phe^{3,4}, Lys⁹/Orn⁹ and Tyr⁷/Phe⁷/Trp⁷ [14, 15]. The production of Trcs and Trc analogues with *Bacillus aneurinolyticus* in aromatic amino acid supplemented media results in a shift of the peptide production profile favouring the production of Trc analogues, such as the

tryptocidines (Tpcs) and phenycidines (Phcs), not commonly found in abundance in the control cultures [6, 15].

In this study the Trcs and Trc analogues were produced by *B. aneurinolyticus* 8185 (*Bb. parabrevis* ATCC 8185) in media supplemented with Trp, Tyr and Phe and extracted from the microbial biomass. Peptide extracts were analysed with ultra-performance liquid chromatography (UPLC) linked electrospray mass spectrometer (MS) to determine the identity of peptides present and also contribution of each Trc and Trc analogue to the total cyclic peptide present within the sample. A control extract obtained was further purified using absorption chromatography separating the more non-polar linear gramicidins from the cationic cyclic decapeptides present in the sample. Each fraction obtained was analysed with UPLC-MS, to determine the identity of the peptides present within the sample, and analytical reverse phase high performance liquid chromatography (HPLC), to determine the percentage purity. The extract was further fractionated with semi-preparative reverse phase HPLC to determine the relative mass percentage of Trcs and linear gramicidins in the extract.

2.2 Materials

The National Collection of Type Cultures (NCTC, Porton Down, Salisbury, United Kingdom) provided the *Micrococcus luteus* NCTC 8340 cultures used in all antibacterial assays. Agar, sodium chloride, hydrochloric acid, tryptone, yeast extract and KH_2PO_4 were obtained from Merck (Darmstadt, Germany). Tyrothricin, gramicidin S, polypropylene microtiter plates and trifluoroacetic acid (TFA, >98%) were obtained from Sigma (St. Louis, USA). The L-amino acids, tyrosine, tryptophan and phenylalanine were supplied by Sigma-Aldrich (Darmstadt, Germany). Romil Ltd. (Cambridge, UK) provided the methanol (>99.9%) and acetonitrile (HPLC-grade, far UV cut-off). Petri dishes were obtained from Greiner bio-one (Frickenhausen, Germany), Falcon ® tubes were obtained from Becton

Dickson Labware (Lincoln Park, USA), potassium chloride was obtained from Capital Enterprises (Hillcrest, South Africa). Saarchem (Krugersdorp, South Africa) provided the diethyl ether and acetone. Biolab Diagnostics (Midrand, South Africa) supplied the brain heart infusion broth and 0.22 µm polycarbonate syringe filters were supplied by Lasec (Cape Town, South Africa). Analytical grade water was used for all solvents and wash steps and was obtained with a Millipore Milli-Q water purification system (Milford, USA) and was filtered from a reverse osmosis water purification plant. The Nova-pak-C₁₈ reverse phase semi-preparative HPLC column (6 µm particle size, 60 Å pore size, 7.8 mm x 300 mm) and analytical HPLC column (5 µm particle size, 60 Å pore size, 3.9 mm x 150 mm) was also supplied by Waters-Millipore (Milford, USA).

2.3 Methods

2.3.1 Production of peptide with *B. aneurinolyticus* in amino acid supplemented media

B. aneurinolyticus 8185 was streaked out onto TGS agar plates (1.5 % (w/v) tryptone, 3% (w/v) glucose, 0.07% (w/v) KH₂PO₄, 0.035% (w/v) KCl, 0.05% (w/v) MgSO₄, 0.025% (w/v) CaCl₂, 0.002% (w/v) FeSO₄, 0.002% (w/v) MnSO₄, 1.5% (w/v) agar) from freezer stocks and incubated for ±48 hours at 37°C. Single colonies were transferred to blood plates (2% haematocrit in Brain Heart Infusion Agar) and incubated at 37°C overnight (±16 hours) to confirm peptide production. Peptide producing colonies, where haemolysis was seen around the colony, were transferred to TGS broth (20 mL) in a Falcon® tube and incubated on a shaker at an angle (150 rpm) at 37°C until an absorbance A₆₂₀ 0.8-1.2 was reached (±16 hours). The optimised culture method in large volumes can only be briefly described since it is currently protected under a non-disclosure agreement (NDA) as has been classified as trade-secret (BIOPEP™, University of Stellenbosch). The starter culture was used to inoculate fresh TGS media and amino acid supplemented media in culture flasks. Cultures were incubated at 37°C for up to 14 days after which the peptide was extracted and purified.

Amino acid supplemented media consisted of either 2.5mM Tyr, 5mM Trp, 10mM Trp, 5mM Phe or 10mM Phe. The media was filter sterilized with a 0.22 μm filter. In total seven cultures were inoculated: five filter sterilised amino acid supplemented, one TGS filter sterilised and one TGS media that was autoclaved.

2.3.2 Extraction and purification of peptides produced in culture

The produced peptide was extracted according to an optimised method based on Dubos and Hotchkiss [16]. The optimised purification method of crude peptide can only be briefly described since it is currently protected under a NDA as it has been classified as trade-secret (BIOPEPTM, University of Stellenbosch). The biomass was extracted using an extreme pH step, an organic extraction and finally a precipitation step. The crude peptide in the precipitate was dissolved in 90% (v/v) acetonitrile (containing 0.01% (v/v) TFA), centrifuged at 3 000 $\times g$, the supernatant transferred to a round bottom flask and freeze dried. This was done twice. The remaining fraction was dissolved in 90% (v/v) acetonitrile and mixed with activated charcoal, centrifuged at 3 000 $\times g$ and the supernatant transferred to a round bottom flask and dried under vacuum. This procedure was followed for all seven culture peptide production runs and all solvents were made with analytical grade water.

Further purification was only completed on the peptide obtained from the autoclaved TGS media culture. Purification entailed a chromatography step with a mixed polar-hydrophobic* resin matrix (*NDA, BIOPEPTM, University of Stellenbosch) denoted PoHy column for the purpose of this discussion. The PoHy column (pore diameter 100 \AA) was washed five times with analytical grade water followed by 10 minute regeneration with 100% methanol. This was followed by two wash steps with solvent B (90% (v/v) acetonitrile containing 0.01% (v/v) TFA), followed by a wash step of 40% solvent B to solvent A (0.1% (v/v) TFA). Subsequently, the prepared crude peptide extract was allowed to bind to the column for 30

minutes, after which most of the solution was drained from the column. Next multiple elution steps were used to elute the bound peptide and contaminant from the PoHy column. Four elution steps (10 mL each) of varying concentrations of solvent B mixed with solvent A (40% B, 60% B and 100% B) were performed and fractions (denoted PoHy fractions) were collected for further analysis and treatment of solid surfaces.

2.3.3 Purification of commercially produced tyrocidine

Commercial tyrothricin was washed eight times with a ether:acetone (50:50, v/v) solution [13] and dried under vacuum to be used for further analysis and treatment of solid surfaces. Each step consisted of adding the ether:acetone solution to the peptide, sonication to homogenize the solution followed by centrifugation at 3 000×g for 10 minutes to collect the pellet. The Trc containing pellet was first dried with nitrogen gas to remove the ether:acetone solution before it was re-suspended in 50% (v/v) acetonitrile and freeze-dried for further analysis and used as positive control.

2.3.4 Characterisation and analysis of purified extract

The relative purity of the purified fraction of each of the amino acid supplemented cultures and controls, as well as the fractions obtained from the column purification was determined against that of a pure Trc standard. HPLC analysis of fractions was done using a chromatographic system controlled by Millennium software comprising of a Waters 717 Plus Autosampler, two Waters 510 pumps and a Waters 440 UV detector set at 254 nm. Samples were dissolved in 50% v/v acetonitrile (1 mg/mL), centrifuged at 8600×g to remove particulate and then injected (20 µL) onto a reverse phase analytical C₁₈ Nova-Pak[®] column (150 mm x 3.9 mm, 6 µm particle size, 60Å pore size). A gradient of solvent A (0.1% v/v TFA) to solvent B (90% v/v ACN, 0.01% v/v TFA) was used for the separation of the peptide in the Trc extract at 1.0 mL/min flow rate (Table 2.2).

Characterisation of the mass contribution of Trcs to an extracted sample was done by using the same methods as described above with the exception of the use of a reverse phase semi-preparative column C₁₈ Nova-Pak® column (300 mm x 7.8 mm, 5 µm particle size, 60Å pore size), an injection volume of 100 µL, peptide concentration of 8 mg/mL and flow rate of 3mL/min.

Table 2.2: Gradient of Eluent A to Eluent B used during analysis and characterisation of samples using analytical or semi-preparative RP-HPLC

Time (min)	% Eluent A ^a	%Eluent B ^b	Curve Type ^d
0.0	50	50	
0.5/1.0 ^c	50	50	6 (linear)
23.0	20	80	5 (concave curve)
24.0	0	100	6 (linear)
26.0	0	100	6 (linear)
30.0	50	50	6 (linear)
35.0	50	50	6 (linear)

^a 0.1% v/v TFA in water

^b 0.01% v/v TFA in 90% v/v acetonitrile

^c 0.5 minutes during semi-preparative RP-HPLC separation; 1.0 min during analytical RP-HPLC separation

^d Gradient curves on Millennium software Version 32, WatersTM

2.3.5 Analysis and identification of purified peptide extracts

The relative purity of the extracts were calculated based on the data obtained from UPLC-MS whereas the identity of peptides present in purified extracts were confirmed using high resolution MS. During UPLC-MS 3µL of the peptide sample (250 µg/mL in water) was injected onto a Waters Acquity UPLC® HSS T3 C₁₈ column (2.1 x 150mm, 1.8µm particle size) with a 0.450 mL/min flow rate. Separation was obtained with a gradient from solvent A (0.1% v/v formic acid) and solvent B (acetonitrile). The gradient was as follows: 100% A from 0 to 0.5 minutes, 0% to 58% B from 0.5 to 12 minutes and lastly 58% to 90% B from 12 to 13 minutes. In line high resolution MS was done the same as with direct injections, with a Waters Synapt G2 quadrupole TOF mass spectrometer (Milford, MA, USA) with an electrospray ionisation source. Samples were exposed to a capillary voltage of 3.0 kV with a

cone voltage of 15V and source temperature of 120°C. Nitrogen was selected as the desolvation gas (650 L.h⁻¹) at a desolvation temperature of 275°C. All data was collected scanning over an *m/z* range of 300-2000 in the positive mode. Samples injected directly into the MS were prepared in the same manner as with UPLC-MS and with the same injection volume.

2.3.6 Determination of activity of purified peptides

M. luteus (NCTC 8340) was streaked out from a freezer stock on Luria Bertani plates (LB; 1% *m/v* NaCl, 1% *m/v* tryptone, 0.5% *m/v* yeast extract, 1.5% *m/v* agar in water) and incubated for ±48 hours at 37°C. LB media (20 mL) was inoculated with a single colony and incubated at 37°C on a shaker at 150 rpm until an OD₆₂₀ of 0.8-1.2 was reached (±16 hours). Fresh TSB media (20 mL) was incubated with 500 µL of the original culture and incubated at 37°C on a shaker at 150 rpm until an OD₆₂₀ of 0.6-0.8 was reached (±5-6 hours). This culture was diluted to an OD₆₂₀ 0.20, of which 90 µL was transferred to each well of a polystyrene 96-well plate. Media (90 µL), serving as a blank, was also transferred into a number of wells. The peptide (10 µL) was also added to the plate as well as 10 µL of 15% (*v/v*) ethanol to the blank and growth control. The purified peptide PoHy fractions and Trc commercial standard was prepared in a polypropylene plate in 15% (*v/v*) ethanol to a concentration of 1 mg/mL. Double dilutions of the Trc standard were performed, whereas the PoHy fractions were diluted ten times. Gramicidin S (GS) (100 µg/mL), as positive control, was added to all plates. The cultures were subsequently incubated at 37°C for 16 hours after which the optical density (OD) was determined at 595 nm using a Model 680 Microplate reader (Biorad) and relevant software. The antimicrobial activity was calculated using the following equation:

$$\% \text{ Growth} = 100 \times \frac{\text{Abs. of well} - \text{Average Abs. background}}{\text{Average Abs. Growth} - \text{Average background}}$$

The purity of the PoHy fractions was determined by comparing the obtained antimicrobial activity to that of the Trc standard.

2.3.7 Graphs and statistical analysis

Graphs and all statistical analysis were done using GraphPad Prism ® 4.03 (GraphPad Software, San Diego, USA). Statistical analysis included 95% confidence levels, standard error of the mean, absolute sum of squares and One-way Anova using Bonferroni's Multiple Comparison test.

2.4 Results

2.4.1 Purification of commercial tyrocidine

The cationic cyclic decapeptide fraction was successfully purified from commercially obtained tyrothricin by removing the linear gramicidins produced with Trcs as part of the tyrothricin complex. A high purity extract was obtained with the only peaks detected with UPLC-MS analysis, other than the solvent background, were Trcs namely TrcC (1348.6848), TrcB₁ (1323.6860), TrcB (1309.6682), TrcA (1270.6683) and TrcA₁ (1284.6704), and Tpcs namely TpcC (1371.6927) and TpcB (1332.6829) (Fig. 2.1). As the UPLC-MS methodology is very sensitive, a very small amount of the linear gramicidin VGA (1881.0643) was also detected. Based on the abundance of the peptides calculated from the sum of the peak areas of the detected peaks obtained from the UPLC-MS analysis, the extract had a purity of >95% and was used in dose response assays and treatment of solid surfaces described in the following chapters. A yield of 67% (120 mg) clean Trcs was obtained.

The results for the commercial extract in terms of retention times and percentage abundance compared well with those previously reported [6, 11, 15]. The retention times of each of the peptides were comparable to that found by Vosloo *et al.* [6] (Table 2.1) with a similar profile and abundance. The observed abundance of each Trc and Trc analogue within the

commercial extract purified in this study was comparable with that reported in literature [11, 15]. The most abundant peptides found in the commercial extract were TrcB₁ (19.2%), TrcB (18.2%), TrcA₁ (15.8%) and TrcC (14.8) whereas the most abundant peptides found in the extracts analysed by Tang *et al.* [15] and Troskie *et al.* [11] were TrcA (18.0% and 17.6%), TrcB (22.3% and 21.3%) and TrcC (20.5% and 14.3%) (Table 2.1).

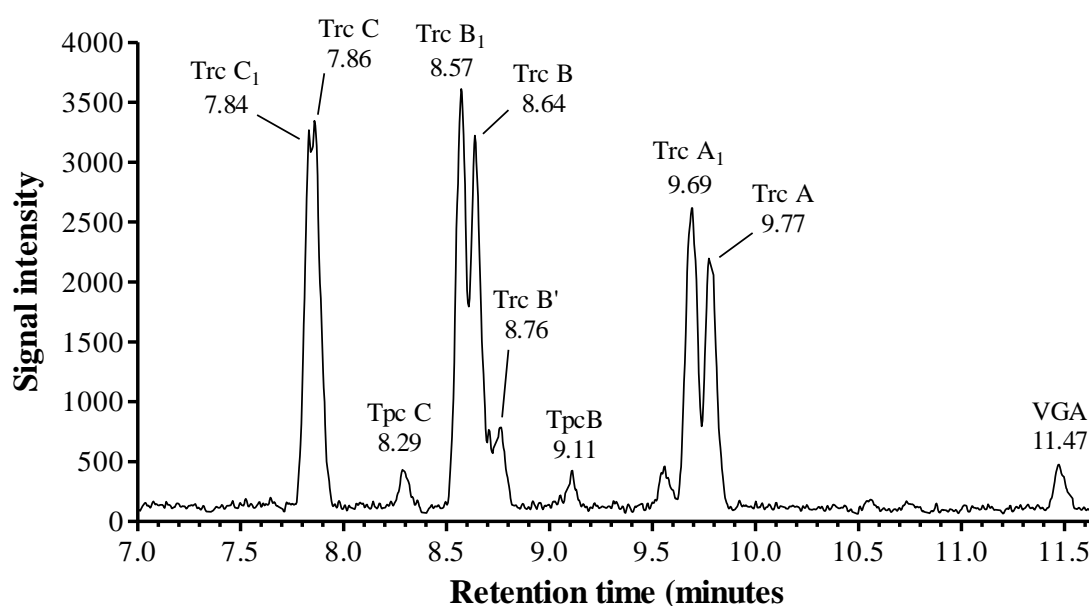


Figure 2.1: UPLC-MS analysis of the Trc extract obtained from the organic extraction of the commercially obtained tyrothricin complex showing the detected peaks of the major Trcs and Trc analogues.

Comparing the three extracts based on the combined abundance of Lys- and Orn-containing Trcs, it was found that in all three TrcB/B₁ had the highest abundance, followed by similar abundances of TrcA/A₁ and TrcC/C₁. There was $\leq 3\%$ abundance of Tpcs present in the commercial extract for this study, while about 7% abundance of Tpcs was observed by Troskie *et al.* [11] and $\pm 13\%$ abundance for the Tang *et al.* [15]. The differences in Trcs and Tpcs abundances can be attributed to different tyrothricin sources and extraction procedures.

Table 2.1: Summary of the UPLC-MS detected peptides in the commercial Trc peptide extract regarding peptide identity, monoisotopic M_r , observed retention time and percentage abundance of the Trc in the total extract. The theoretical M_r , Retention time and percentage abundance of the respective peptides as found by Troskie *et al.* [11] and Tang *et al.* [15].

Peptide	Observed M_r (Theoretical M_r) ^a	R_t (min) ^b	% Abundance		
			Trc in commercial extract ^c	Troskie et al (2013)	Tang et al. (1992)
TrcC ₁	1361.6888 (1361.6921)	7.84	12.5	10.3	6.1
TrcC	1347.6743 (1347.6764)	7.86	14.8	14.3	20.5
TpcC	1370.6833 (1370.6924)	8.29	1.7	3.5	4.7
TrcB ₁ /B ₁ '	1322.6722 (1322.6812)	8.57	19.2	15.7	9.0
TrcB	1308.6564 (1308.6555)	8.64	18.2	21.3	22.3
TrcB'	1308.6572 (1308.6555)	8.76	4.0	2.9	2.9
TpcB	1331.6744 (1331.6815)	9.11	1.0	3.1	5.3
TrcA ₁	1283.6704 (1283.6703)	9.69	15.8	13.2	8.0
TrcA	1269.6588 (1269.6538)	9.77	12.9	17.6	18.0
TpcA	1292.6885 (1292.6479)	10.19	Trace	<1	3.1

^aTheoretical monoisotopic M_r calculated from the molecular masses of all the constituent amino acids.

^bRetention times obtained from UPLC-MS analysis

^cPercentage abundance calculated as the percentage peak areas contributed by each individual Trc and analogue for the total percentage Trcs present in the sample, with the assumption that all the peptides have similar MS signal intensities (response factors) due to their analogous structures.

2.4.2 Peptide extracts obtained from *B. aneurinolyticus* cultures

The aim of the amino acid supplementations was to obtain different ratios of Trcs and Trc analogues (Tpcs and Phcs) that could be used for different applications of peptide impregnated solid surfaces. An extract containing predominantly TrcB and TrcC can be applied, for example, to the development of an antibacterial surface due to its high antibacterial activity against Gram-positive organisms compared to that of TrcA [7, 8].

Peptide extracts from amino acid supplemented and control cultures produced by *B. aneurinolyticus* 8185 were further fractionated. Supplementation of culture broth included 2.5 mM Tyr, 5 mM and 10 mM Phe and 5 mM and 10 mM Trp and as a control to the filtered media, an autoclaved media control was added. Trp is destroyed by high temperatures [17] and as a result the Trp supplemented media could not be autoclaved to obtain sterility. Therefore all amino acid containing media plus a control medium (not supplemented) were filter sterilized. The autoclaved control medium was added based on the hypothesis that autoclaving the media would aid in the breakdown of tryptone in the media which in turn would lead to the release of amino acids into the media. This would make the amino acids more accessible to the growing organism which subsequently would lead to higher biomass and peptide production.

The peptide extracts were compared based on wet biomass (producer organism growth), mass yield per litre of culture after purification and percentage abundance of Trcs within the sample (Table 2.2). There was no statistical significant difference observed between each of the cultures in terms of the wet biomass and mass yield, correlating with results obtained by Vosloo *et al.* [6], indicating that the method of sterilization has very little effect on the production of Trcs. The highest abundance of Trcs, based on UPLC-MS analysis, was observed for the culture extracts from media supplemented with 10 mM Trp, then the filtered media and third the 5 mM Trp supplemented media. The lowest abundance of Trcs observed was for the extracts from the 10 mM Phe supplemented culture.

In order to compare the peptide profile for each of the extracts, the percentage abundance for each of the Trcs and analogues was calculated by using the UPLC-MS analysis determining retention times, peak identity and peak area for each of the extracts. The extract from cultures grown in autoclaved media (autoclaved extract) was used as an example (Fig. 2.2) where

each peak of the Trc peptide profile was identified and peak area calculated. The assumption was made that the Trcs and analogues showed similar ion signal response due to the high similarity in peptide structure.

Table 2.2: Summary of the wet biomass (producer growth), mass yield of purified peptide per litre of culture and the percentage abundance of Trcs within the extract as determined by UPLC-MS analysis ($n =$ biological repeats)

Amino acid supplementation	Wet biomass (g) ($n=2$)	Extract Yield (g/L) ($n=2$)	% Abundance of Trc in the extract
None – Autoclaved	4.5 \pm 3.3	0.82 \pm 0.34	65.8
None – Filtered	4.0 \pm 3.1	0.81 \pm 0.08	74.0
2.5 mM Tyr	5.0 \pm 3.3	1.19 \pm 0.21	67.6
5 mM Phe	5.2 \pm 2.9	0.83 \pm 0.06	66.4
10 mM Phe	6.3 \pm 4.4	0.75 \pm 0.14	58.5
5 mM Trp	5.7 \pm 2.5	1.26 \pm 0.07	72.4
10 mM Trp	6.3 \pm 4.4	1.31 \pm 0.36	76.0

UPLC-MS analysis of the peaks detected for the autoclaved extract is described in Addendum A and all the remaining cultures were analysed similarly. The detected peak identities and retention times are summarised in Table 2.3. The peaks containing impurities such as the pigment produced with Trcs or non-peptide compounds are not shown. Comparing the percentage peptide abundance observed for the autoclaved extract with that of the commercially extracted Trc mixture it can be seen that the culture extract contain predominantly TrcA and TrcB, whereas the commercial extract contains predominantly TrcB/B₁ with lower amounts of TrcA/A₁ and TrcC/C₁. Also very low amounts of the Lys analogues of the Trcs (TrcA₁, TrcB₁ and TrcC₁) were found, while more tryptocidines (TpcC and Tpc A) are present in the autoclaved extract compared to the commercial Trc mixture.

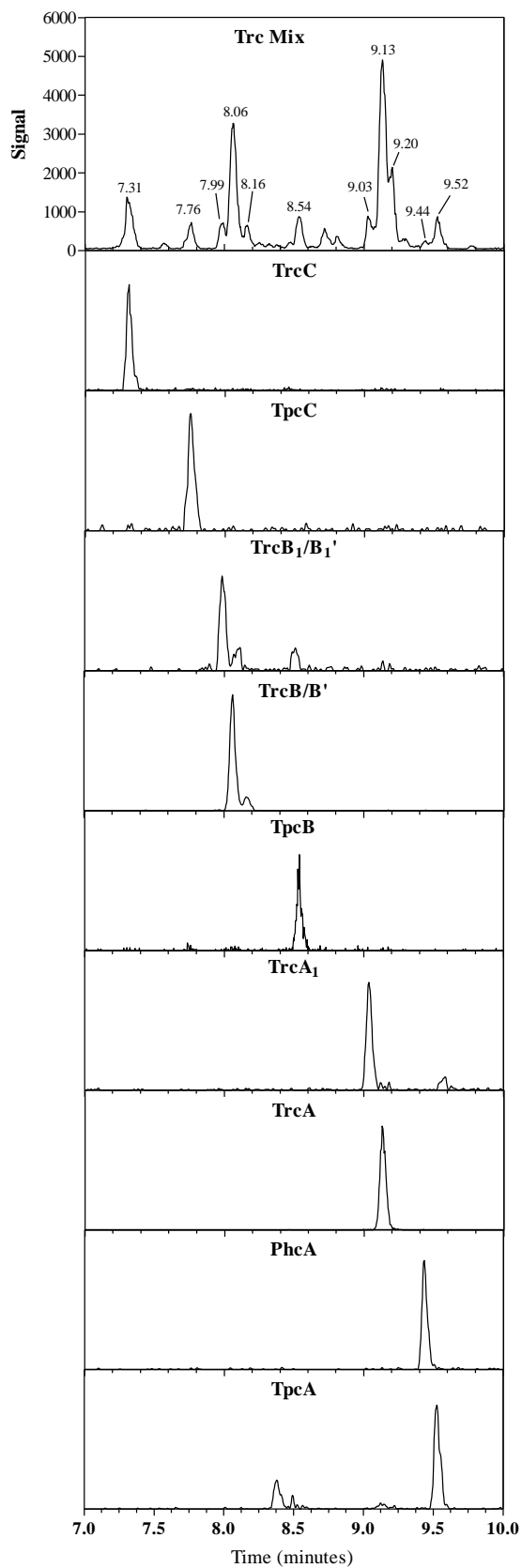


Figure 2.2: UPLC-MS analysis of the autoclaved extract indicating the identity of each Trc and Trc analogues in the UPLC profile.

Table 2.3: Summary of the detected peaks of the autoclaved peptide extract regarding peptide identity, sequence, monoisotopic M_r , observed retention time and percentage abundance of the Trc in the total extract.

Peptide	Observed M_r (Theoretical M_r) ^b	R_t (min) ^c	% Abundance of Trc in culture extract ^c	% Abundance of Trc in commercial extract
TrcC ₁	1361.6782 (1361.6921)	7.30	Trace	12.5
TrcC	1347.6688 (1347.6764)	7.30	8.9	14.8
TpcC	1370.6783 (1370.6924)	7.76	4.0	1.7
TrcB ₁ / B ₁ '	1322.6593 (1322.6812)	7.99	3.7	19.2
TrcB/B'	1308.6507 (1308.6555)	8.06	25.2	22.2
TpcB	1332.6638 (1331.6815)	8.47	0.6	1.0
TrcA ₁	1283.6597 (1283.6703)	9.03	3.9	15.8
TrcA	1269.6332 (1269.6538)	9.13	42.1	12.9
TpcA	1292.6508 (1292.6479)	9.52	4.9	Trace

^aTheoretical monoisotopic M_r calculated from the molecular weights of all the constituent amino acids.

^bRetention times obtained from UPLC-MS analysis

^cPercentage abundance calculated as the percentage peak areas contributed by each individual Trc and analogues considering only the Trcs and analogues present in the sample

The effect of the amino acid supplementation was determined by first analysing the change in chromatographic profile of the peptide extract compared to the chromatogram of the control autoclaved extract by utilising UPLC-MS. When comparing the profile generated for the peptide extract from cultures grown in 2.5 mM Tyr supplemented media (2.5 mM Tyr extract) with the autoclaved control the main Trcs containing peaks were observed (Fig. 2 3:A). Other than a slight increase in the production TrcC₁, TrcC, TrcB₁ and TrcB there was not much difference between the peptide profiles obtained for the 2.5 mM Tyr extract compared to that of the autoclaved extract. When the same comparison was done with the

extracts from cultures grown in 10 mM Trp supplemented media (10 mM Trp extract) the profile had shifted to the left (7.5-8.5 min) to the predominant production of Tpc C (Fig. 2 3:B).

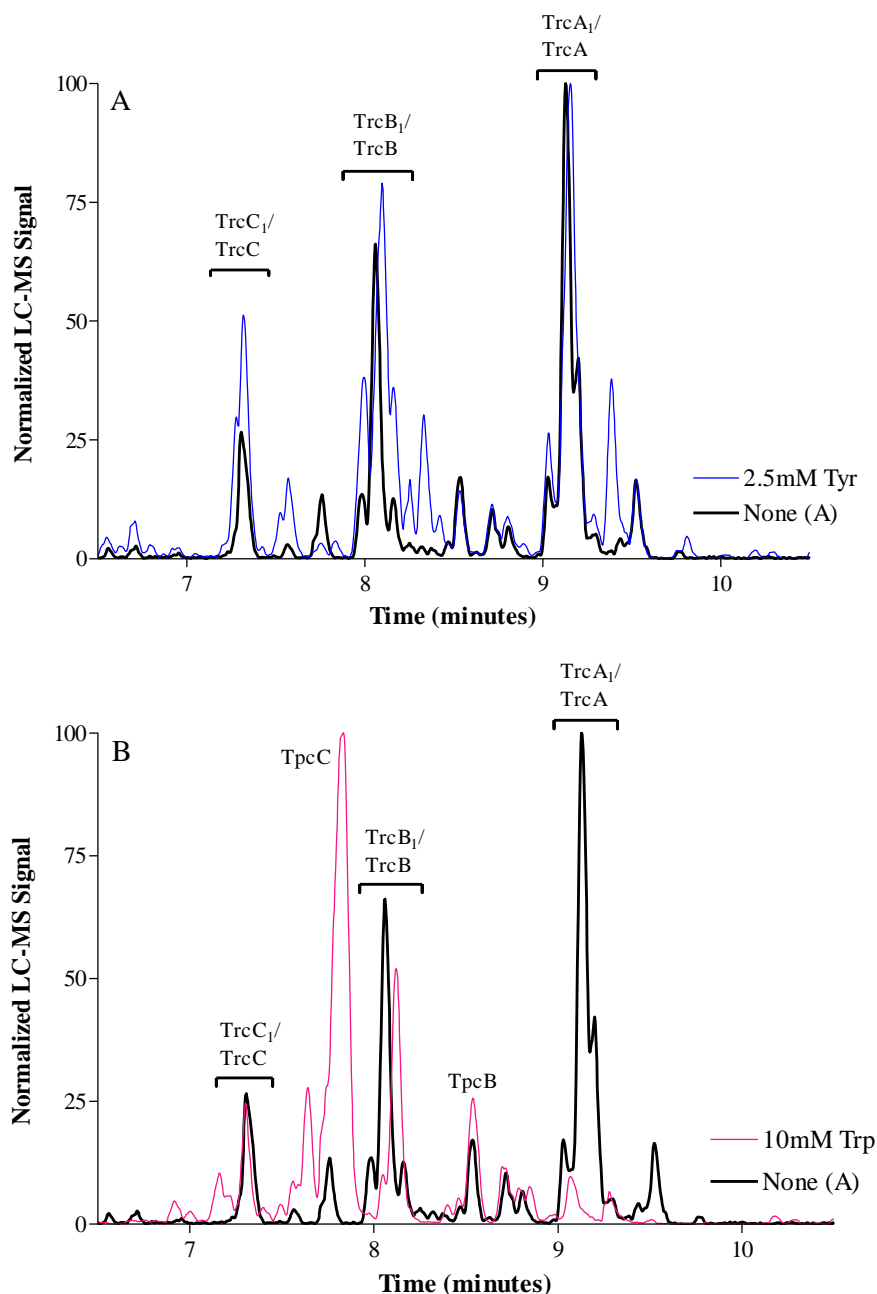


Figure 2.3: Overlay of chromatograms obtained from UPLC-MS analysis for the non-supplemented autoclaved media (None (A)) vs. **A** 2.5 mM tyrosine supplemented media and **B** 10 mM tryptophan supplemented media showing the shift of peptide production profile with the amino acid supplementation of the culture media. The major Trcs and Tpcs contributing to the total peptide in the extract are indicated.

Closer inspection of each individual peptide produced and detected with UPLC-MS analysis (Fig. 2.4) it was observed that the 5 mM Phe extract showed to have more TrcA analogues (TrcA₁, TpcA) with TrcA as the main contributing peptide in the extract. The 10 mM Phe extract had less TrcA and TrcA₁, but showed a shift to the production of Phc A and PhcA₁ and very little TrcB and TrcC analogues were detected. In both cases this is due to the higher levels of Phe available in the growth media resulting to the increased incorporation of Phe into the Trc structure. Substitutions of Phe^{3,4} would result in formation of TrcA analogues and the substitution of Phe⁷ would lead to the formation of Phc analogues.

The 5 mM Trp extract had a low abundance of TrcA analogues, some TrcB analogues with TrcC and TpcC as the main contributors to the Trc content (Fig. 2.4). The extract from 10 mM Trp supplemented medium showed a shift to the production of predominantly Trc C analogues (TrcC₁, TrcC, TpcC), containing only 12% TrcB analogues (TpcB and TrcB₁) (Fig. 2.4). This is due to the higher levels of Trp available in the growth media, as with the Phe supplementation, resulting in the increased incorporation of Trp into the Trc structure.

The substitution of Trp^{3,4} would result in the formation of TrcC analogues and the substitution of Trp⁷ would lead to the formation of Tpc analogues. These results correlate to previous studies on the effect of amino acid supplementation on the types of Trcs and Trc analogues produced. Vosloo *et al.* [6] found Phe and Trp to be the only amino acids that affect the peptide production profile. Supplementation with Phe led to subsequent production of mainly TrcA and analogues were as the supplementation of Trp lead to the predominant production of TrcC and TpcC. The latter was also observed by Ruttenberg & Mach [18].

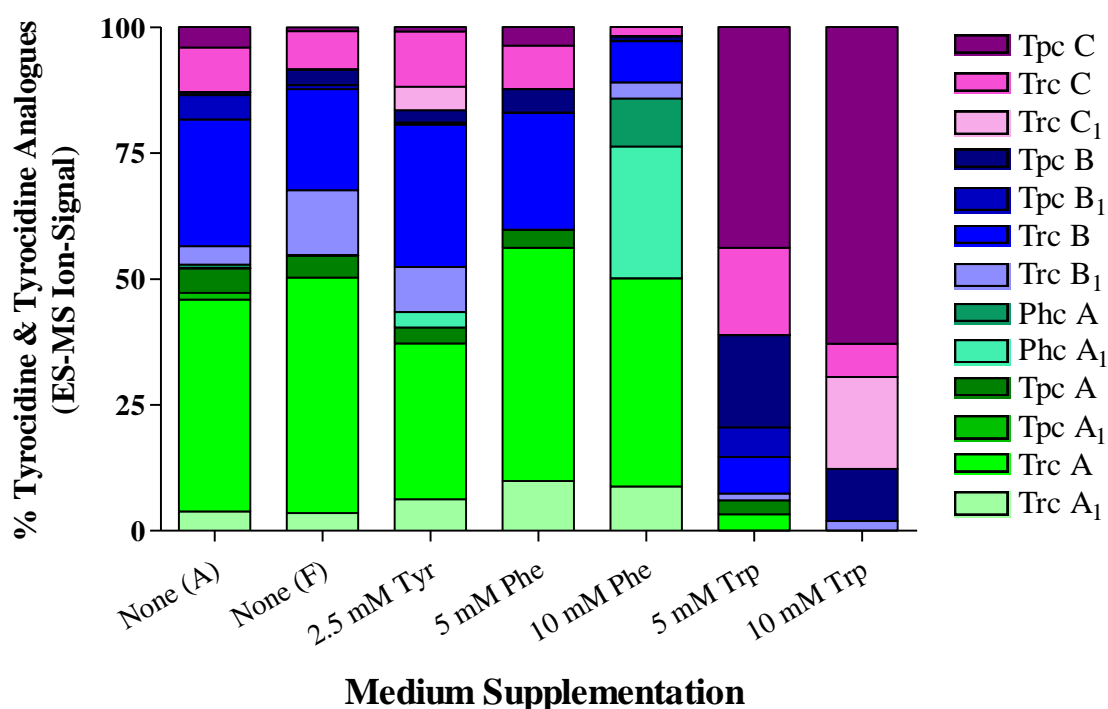


Figure 2 4: Comparison of the contribution of Trcs and analogues within the various extracts from culture grown in supplemented and non-supplemented media as detected with UPLC-MS analysis.

The autoclaved extract was selected for further chromatographic purification based on its similarity to commercial Trc mixture, in comparison to the other extracts (Table 2.3). The Trcs and linear gramicidins differ in polarity, thus multiple wash steps with varying solvent polarities were used to separate Trc and analogues from the more non-polar neutral linear gramicidins. Each of the fractions obtained were analysed according to the percentage peptide recovered, the peptides detected with MS analysis, the relative purity in comparison with a commercial Trc standard achieved with analytical RF-HPLC and relative activity based on the antimicrobial activity against *M. luteus* of each fraction.

Relative purity was calculated by firstly identifying the Trcs containing peaks in the PoHy fraction based on the peaks identified in the Trx mixture (Fig. 2.5) and then using the peak areas, as calculated by the analytical HPLC analysis, to determine the percentage of the

sample comprising of Trcs. It was found that the 60% solvent B fractions were the purest extracts containing predominantly Trcs and Trc analogues.

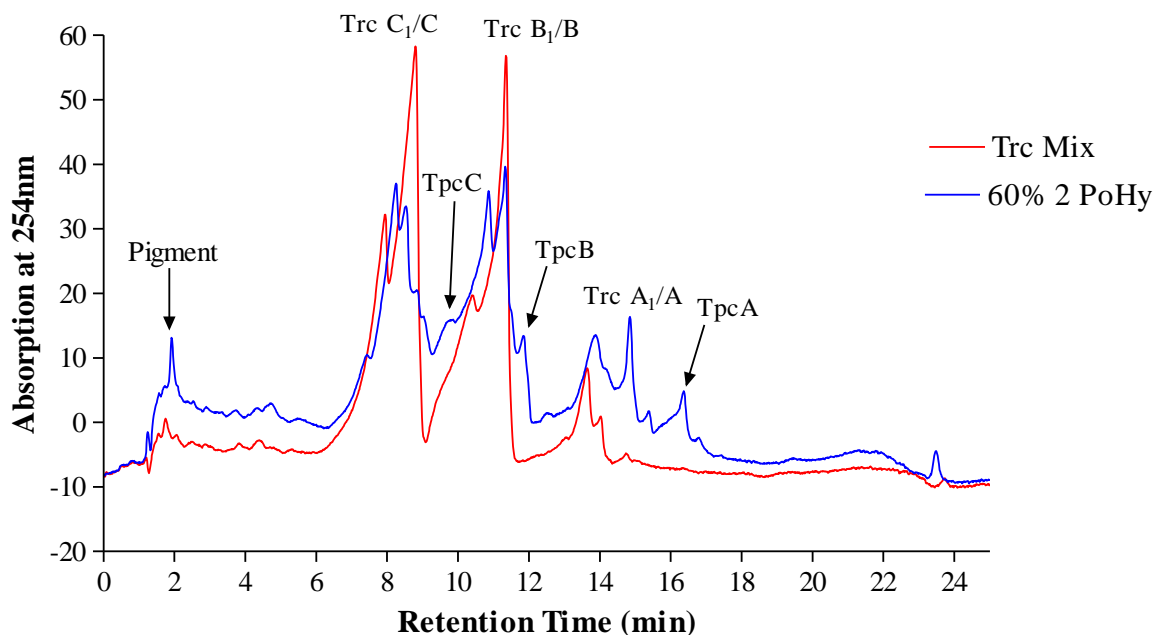


Figure 2.5: Chromatograms obtained from the analytical HPLC analysis of the 2nd PoHy fraction at 60% (v/v) solvent B wash step (60% 2 PoHy) and purified Trc mixture as control. Relative purity can be calculated based on the abundance of overlapping peaks of 60% 2nd PoHy fraction with that of Trc mixture

Percentage activity was determined as the percentage activity observed for each fraction at a set concentration (10 µg/mL) as a function of the activity observed at the same concentration for the commercial Trc mixture standard. A rudimentary standard curve was compiled from the antibacterial activities of known concentrations of Trc mixture against *M. luteus* (Fig. 2.6).

The percentage activity was calculated based on known concentration of the extracts, i.e. if it was calculated that the fraction had the activity of 8 µg/mL purified commercial Trc, as calculated from the standard curve, it would have a percentage activity/purity equating to 80%. It was found that the 60% solvent B fractions had the highest antimicrobial activity (Table 2.4). Therefore the 60% solvent B wash step yielded both the most relatively pure and

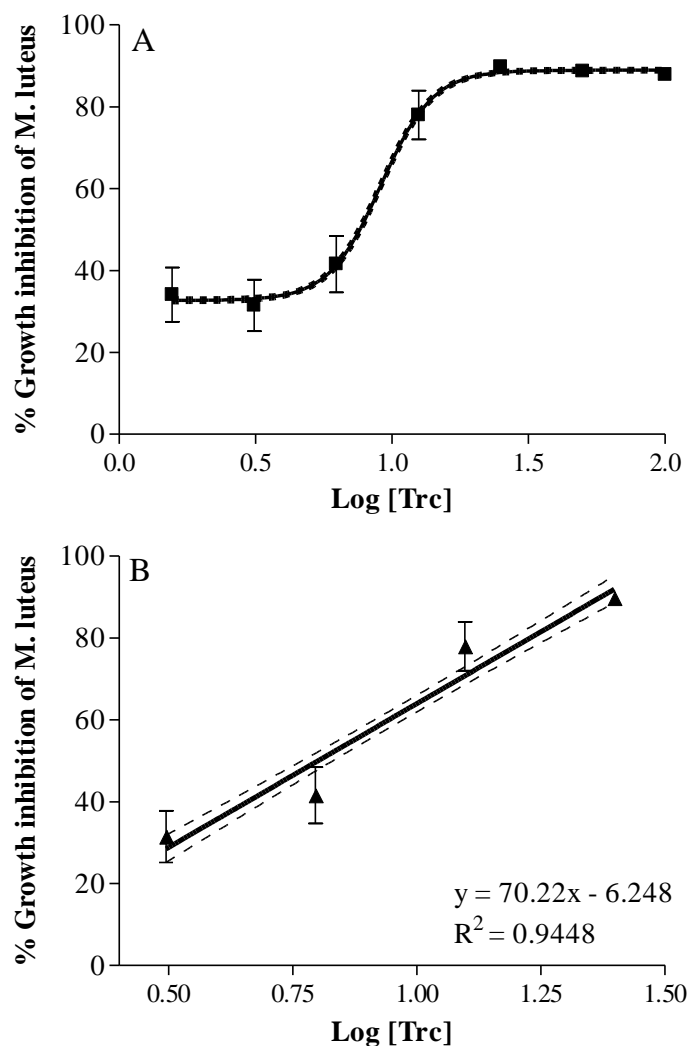


Figure 2.6: **A** Dose response curve as compiled from the antimicrobial activity of the commercial Trc extract against *M. luteus*, **B** The linear part of the dose response curve used to determine the approximate % activity of the PoHy fractions compared to the standard Trc Mix

antimicrobial fractions and contained predominantly Trcs and analogues. The 2nd 60% fraction had the highest purity and both the highest observed antimicrobial activity and peptide yield and could thus be used for the treatment of the selected solid surfaces discussed in later chapters. The 3rd and 4th 60% fractions showed slightly higher or similar purities to that of the 2nd 60% fraction, but lower antimicrobial activities possibly because of aggregation due to the high purity of these samples [19]. We previously found that some pigment in the sample actually improves solubility and activity (personal communication JA

Table 2.4: Summary of purification data of PoHy column fractions. The purity of each fraction based on analytical HPLC and relative activity of each fraction based on the antibacterial activity against *M. luteus* compared to a Trc standard (Fig. 2.6). The major peptides within each fraction are indicated in bold and (-) indicates the absence of the peptide within the fraction.

Identity (Monoisopic M_r)	Detected M_r values of compounds in column fraction eluted with increasing % solvent B							
	40% 1	40% 2	60% 1	60% 2	60% 3	60% 4	100% 1	100% 2
TrcA (1269.65)	1269.63	1269.65	1269.65	1269.65	1269.65	1269.65	1269.64	1269.65
TrcA ₁ (1283.67)	1283.65	1283.66	1283.66	1283.66	1283.67	1283.67	1283.66	1283.67
TpcA (1292.67)	1292.63	1292.61	1292.64	1292.64	1292.64	1292.64	1292.64	1292.65
TrcB/ B' (1308.65)	1308.65	1308.65	1308.65	1308.65	1308.65	1308.65	1308.65	1308.65
Trc B ₁ /B ₁ ' (1322.68)	1322.66	1322.66	1322.66	1322.68	1322.67	1322.66	1322.66	1322.68
TpcB/B' (1331.68)	1331.62	1331.65	1331.66	1331.66	1331.66	1331.67	1331.66	1331.66
TrcC (1347.67)	1347.64	1347.66	1347.67	1347.67	1347.67	1347.67	1347.67	1347.67
TrcC ₁ (1361.69)	1361.67	1361.67	1361.68	1361.67	1361.67	1361.68	1361.67	1361.68
TpcC (1370.69)	-	1370.67	1370.67	1370.67	1370.68	1370.68	1370.68	1370.68
TpcC ₁ (1384.70)	-	1384.65	1384.67	1384.68	1384.68	1384.69	1384.68	1384.69
VGA (1881.07)	-	-	-	-	-	1881.08	1881.07	1881.07
VGB (1842.06)	1842.10	-	-	-	-	-	-	-
% Mass recovery	8.5	4.5	20.4	26.2	9.9	3.7	8.7	3.9
% Peptide purity ^a	5.8	26	70	84	86	84	66	35
% Activity ^b (n=9)	18	20	67	83	69	72	24	16

^a Purity calculated based on the combined Trc peak areas obtained at 254 nm from reverse phase analytical HPLC compared to the peaks areas obtained for the purified Trc mixture standard

^b Activity calculated based on the inhibition observed against *M. luteus* compared to the purified Trc mixture standard

Vosloo). The lower activity in the 4th 60% fraction could also be due to the presence of VGA (see discussion below). The 40% fractions showed both low purity and low antimicrobial activity. The 100% solvent B wash gave a total recovery of 17% and contained

predominantly linear gramicidins. Not shown on the table (Table 2.4) is the 3rd 100% fraction having 2% recovery and 4th 100% fraction also having 2% recovery.

Even though the 1st 100% wash fraction had a high purity based on the analytical HPLC analysis and contained Trc and analogues, the antibacterial activity observed was very low. This could be explained by the negative effect linear gramicidin VGA has on the activity of Trcs and its ability to reverse the effect of Trcs on a target cell [2]. Consequently it would be of great importance to obtain a Trc extract with as little linear gramicidins present as possible. It can be concluded that the column method separates the Trc analogues from the linear gramicidins found in the extract to produce a relatively clean Trc extract that can be used for the treatment of solid surfaces.

Since the aforementioned methods rely on the absorbance and ionisation of the peptides present, which can lead to an overestimation, it is of great importance to determine the mass percentage of each peptide group present in the autoclaved extract. Thus the peptide extract was analysed with semi-preparative HPLC and three fractions collected: the Trc fraction (fraction 2) and then the fractions before (fraction 1) and after (fraction 3) the Trc containing fraction (Fig. 2.7: A).

After the fractions were collected it was dried under vacuum and weighed analytically. The masses obtained for each of the fractions were: Fraction 1 (0.932 mg), Fraction 2 (3.942mg) and Fraction 3 (0.801mg). Fraction 2 was predominantly Trcs (Fig. 2.7: B) (TrcA, TrcB, TrcC and TpcC) and it can thus be concluded that Trcs contribute 70% of the mass of the sample. A small amount of VGA was detected within fraction 2 (Fig. 2.7). Since it elutes close to the TrcA analogues it is possible that a small fraction was collected as the gramicidins tend to give broad fronting peaks [20]. This result correlates closely to the purity of this fraction as determined using the standard curve derived from activity against *M. luteus* (Table 2.4).

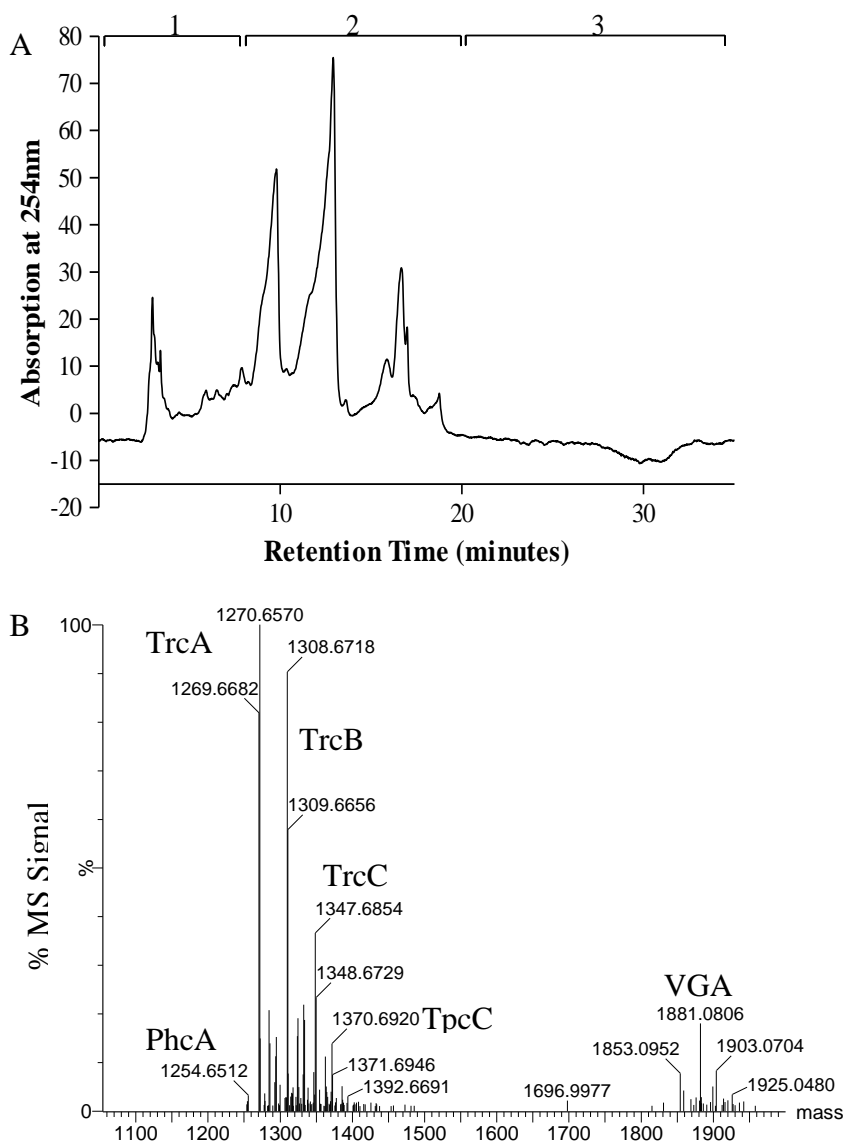


Figure 2.7: Semi-preparative RP-HPLC analysis on the peptide extract from cultures grown in autoclaved TGS. **A** The three fractions, collected to determine the mass contribution of Trcs to the total sample, are indicated. **B** MaxEnt 3 analysis of MS analysis of fraction 2 showing the presence of the major Trcs (Trc A, Trc B and Trc C) and a minor amount of Tpc C and linear gramicidin A (VGA).

2.5 Conclusions

Tyrocidine extraction from the commercially obtained tyrothricin produced a high purity extract (>95%) as determined by the percentage abundance of Trcs and Trc analogues from UPLC-MS analysis. This extract (purified commercial Trc mixture) is to be used in later chapters as a control in antimicrobial and haemolytic assays to correlate the activity observed

for Trc treated solid surfaces. Amino acid supplementation of the growth medium in this study produced the similar shifts in peptide production profile as can be found in literature [6]. It was found that only the addition of phenylalanine and tryptophan to the culture medium could change the types of Trcs and Trc analogues produced. The addition of phenylalanine caused a shift to predominantly Trc A analogues (TrcA, TrcA₁, PhcA and PhcA₁) to be produced whereas the addition of tryptophan showed a shift to the production of mainly tryptocidines (Tpcs).

Further purification of the autoclaved extract yielded an extract containing only Trcs and Trc analogues, which had a good purity based on analytical HPLC and potent antimicrobial activity against *M. luteus*. Analytical HPLC analysis of the autoclaved extract showed that Trcs contributed to about 70% of the sample weight which can have an effect on the observed antimicrobial activity of the Trc treated solid surfaces although a small amount of linear gramicidins may be present within the sample. A relatively pure peptide extract was obtained and in the rest of the study we will report the use of this autoclaved extract to produce antimicrobial surfaces, although the tailored extracts from amino acid supplemented cultures can be used to produce a variety of antimicrobial surfaces with different characteristics.

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Addendum A

In order to compare the peptide profile of each of the supplemented and unsupplemented media, the peptide extracts were analysed with UPLC-MS determining retention times, peak identity and peak area for each of the extracts. The analysis of the autoclaved extract is shown as an example of the analysis done to compile the data presented in the comparative bar graph (Fig. 2.4) discussed in the chapter.

An MS spectrum was obtained for each peak in the UPLC profile (Fig. A1) and was analysed with MaxEnt 3 to determine the M_r of compound in the peak. Most of the peaks contained only one major compound and showed the typical doubly ($[M+2H]^{2+}$) and singly charged ($[M+H]^+$) molecular ion species that are generally found for the Trcs and analogues in their spectra. There were some minor early eluting peaks with unknown compounds also showing the typical Trc spectra that were tentatively attributed to partially degraded peptide products.

The Trcs and Trc analogues identified within the sample were TrcC₁ (Fig. A5), TrcC (Fig. A6), TpcC (Fig. A8), TrcB₁/B₁' (Fig. A9), TrcB/B' (Fig. A10), PhcB (Fig. A11), TpcB/B' (Fig. A12), TrcA₁ (Fig. A15), TrcA (Fig. A16), PhcA (Fig. A17) and TpcA (Fig. A18). The peak areas were used to determine the percentage of each Trc that contributes to the entire Trcs present in the sample. Some of the peaks contained unknown compounds (Fig. A2, A3, A4, A7, A14) which could either be uncompleted or degraded tyrocindes.

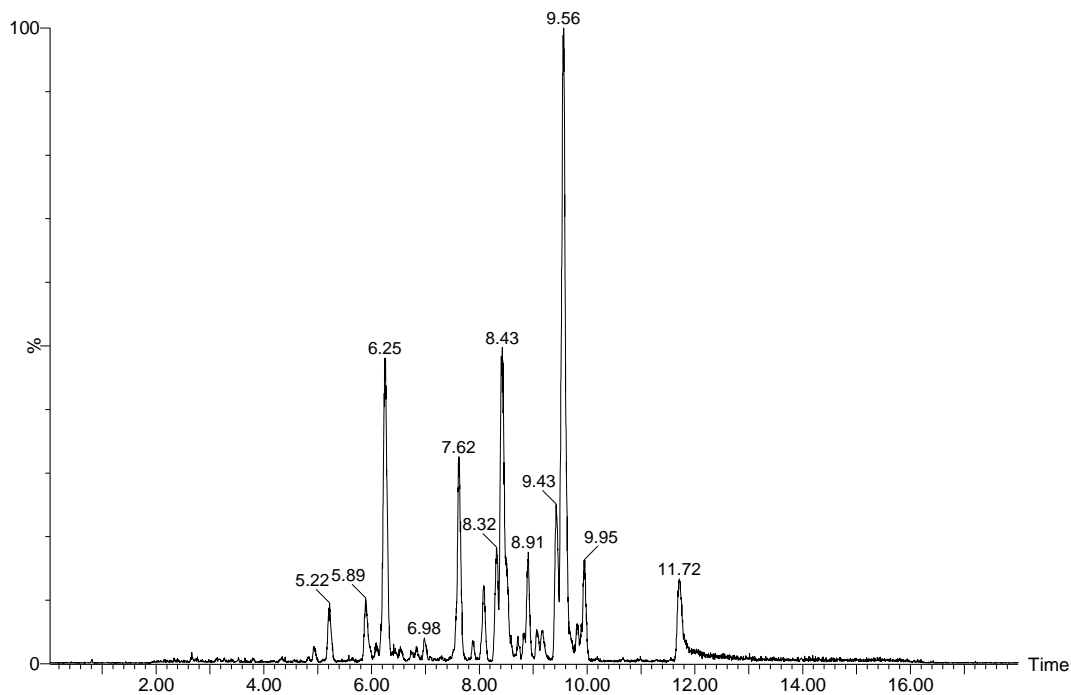


Figure A1: Complete UPLC profile obtained for the autoclaved extract.

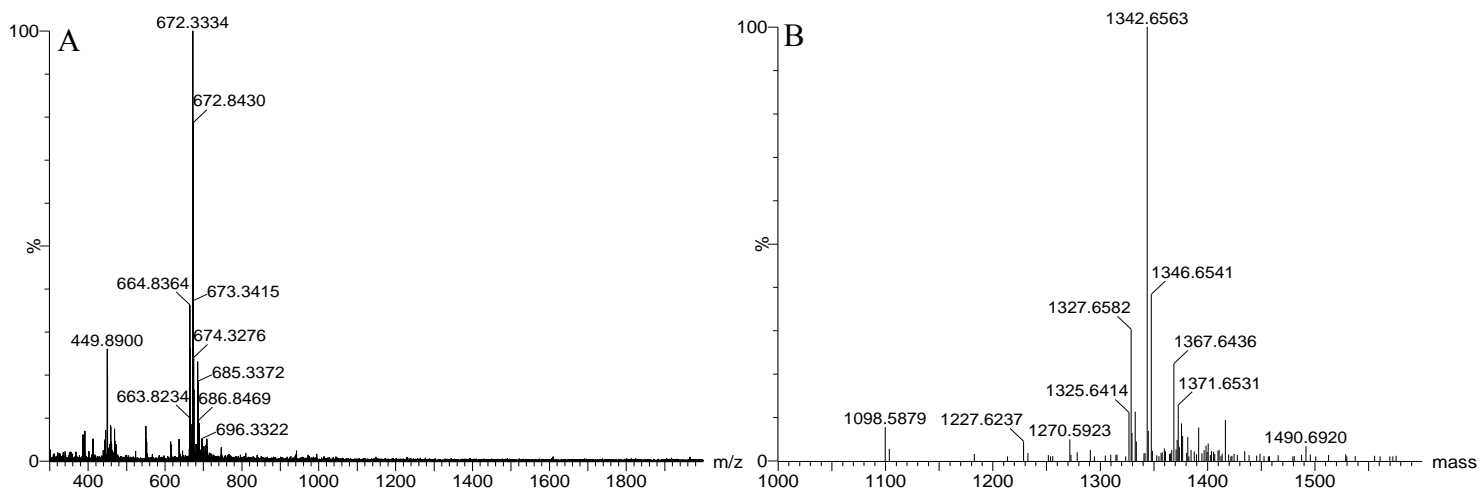


Figure A2: **A** An MS spectrum of the peak at 4.93min and **B** MaxEnt 3 analysis of MS spectrum indicating an unknown compound/peptide + H⁺ (1342.6563), possibly a linear deaminated TrcB₁, (expected M_r: 1341.7040)

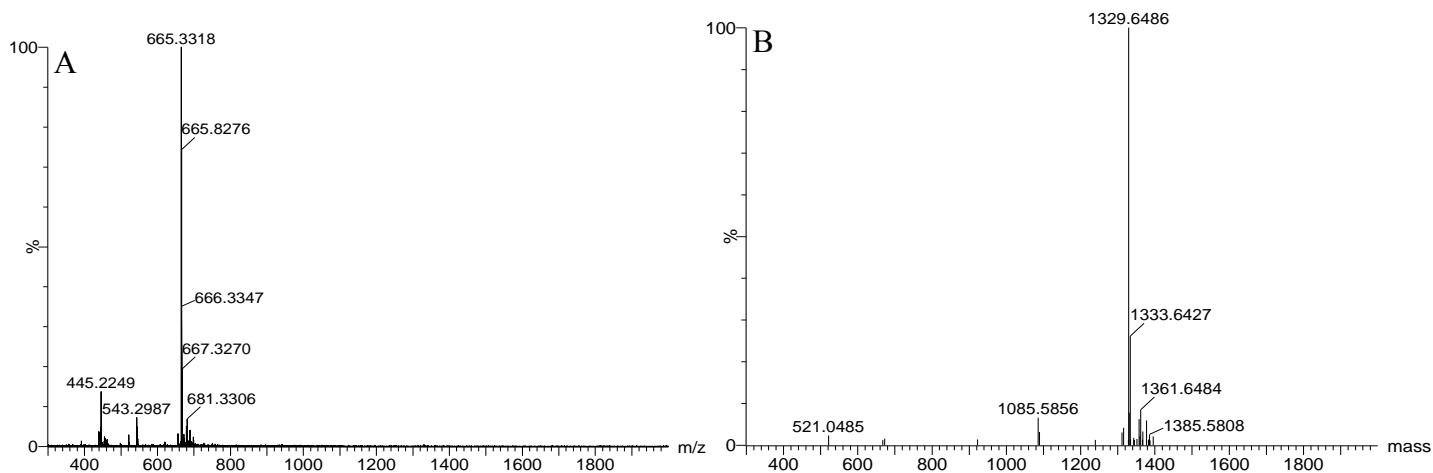


Figure A3: An MS spectrum of the peak at 5.22 min and **B** MaxEnt 3 analysis of MS spectrum indicating an unknown compound/peptide + H⁺ (1329.6486)

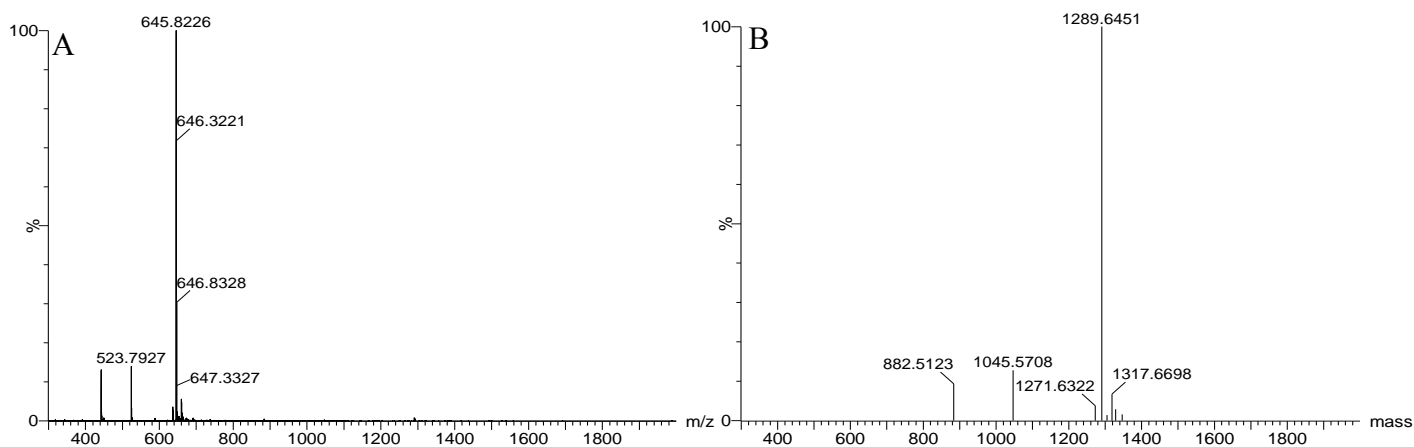


Figure A4: An MS spectrum of the peak at 6.25 min and **B** MaxEnt 3 analysis of MS spectrum indicating an unknown compound/peptide + H⁺ (1289.6451), possibly a linear deaminated TrcA (expected M_r: 1288.6775)

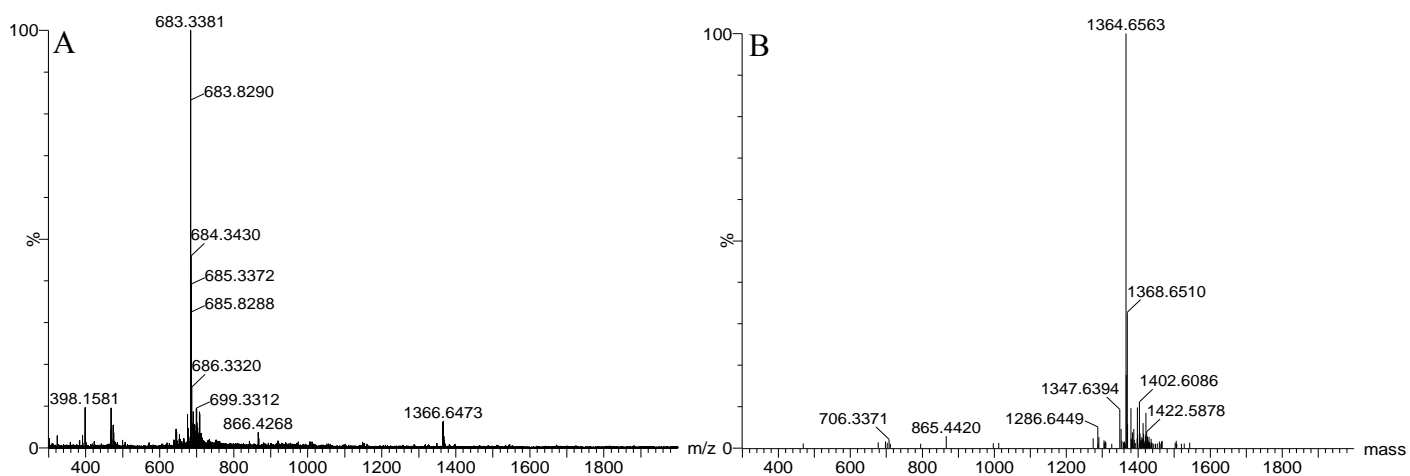


Figure A5: An MS spectrum of the peak at 6.98 min and **B** MaxEnt 3 analysis of MS spectrum indicating a unknown compound/peptide + H⁺ (1364.6563), possibly a hydroxylated TrcC (expected M_r: 1363.6758) .

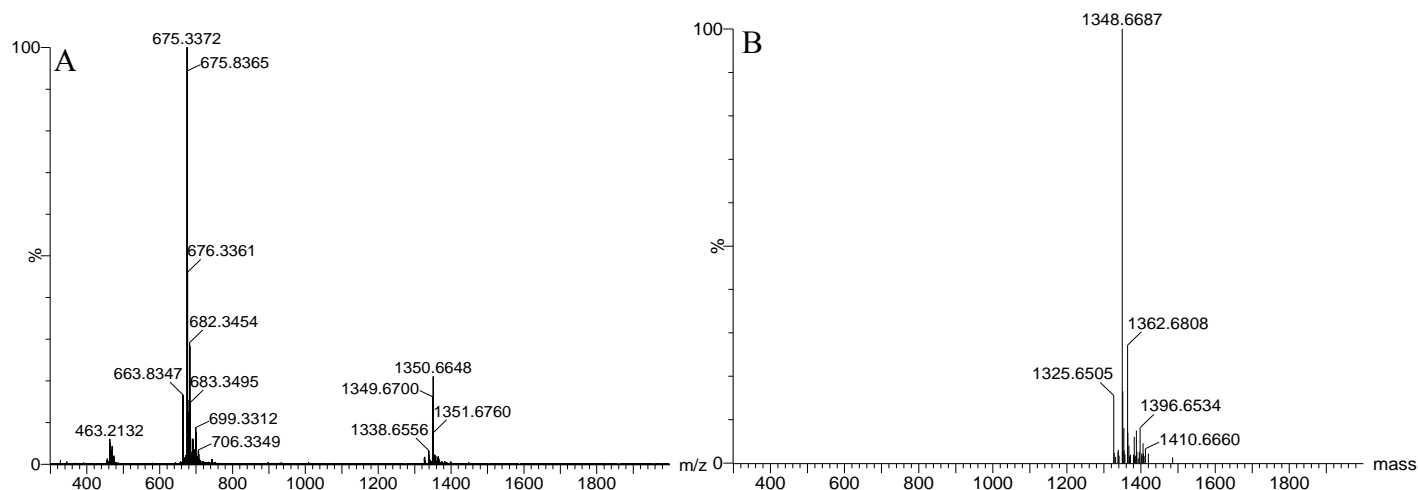


Figure A6: An MS spectrum of the peak at 7.62 min and **B** MaxEnt 3 analysis of MS spectrum indicating a TrcC + H⁺ (1348.6687)(expected M_r: 1347.6764)

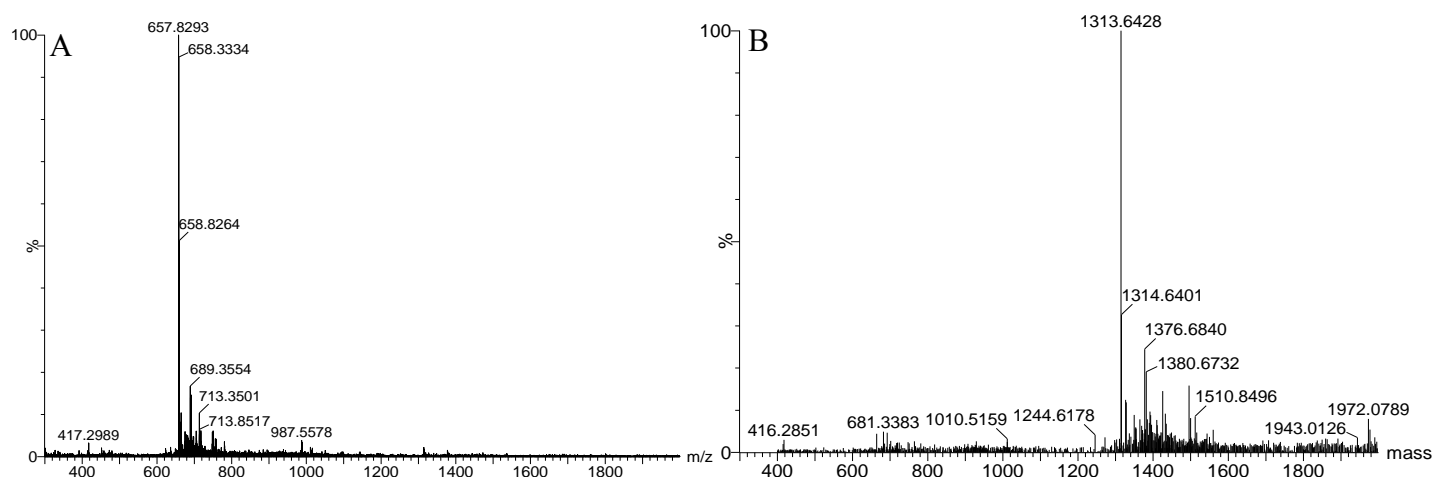


Figure A7: An MS spectrum of the peak at 7.88 min and **B** MaxEnt 3 analysis of MS spectrum indicating an unknown compound/peptide +H⁺ (1313.6428), possibly a linear deaminated TpcA (expected M_r: 1312.7013)

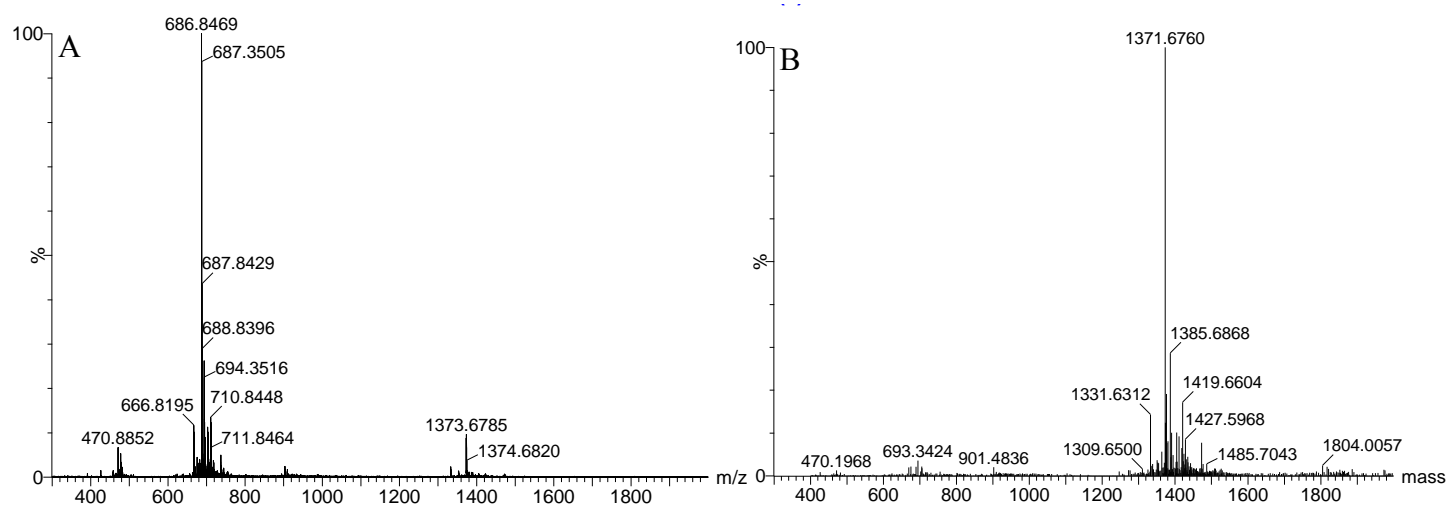


Figure A8: An MS spectrum of the peak at 8.08 min and **B** MaxEnt 3 analysis of MS spectrum indicating a TpcC + H⁺ (1371.6760)(expected M_r: 1370.6924)

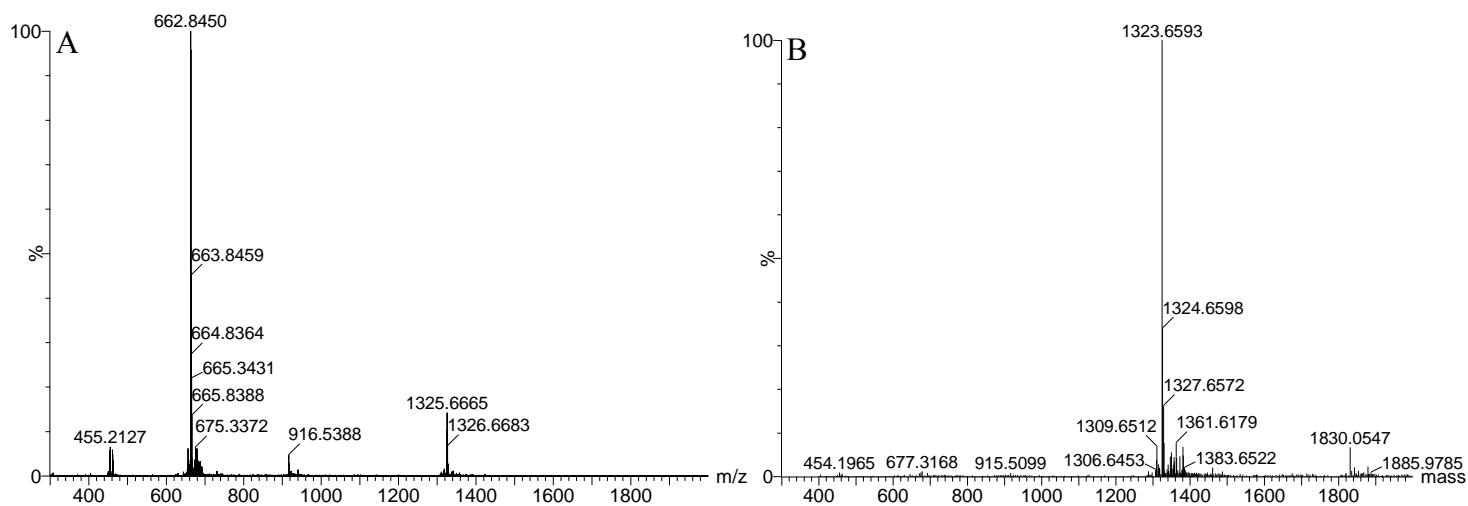


Figure A9: An MS spectrum of the peak at 8.32 min and **B** MaxEnt3 analysis of MS spectrum indicating a $\text{TrcB}_1/\text{B}_1' + \text{H}^+$ (1323.6593)(expected M_r : 1322.6812)

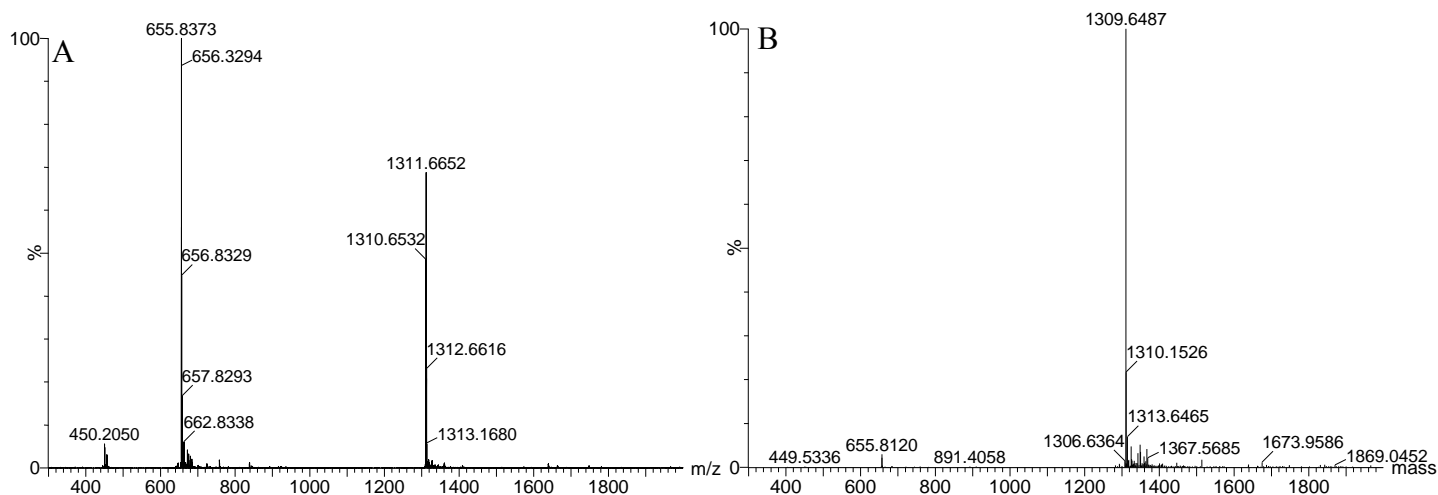


Figure A10: An MS spectrum of the peak at 8.43 min and **B** MaxEnt 3 analysis of MS spectrum indicating a $\text{TrcB}/\text{B}' + \text{H}^+$ (1309.6487)(expected M_r : 1308.6655)

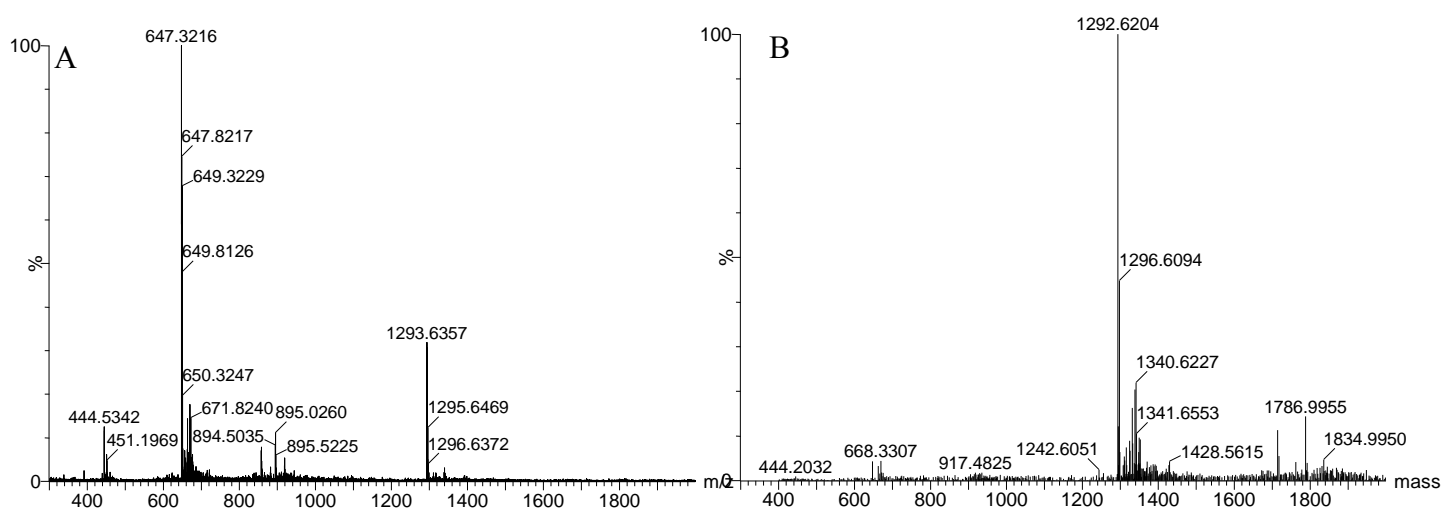


Figure A11: An MS spectrum of the peak at 8.72 min and **B** MaxEnt 3 analysis of MS spectrum indicating a $\text{TpcA} + \text{H}^+$ (1292.6204)(expected M_r : 1292.6706)

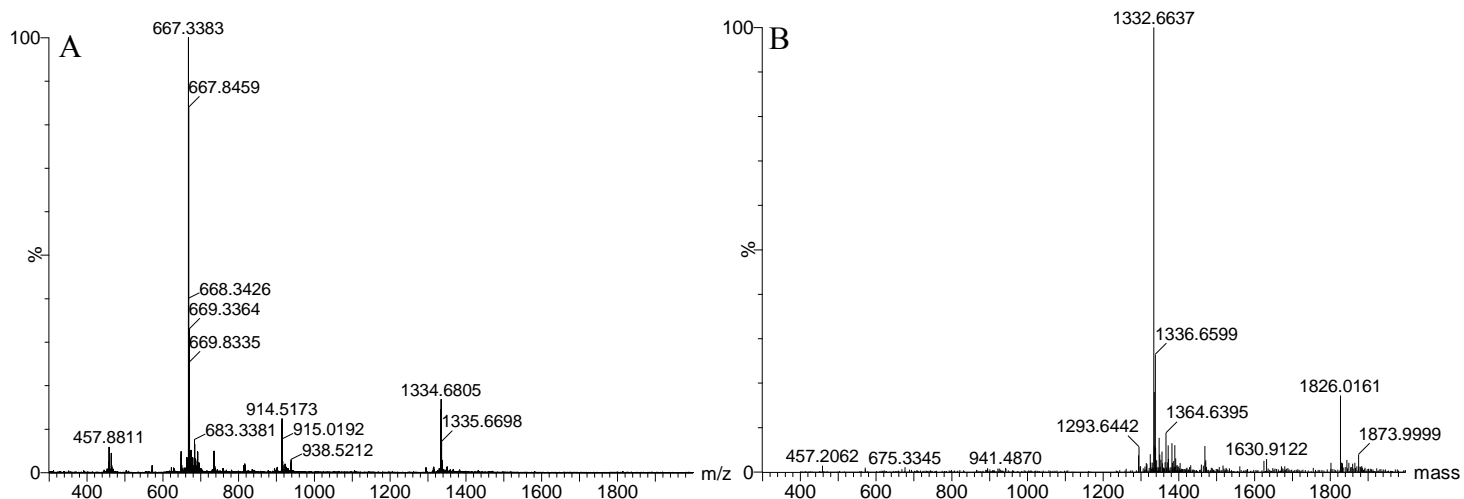


Figure A12: An MS spectrum of the peak at 8.91 min and B MaxEnt 3 analysis of MS spectrum indicating a TpcB/B' + H⁺ (1332.6637)(expected Mr: 1331.6815)

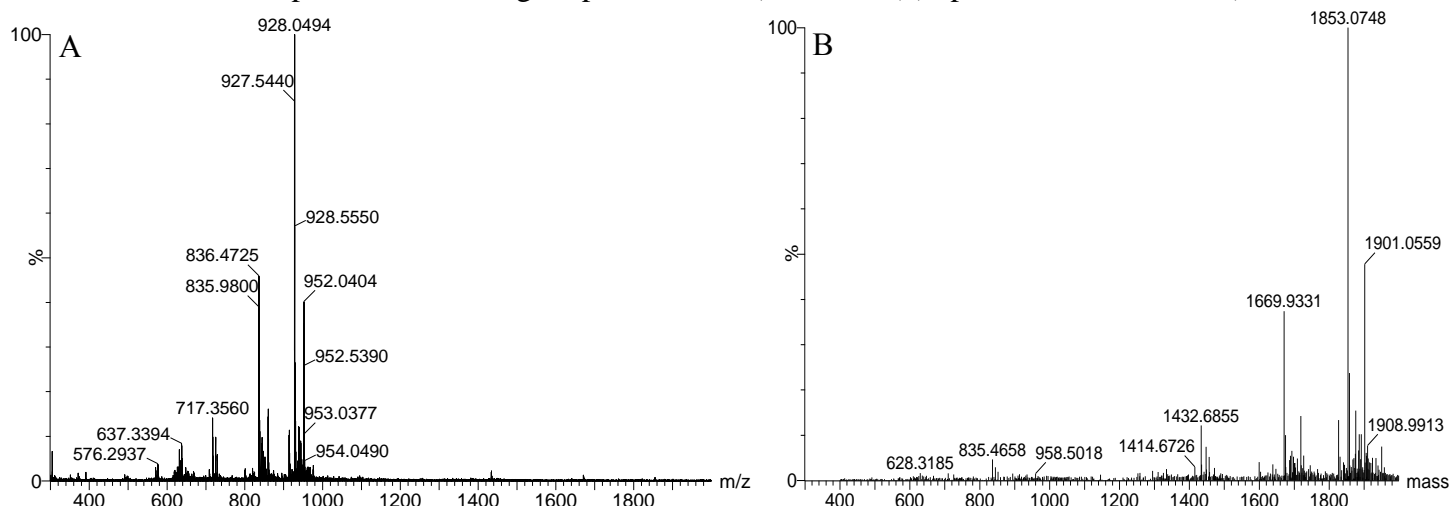


Figure A13: An MS spectrum of the peak at 9.06 min and B MaxEnt 3 analysis of MS spectrum indicating possibly a modified VGA (VGA-CO; 1853.0748) (expected M_r: 1853.0790)

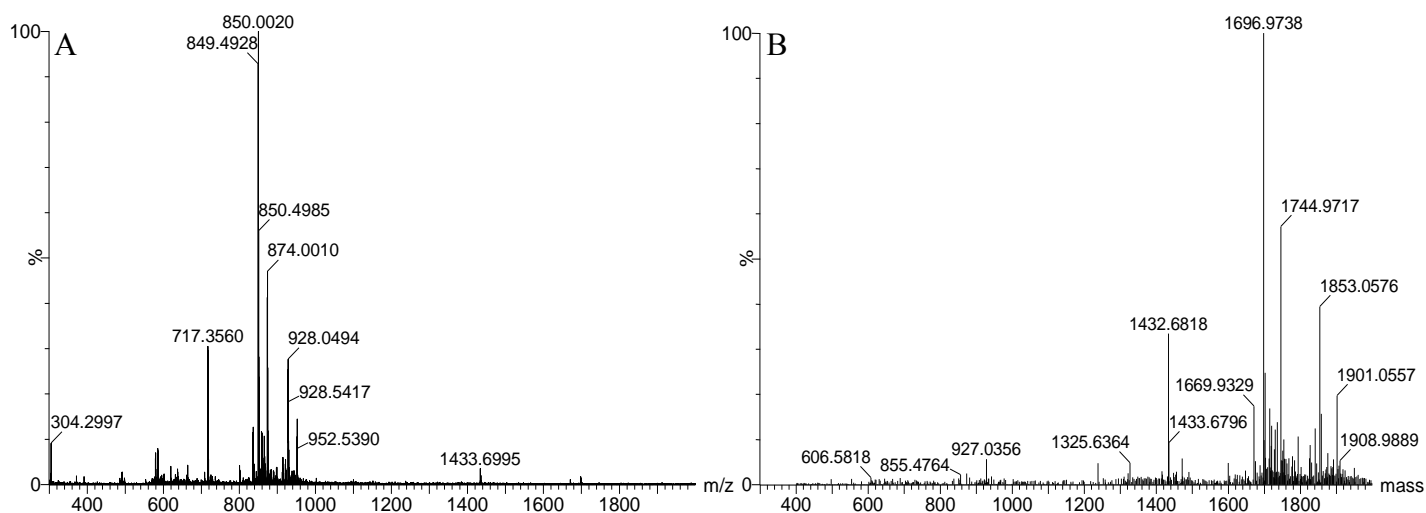


Figure A14: An MS spectrum of the peak at 9.18 min and B MaxEnt 3 analysis of MS spectrum indicating an unknown compound + H⁺ (1696.9738)

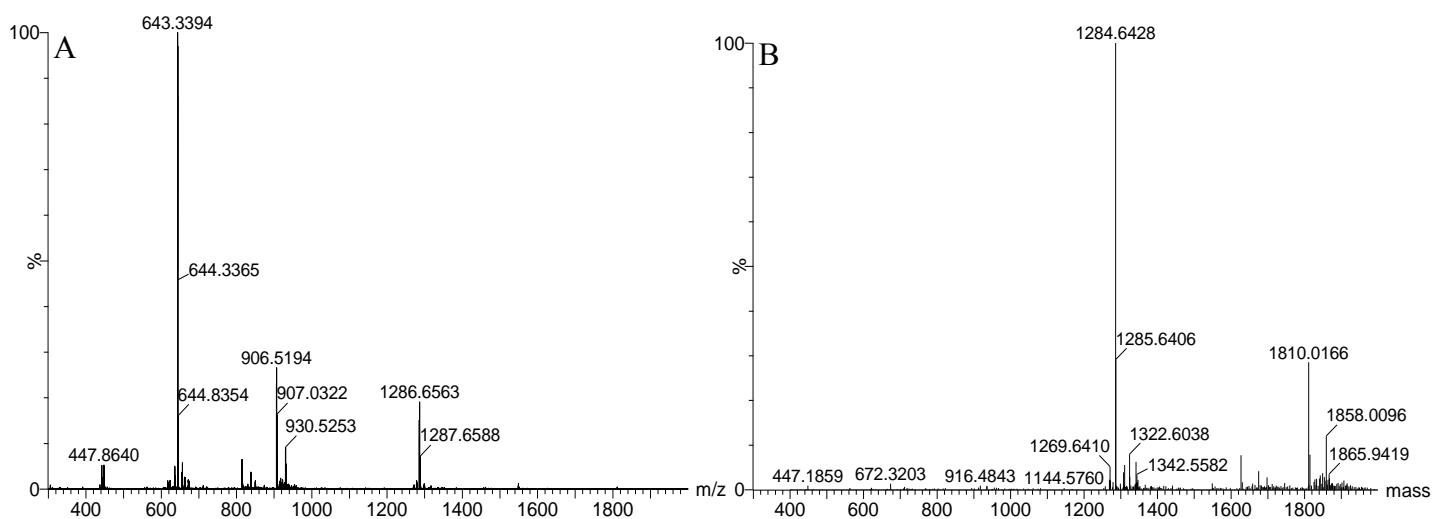


Figure A15: An MS spectrum of the peak at 9.43 min and B MaxEnt 3 analysis of MS spectrum indicating a TrcA₁ + H (1284.6428)(expected Mr:1283.6703)

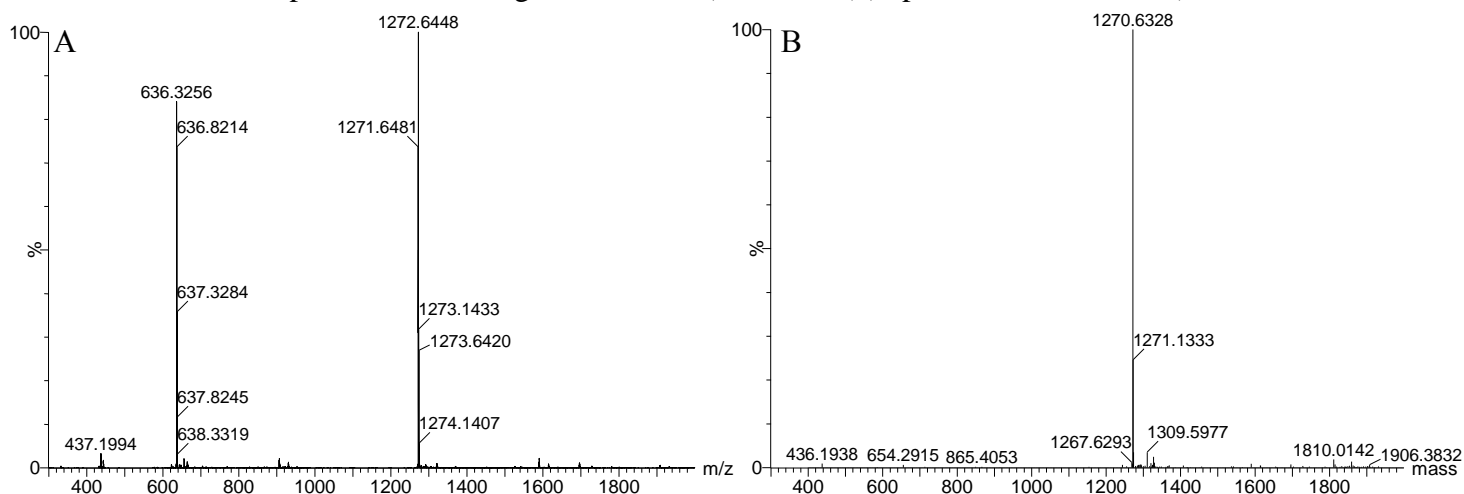


Figure A16: An MS spectrum of the peak at 9.56 min and B MaxEnt 3 analysis of MS spectrum indicating a TrcA + H⁺ (1270.6328)(expected M_r:1269.6546)

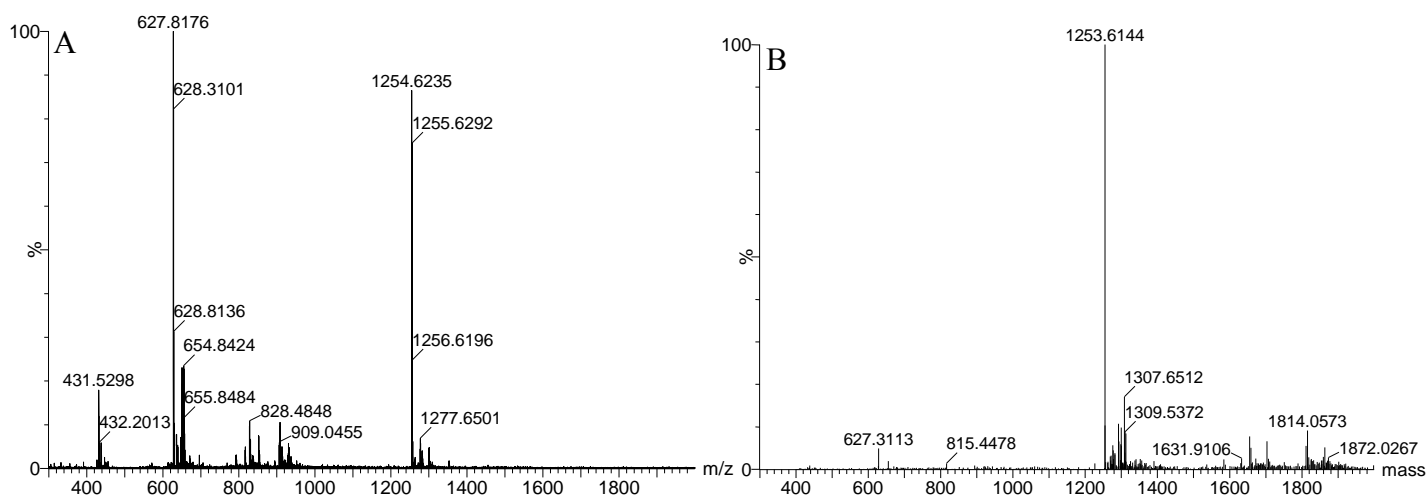


Figure A17: An MS spectrum of the peak at 9.82 min and B MaxEnt 3 analysis of MS spectrum indicating a PhcA + H⁺ (1253.6144)(expected M_r: 1253.6597)

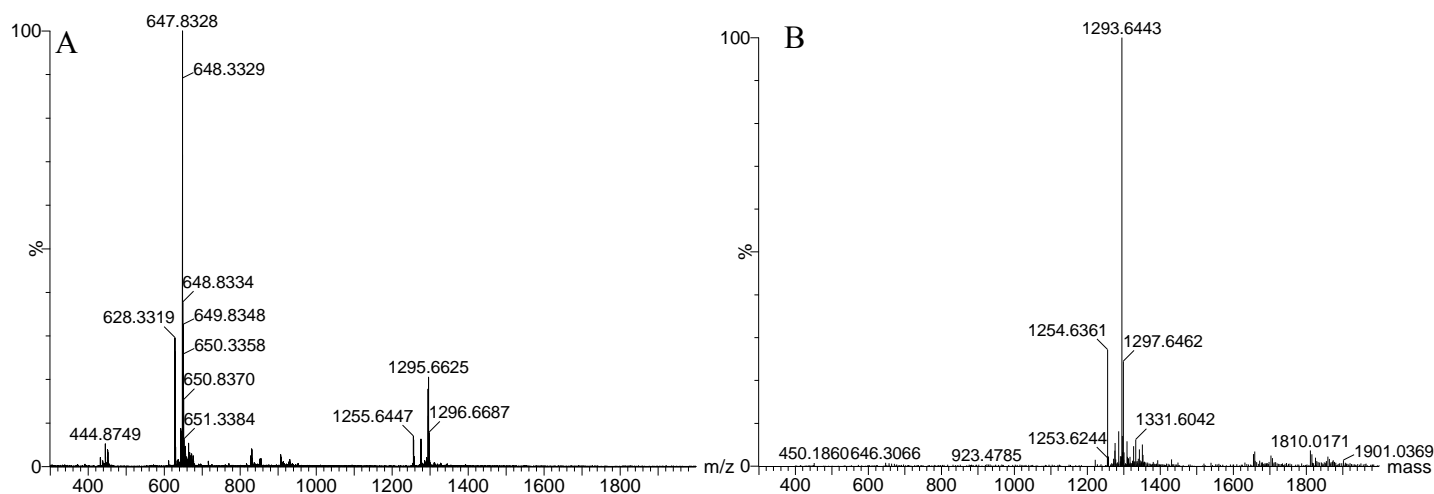


Figure A18: An MS spectrum of the peak at 9.95 min and B MaxEnt 3 analysis of MS spectrum indicating a TpcA + H⁺ (1293.6443)(expected M_r: 1292.6706)

Chapter 3

Characterisation of solid surfaces treated with antimicrobial peptides

3.1 Introduction

The emerging resistance of pathogens to currently available antibiotics, of which multidrug resistant pathogens is of the greatest concern, have forced researchers to establish how resistance develops and how it can be controlled. In most cases, in both medical and non-medical environments, biofilms seem to be the main cause of this predicament. Since biofilms are mostly resistant to treatments, the focus has shifted to prevent the colonisation of pathogens altogether. As a result much effort is put into developing antimicrobial solid phases or coatings. Antimicrobial coatings include non-fouling coatings, non-antibiotic substances such as silver, nitric oxide etc. and antibiotics. However, all these options presents its own problems ranging from narrow spectrum of organisms against which it is effective, cytotoxicity, inconsistent antimicrobial activity and the development of resistance. Consequently antimicrobial peptides are investigated as an alternative option. As an example to the use of antimicrobial peptide in the sterilisation of surfaces and the development of antimicrobial surfaces a synthetic peptide named Tet-20 (KRWRIRVRVIRKC) has shown a broad spectrum of activity *in vitro* and *in vivo*, prevented biofilm formation and was not cytotoxic to the host cells [1].

Tyrocidines (Trcs), the cyclic decapeptides produced by *Bacillus aneurinolyticus*, with linear gramicidins, are part of a peptide complex named tyrothricin. Tyrothricin was the first antimicrobial agent in clinical use and was limited to throat lozenges and topical applications due to its haemolytic activity. Trcs have a broad spectrum of activity ranging from Gram positive biofilm forming *Listeria monocytogenes* [2,3], the human malaria parasite *Plasmodium falciparum* [4] and fungi such as *Neurospora spp.*, *Fusarium solani*, *Botritus*

cinerea [5] and *Candida albicans* [6]. It has also been shown to prevent the formation of *C. albicans* biofilms and disrupt mature biofilms [6].

Peptide loss through purification and handling steps have indicated a tendency of Trcs to adsorb to various surfaces. This inherent characteristic can be harnessed to develop antimicrobial surfaces with a broad spectrum of activity. Based on the study done by Bagheri *et al.* [7] the immobilisation of peptides to solid surfaces are better suited for membrane active antimicrobial peptides especially when the peptides are covalently bound to the surface. Trcs have been observed to be membranolytic, but also have other intracellular targets [8], thus it would be better to adsorb it to a range of solid surfaces and determine its antimicrobial activity opposed to covalently binding the peptides to the surface. Moreover, the Trcs can still have a membranolytic activity, but also diffuse off the solid surface to act against the intracellular targets should it be required.

To date Trc activity studies have been predominantly done using liquid media and not solid phase interaction and activity. Therefore various solid surfaces were treated with a Trc extract and the amount of peptide adsorbed, quantified with change of absorbance of the incubation solution, ninhydrin staining, Sanger and Kaiser tests, amino acid analysis, wettability assay, Fourier transform infrared spectroscopy (FTIR), matrix assisted laser desorption ionization mass spectrometry (MALDI-MS), desorption of the peptide from matrices followed by UPLC-MS and scanning electron microscopy (SEM).

3.2 Materials

Pyridene, phosphorus pentoxide, trifluoroacetic acid (TFA, >98%), KCN, tyrothricin (extract from *Bacillus aneurinolyticus*) and gramicidin S (from *Brevibacillus brevis*) was supplied by Sigma-Aldrich (St. Louis, MA, USA). EDTA, acetic acid, sodium acetate was obtained from Merck (Wadeville, Gauteng). Analytical grade water was obtained from a reverse osmosis

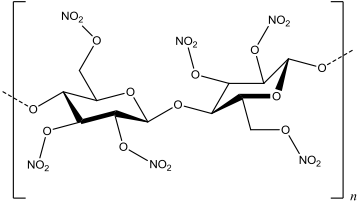
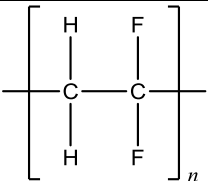
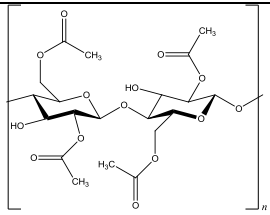
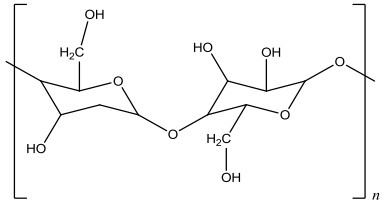
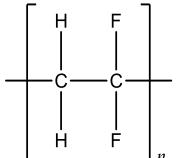
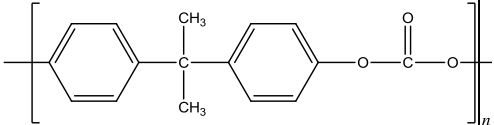
purification plant filtered through a Millipore MilliQ® water purification system (Milford, USA). Mixed cellulose ester filters (HAWP, GSWP), Polyvinylidene filters (HVLP, GSWP), 0.22µm polycarbonate syringe filters, Pico-Tag™ solution and analytical HPLC column (5µm particle size, 60 Å pore size, 3.9 mm x 150 mm) was supplied by Waters-Millipore (Milford, USA). Merck (Darmstadt, Germany) supplied ninhydrin, 1-fluoro-2,4-dinitrobenzene, phenol, absolute ethanol (>99.8%), hydrochloric acid and triethylamine (> 99.5%). Acetonitrile (ACN, HPLC-grade, far UV cut-off) was provided by Romil Ltd. (Cambridge, UK). Polycarbonate filters was supplied by Nuclepore Corp (Plesanton, CA, USA). Cellulose acetate and high density cellulose filters were provided by Sartorius (Göttingen, Germany). Cellulose filters (Paper) (MN 615/No 1) were obtained from Macherey-Nagel (Düren, Germany), phenylisothiocyanate (Edman's Reagent) was supplied by Life Technologies (Johannesburg, South Africa) and blue food colouring E133 was supplied by Moir's (Cape Town, South Africa)

3.3 Methods

3.3.1 Treatment of solid surfaces with tyrocidine extract

Solid surfaces were incubated with a 50 µg/mL peptide solution (± 1 hour) and rinsed with analytical grade water to remove any excess peptide. Filters were then dried overnight (± 16 hours) in a low temperature oven. Filters (diameter 47 mm) used were mixed cellulose esters (HAWP, GSWP), polyvinylidene fluoride (HVLP, GSWP), polycarbonate (PC), cellulose acetate (CA), high density cellulose (HDC)(commonly used as an insert between filter packaging) and cellulose filters (CL, diameter 47 mm or 100 mm) (Table 3.1). Filters were pre-sterilized using either 50% acetonitrile or autoclaved based on the characteristics of the filters and which method least affected the filter integrity.

Table 3.1: Summary of the characteristics and conventional use of selected filters grouped based on hydrophilic/hydrophobic nature as classified by manufacturing companies.

Filter name	Monomer structure(s)	Protein binding	Conventional use
Hydrophilic character			
Mixed cellulose ester - cellulose acetate and nitrocellulose (GSWP, HAWP)		High Binding	Buffer/solvent filtration, unsuitable for protein recovery
Polivinyldene fluoride (HVLP)		Low non-specific binding	General biological filtration where high protein recovery is desired
Cellulose acetate (CA)		Very low binding	Filtration of tissue culture media and sensitive biological samples
Cellulose (CL)		Extremely low non-specific binding	Applications where low non-specific binding is desired
High density cellulose (HDC)		Unknown	Packaging
Hydrophobic character			
Polivinyldene fluoride (GVHP)		Low non-specific binding	General biological filtration where high protein recovery is desired
Polycarbonate (PC)		Low non-specific binding	General sterile filtration

3.3.2 Detection and quantification of peptide adsorbed to filters

3.3.2.1 Spectrophotometric analysis

Absorbance readings were taken before and after incubation of each of the 50 µg/mL peptide solution at wavelengths 230 nm, 256 nm and 280 nm. The wavelengths were chosen to detect peptide bonds, aromatic rings and tryptophan respectively. The amount of peptide adsorbed to each filter was calculated based on a standard curve of a known concentration Trc extract and the change of absorbance between the before and after readings.

3.3.2.2 Ninhydrin visualisation

Ninhydrin staining was used to detect primary and secondary amines present on the filter. A solution of ninhydrin (0.2% w/v in absolute ethanol) was used to spray treated and untreated filters after which it was dried and heated to 50-60°C in an oven.

3.3.2.3 Sanger's Test

The Sanger's Reagent is used to detect the presence of primary amines within a solution [9]. Sanger's Reagent (1-fluoro-2,4-dinitrobenzene) was prepared according to an established protocol at a concentration of 1.0 g/L in 95% ethanol. The concentration of 1-fluoro-2,4-dinitrobenzene in the stock solution was adjusted to an absorbance value of 0.200 at 381 nm for a mixture of equal volumes of ethanol and Sanger's reagent. A small treated and untreated punch filter (5 mm diameter), was incubated with 500 µL ethanol and Sanger's reagent for 30 minutes in the dark, after which the absorbance of the solution was measured at 381 nm. Each sample set was incubated with a blank sample (500 µL ethanol and Sanger's reagent) and positive control of Trc extract (2.00 mg/mL).

3.3.2.4 Kaiser test

The Kaiser test is used in protein synthesis to detect primary amines within a solution [10]. Test solutions were prepared according to an established protocol: ninhydrin (5% w/v in 95% ethanol), phenol (40% w/v in 95% ethanol) and KCN (2% v/v in freshly distilled pyridine). Equal volumes of each solution (100 μ L) was incubated with a small treated and untreated punch filter (5 mm diameter) in an 80°C water bath for 5 minutes after which the colour of the solution and the filter surface was visually assessed to determine the presence of peptide. Both treated and untreated filters were tested including Trc extract (2.00 mg/mL) as positive control.

3.3.2.5 Amino acid analysis

Amino acid analysis was performed to quantify the amino acids present on the filter. A punch filter (5 mm diameter) of each filter type, treated and untreated, was placed in analysis tubes and dried under vacuum after which it was placed in a hydrolysis flask with 6 M HCl containing 1% phenol to be hydrolysed for 1 hour at 150°C. Following hydrolysis the glass tubes were removed and wiped clean to remove any acid on the outside of the tubes. Next re-drying agent (10 μ L, ethanol:water:TEA, 1:2:1) was added to all the samples to obtain the optimal pH for derivatisation and dried under vacuum. Derivatisation solution was added to all the samples (20 μ L, methanol:TEA:water:phenylisothiocyanate (PITC) (7:1:1:1), left at room temperature for 20 minutes after which it was dried under vacuum. Lastly Pico-Tag solution (50-300 μ L) was added to each sample and was filtered through HVLP (0.45 μ m) filters.

Amino acid separation was obtained with a reverse phase analytical C₁₈ Nova-Pak® column (150 mm x 3.9 mm, 6 μ m particle size, 60Å pore size) using a chromatographic system controlled by Millennium software (Version 32) comprising of a Waters 717 Plus

Autosampler, two Waters 510 pumps and a Waters 440 detector set at 254 nm. A gradient of Eluent A and Reagent was used for the column separation at 1,0 mL/min flow rate (Table 3.2). Eluent A consisted of 19.0 g sodium acetate, 1L analytical grade water, 0.500 μ L triethylamine, 0.200 μ L EDTA (1000 ppm) at a pH of 6.4 (adjusted with acetic acid and KOH) and was filtered with a HVLP filter. Acetonitrile (20 mL) was added to the mixture (940mL) and sonicated for 20 seconds. Eluent B consisted of acetonitrile (600 mL), analytical grade water (400 mL) and 0.200 μ L EDTA (1000 ppm). Eluent B was also filtered using a HVLP filter and sonicated for 20 seconds.

Table 3.2: Gradient of Eluent A and Eluent B used for amino acid separation [11]

Time (min)	% Eluent A ^a	% Eluent B ^b	Curve Type
0.0	99	1	
10.0	50	50	5 (curve)
10.5	0	100	6 (linear)
12.2	0	100	6 (linear)
12.7	0	100	6 (linear)
13.2	99	1	6 (linear)
20.7	99	1	6 (linear)
21.0	99	1	6 (linear)

^a 0.14 M sodium acetate, 10 mM EDTA, 0.5mL TEA/L + 6% Acetonitrile

^b 60% v/v Acetonitrile, 10mM EDTA

3.3.2.7 Fourier transform infrared spectroscopy

FTIR was performed to detect compounds present on the filters based on an increase of absorption at different wavelengths. FTIR spectra were obtained with a NEXUS model custom made by Thermo Nicolet instruments for the Department of Polymer Science at the University of Stellenbosch. The spectra were obtained in reflectance mode using the Golden Gate Smart Performer ATR (ThermoScientific) with ZnSe lenses. Treated and untreated CL filters were dried overnight to remove all moisture, placed in the instrument and scanned with

a resolution 4 cm^{-1} representing a data point spacing of 2 cm^{-1} . The final spectra were recorded over the wavelength range from $4000 - 650\text{ cm}^{-1}$. The spectrometer was equipped with a Ge-on-KBr beamsplitter and DTGS/CsL detector and not purged with high purity nitrogen gas during recording. The data was collected and manipulated with the basic OMNIC software package (ThermoScientific).

3.3.2.8 Matrix assisted laser desorption ionization mass spectrometry

MALDI-MS was performed to confirm the presence of Trcs on the filter. CL filter, treated and untreated, was pulled apart to fibre level in 50% (v/v) acetonitrile after which $1.0\text{ }\mu\text{L}$ was spotted onto a 384 MTP MALDI polished steel target plate and air dried. MALDI matrix, α -cyano-4-hydroxycinnamic acid, was prepared in 50% (v/v) acetonitrile, 2.5 % (v/v) TFA at 10 mg/mL and $1\text{ }\mu\text{L}$ was placed over the dried sample and air dried. Analysis was done on the UltrafleXtreme MALDI TOF/TOF (Bruker Daltonics) mass spectrometer at the Proteomics Research & Services at the University of the Western Cape. Data was collected in the linear positive mode from 0 to $3\text{ }000\text{ Da}$ and the initial laser power set to 40%. The spectrum was acquired with the software FlexControl (version 3.4; Bruker Daltonics, Bremen, Germany) by rastering the target spot 7 times with accumulated peptide spectra of $4\text{ }000$ shots with a laser frequency of 1000 Hz . A peptide calibration standard (Bruker Daltonics) was used to externally calibrate the spectrum.

3.3.2.9 Desorption of peptide from CL and analysis

Desorption of peptide was completed to confirm the presence of Trcs on the CL filters. The peptide present was desorbed by incubating CL filters in 50% (v/v) acetonitrile for 5 days after which the solution was removed, centrifuged at $3\text{ }000\text{ rpm}$ for 10 minutes, the supernatant collected and dried under vacuum. The presence of peptide and peptide identity was detected and confirmed with UPLC-MS.

During UPLC-MS 3 μL of the peptide sample (250 $\mu\text{g}/\text{mL}$ in water) was injected onto a Water Aquity UPLC[®] HSS C₁₈ column (21 x 150mm, 1.8 μm particle size) with a 0.450 mL/min flow rate. Separation was obtained with a gradient from eluent A (0.5% v/v formic acid) and eluent B (acetonitrile). The gradient was as follows: 100% eluent A from 0 to 0.5 minutes, 0% to 58% eluent B from 0.5 to 12 minutes and lastly 58% to 90% eluent B from 12 to 13 minutes. In line TOF-MS was done the same as with direct injections, with a Waters Synapt G2 quadrupole TOF mass spectrometer (Milford, MA, USA) with an electrospray ionisation source. Samples were exposed to a capillary voltage of 3.0kV with a cone voltage of 15V and source temperature of 120°C. Nitrogen was selected as the desolvation gas (650 L.h⁻¹) at a desolvation temperature of 275°C. All data was collected scanning over an m/z range of 300-2000 in the positive mode. Samples injected directly into the MS were prepared in the same manner as with UPLC-MS and with the same injection volume.

3.3.3 Determination of wettability

The wettability of untreated and treated (gramicidin S and Trc) filters were determined to ascertain the effect the peptide have on the hydrophobicity of the filters. Water (50 μL) modified with blue food colour (E133) was pipetted onto untreated and treated filters and the filters studied for the drop size and time to complete absorption of the droplet.

3.3.4 Visualisation with scanning electron microscopy

SEM was performed to determine if the surface structure of treated and untreated filters were changed by treating the filters with a Trc extract. Filters (GSWP, CA and CL) were dried completely to remove any moisture that could interfere with the scanning electron microscopy (SEM) signal and were placed in a desiccator with phosphorus pentoxide to ensure that the samples remained dry for transport. Samples were then mounted on stubs

covered in double sided carbon isolation tape and subsequently coated with a thin layer of gold to make the surface of the sample electrically conducting.

Scanning electron microscope images were obtained with a Leo® 1430VP Scanning Electron Microscope at the Central Analytical Facility at Stellenbosch University. The images obtained show the surfaces of the filters. Conditions during the surface analysis were 7 kV and 1.5 nA for the beam conditions, a spot size of 145 – 155nm and a working distance of 13 mm.

3.4 Results and Discussion

Very little is known about the activity of Trc treated solid surfaces other than tests as part of wound dressings to ascertain whether Trcs would irritate the skin of the patient or hinder the healing of superficial skin injuries [12, 13]. It was therefore decided to test a range of solid surfaces in a pilot study to determine the solid surface activity of Trcs. Solid surfaces included mixed cellulose esters, polyvinylidene fluoride, polycarbonate, CA, HDC and CL (paper) filters (refer to Table 3.1). Solid surfaces were chosen to cover a range of polarities, filter characteristics and conventional applications. Chosen filters can be organized from most polar to least polar (as described by manufacturing companies) as polyvinylidene fluoride > mixed cellulose > CL/HDC (HDC) > CA > polycarbonate. Polyvinylidene fluoride, HDC, polycarbonate and CL filters have a very low protein binding character whereas mixed cellulose filters are known to be high protein binding [14-16].

3.4.1 Chemical quantification of peptide adsorbed to solid surfaces

The activity of peptide attached to any solid surface whether it is via covalent bonds or adsorption is not only related to the amount of peptide attached, but also the orientation of attachment. Quantification of the amount of peptide retained is crucial in the light of possible

application development of the peptide treated filters especially in cases where the dosage plays a key role e.g. wound dressings.

The first line of quantification entailed monitoring the change in absorbance of the incubation solution based on the assumption that a change in absorbance would directly correlate to a change in peptide concentration. This observed change could then be compared to a standard curve (Fig. 3.1), for which the linear relationship between peptide concentration and absorbance has been determined, and the proposed amount of peptide adsorbed to the filter calculated accordingly. The amount of absorbed peptide could not be calculated for the CA, HDC and CL filters since the absorbance of the incubation solution increased after incubation (Table 3.3). This is possibly due to desorption and dissolving of residual compounds on the filters in the aqueous treatment solution. It was found that for the remaining filters that the mixed cellulose ester filters adsorbed the most peptide, followed by the polyvinylidene fluoride filters and lastly the polycarbonate filters. In both cases for polyvinylidene fluoride and mixed cellulose ester filters the change in pore size (0.45 μm & 0.22 μm) showed very little effect on the amount of peptide retained. The change in absorbance can be used to detect the amount of peptide adsorbed, but is limited by the type of filter tested and that the obtained absorbance values must fall within the scope of the standard curve to directly correlate to peptide concentration.

Table 3.3: Summary of amount of Trc or gramicidin S retained as calculated by the observed decrease in absorbance (A_{230}) with (n) amount of repeats per filter. (*) Mixed Cellulose, (#) Polyvinylidene fluoride, PC: Polycarbonate, HDC: High Density Cellulose and CL: Cellulose. HAWP and HVLP have a pore size of $0.45\ \mu\text{m}$, GSWP and GVHP has a pore size of $0.22\ \mu\text{m}$.

Filter (n)	HAWP*	GSWP*	HVLP [#]	GVHP [#]	PC	CA	HDC	CL
[Trc] $\mu\text{g}/\text{cm}^2$ (n)	3.3 ± 0.7 (7)	3.4 ± 2.4 (8)	1.8 ± 1.0 (8)	1.6 ± 0.82 (3)	0.88 ± 0.47 (4)	nd	nd	nd
GS $\mu\text{g}/\text{cm}^2$	nd	Nd	2.8 ± 0.6 (12)	5.3 ± 1.1 (12)	1.2 ± 2.2 (12)	nd	nd	nd

nd – Not determined

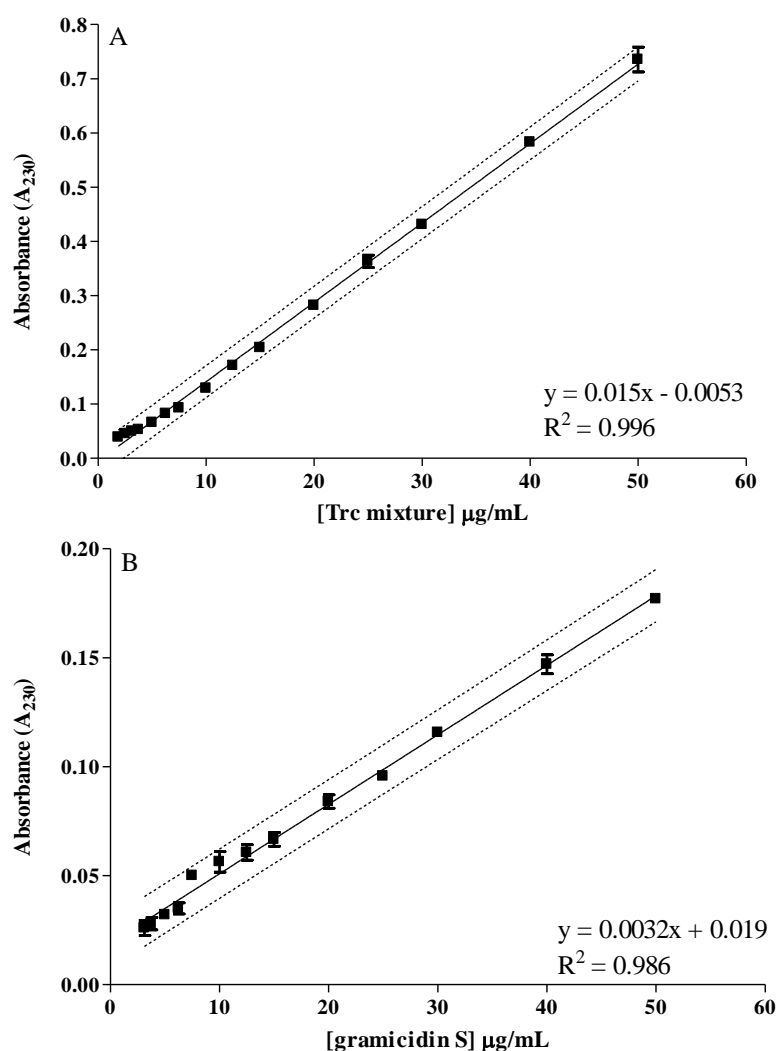


Figure 3.1: Absorbance standard curves as compiled from the **A** Trc extract and **B** gramicidin S used to treat the solid surfaces

The amount of Trc adsorbed was calculated from the standard curve in Fig 3.1 to be between 0.9-34 $\mu\text{g}/\text{cm}^2$ (Table 3.3). The amount of GS retained, based on the absorbance method, was determined to be between 1.2 and 5.3 $\mu\text{g}/\text{cm}^2$ for polyvinylidene fluoride and polycarbonate filters. It was not possible to determine this for the other filters due to the release of an absorbing component from these filters (Table 3.3).

Ninhydrin visualisation and the Kaiser test (also containing ninhydrin) detect primary amines (Orn and Lys side chains) and Sanger test (containing 1-fluoro-2,4-dinitrobenzene) detects the presence of both primary and secondary amines (Orn, Lys, Asn, Gln and Trp side chains). Both treated and untreated filters were analysed with all three methods. Ninhydrin staining only showed results for the treated CL filter (Fig.3.2: B) indicating the presence of primary amines. The CL filter showed uneven staining over the surface of the peptide treated filter (Fig. 3.2: B) which points to irregular coverage of peptide which in turn could have an effect on biological assays and possible application.

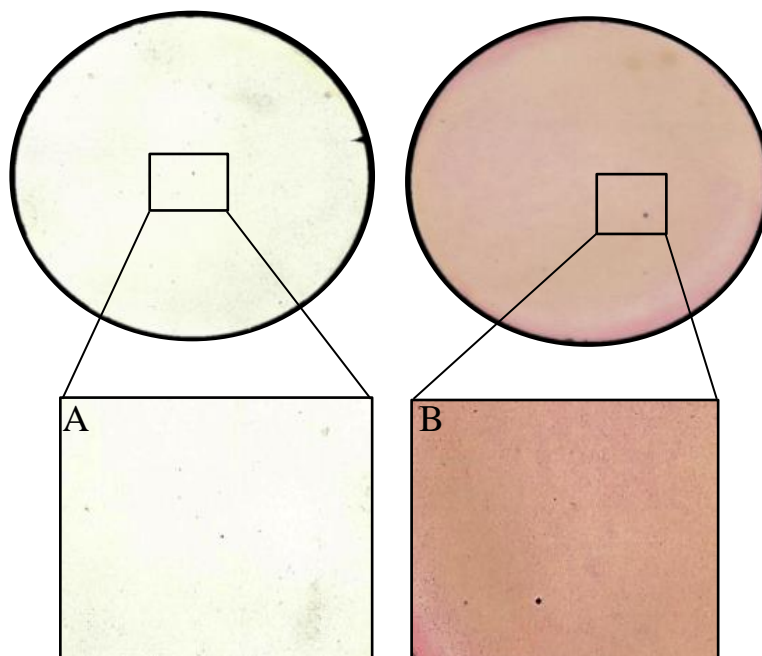


Figure 3.2: Pictures of ninhydrin staining of a representative **A** untreated CL filter and **B** Trc extract treated CL filter.

Although the Kaiser test also depends on ninhydrin for amine detection, very little colour change to green/blue/purple (indicating the presence of amines) was observed in the test solution for the CL filter. There was, however, some colour change on the filter itself but the change was not stable and dissolved in the solution. No overt colour change was observed for the test solutions of the other filters. The Sanger's test should have presented a positive result based on the detected primary amines with the ninhydrin results, however, no change in absorbance (A_{381}) was observed, possibly due to the low peptide concentrations on the filters that were below detection limits. The analyses of the synthetic polymer filters did not yield unbiased results, indicating that either that levels of peptide was too low to detect or that the cationic residue's amino group interacted with the filter and was shielded from the amine active compounds of the various tests.

Amino acid analysis was performed on both treated and untreated filters to quantify the amino acids in the filters, specifically the amino acids present in the Trc structure such as Leu, Phe, Pro, Trp, Asn, Gln, Tyr, Val, Orn and Lys. However this method failed to distinguish between treated and untreated filters as both gave mostly background amino acids. This indicated that the amount of peptide present on the filter samples was too little to be detected over the background amino acid signal of about 50-100 pM (results not shown). This background amino acid signal is possibly due to conventional handling of the filters that caused a transfer of proteins to the filters.

FTIR analysis was used to assess if peptide is adsorbed to CL filters by detecting a change in infrared absorbance at specific wavelengths of amide bonds (peptide bonds) in the Trcs. The FTIR analysis also failed to detect peptide and showed minimal difference between the untreated and treated filters possibly due to the low levels of peptide (Fig 3.3). The IR peaks detected are primarily from the bonds detected within the CL structure of the filters as

summarized in Fig. 3.3. Amide bonds are expected between 3400 – 3250 cm^{-1} and 1650 – 1580 cm^{-1} [17]. No difference between the untreated and treated filters was observed at 1650 – 1580 cm^{-1} (Fig. 3.3). However, some difference was found at 3400 – 3250 cm^{-1} , where the intensity was increased in the treated filters which can be attributed to the retained peptide on the CL filters.

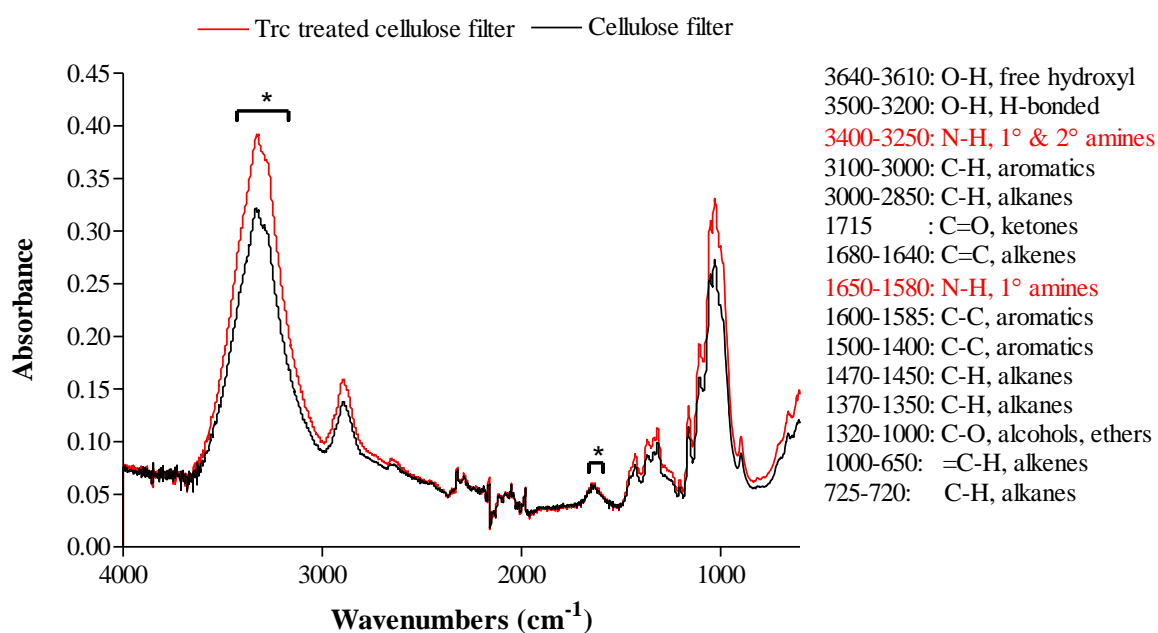


Figure 3.3: Fourier transform infrared spectroscopy of treated and untreated CL filters. Wavenumbers where increases were expected based on amide bonds present in the peptides are in red [17] and on spectra. *Spectral ranges where amide bonds and amines absorb are also indicated on the spectra.

MALDI analysis was performed to confirm the presence of Trcs on the solid surfaces. Analysis done on the CL filter fibres with MALDI-MS indicated the presence of TrcC (1348.590) (Fig. 3.4: B) and some modified peptides namely the dehydrated doubly charged species of TrcC ($m/z = 664.668$) and dehydrated TpcA (1276.896) and possibly a glycosylated TpcA (1418.963).

The presence of TrcC does indicate that Trcs are adsorbed to the CL filter; however none of the other analogues were detected. This could possibly be attributed to the method of sample preparation: the CL filter was decomposed to fibre level in 50% (v/v) ACN in preparation for

analysis. The time that elapsed in doing so, could be ample time for the other analogues to wash off the filter and adsorb to the glass surface used for sample preparation.

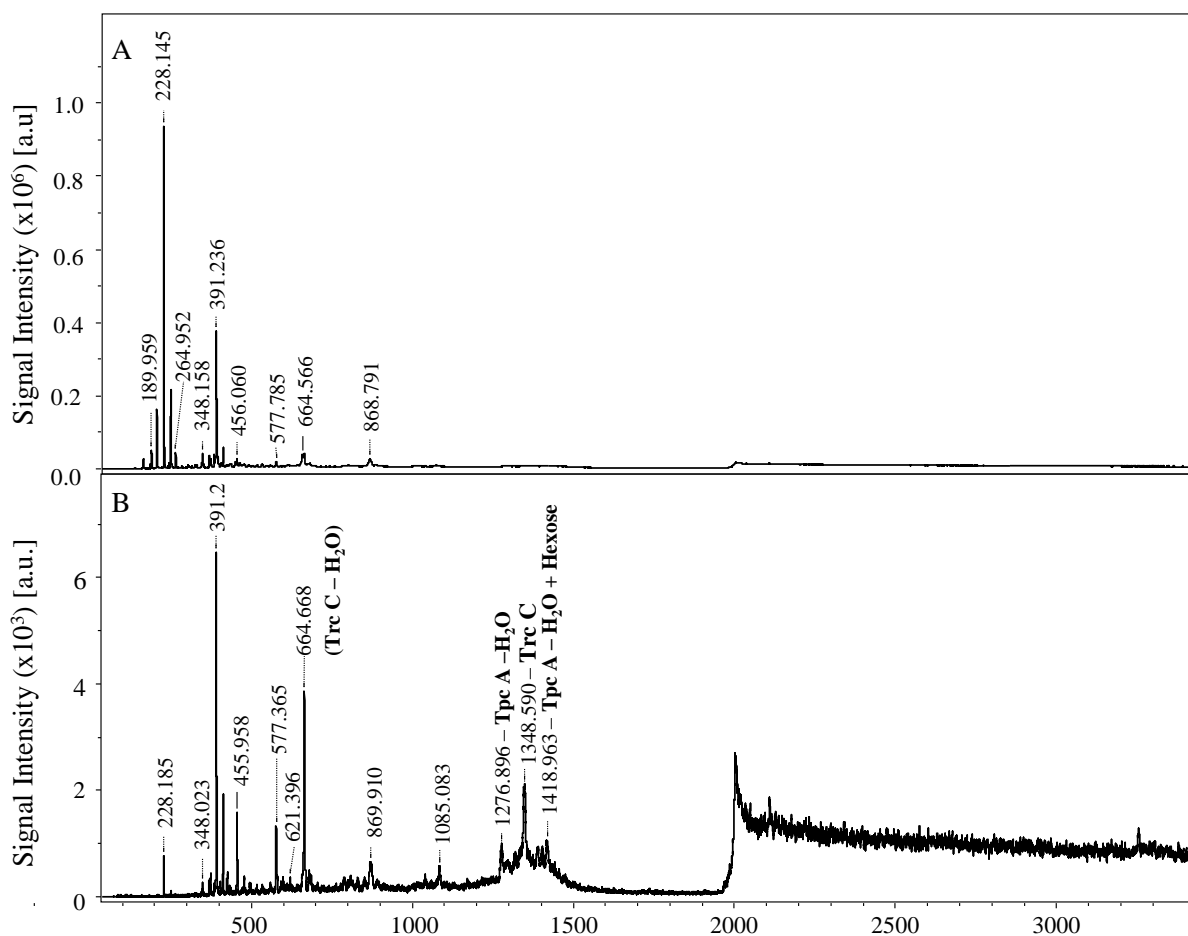


Figure 3.4: Spectrum obtained from MALDI-MS analysis of the **A** untreated CL filter and **B** Trc treated CL filter indicating the presence of Trc C and possibly modified Tpc A (dehydrated and glycosylated) on the filter.

Desorption of the peptide was done to finally confirm the presence and identity of the adsorbed peptide on the filter. It was done in 50% (v/v) ACN since the peptide readily dissolves in the solvent and will accordingly aid desorption of the peptide. The solution was dried and weighed analytically and it was calculated that there was an average of $3.77 \mu\text{g}/\text{cm}^2$ peptide desorbed from the treated CL filter.

The UPLC-MS analysis performed on the desorption extract of peptide from the treated CL filter (Fig 3.5: B) confirmed the presence of TrcC₁ ($m/z = 1361.7014$), TrcC ($m/z =$

1347.6713), TpcC₁ ($m/z = 1384.6908$), TpcC ($m/z = 1370.6882$), TrcB₁ ($m/z = 1322.6886$), TrcB ($m/z = 1308.6627$), TpcB ($m/z = 1331.6710$), TrcA₁ ($m/z = 1283.6704$), TrcA ($m/z = 1292.6750$) and VGA ($m/z = 941.0461$). All of which are present in the original peptide extract used for the treatment of the solid surfaces (Fig 3.5: A). The only observed change was a modified peptide, possibly a deaminated and hydroxylated TrcB₁ ($m/z = 1340.6508$). However, no other peptides with modifications, such as hydroxylation, oxidation, deamination and glycosylation (via Maillard reaction with degraded/hydrolysed CL) were observed.

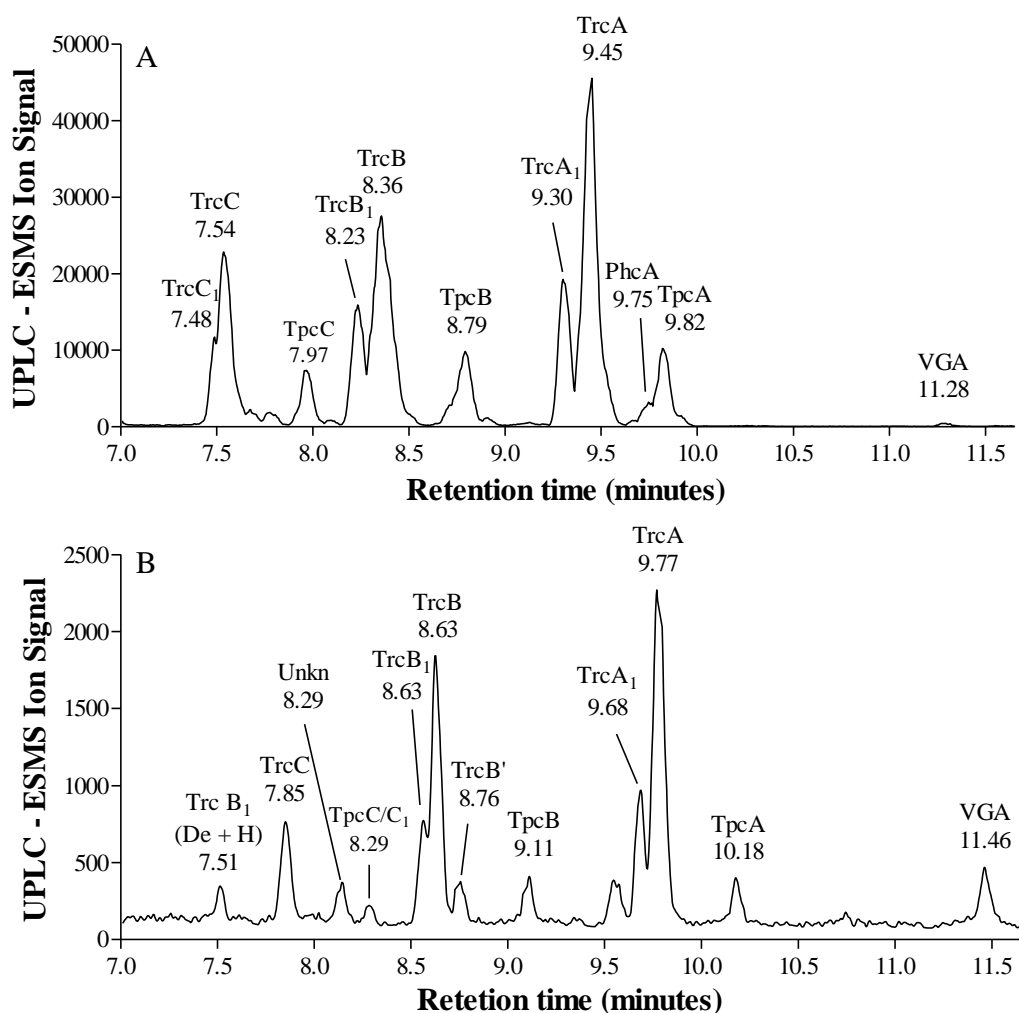


Figure 3.5: UPLC-MS analysis with peak identities and retention times of **A** Trc extract used to treat filters and **B** Trc extract obtained from the desorption of the peptide from the CL filters with 50% (v/v) acetonitrile.

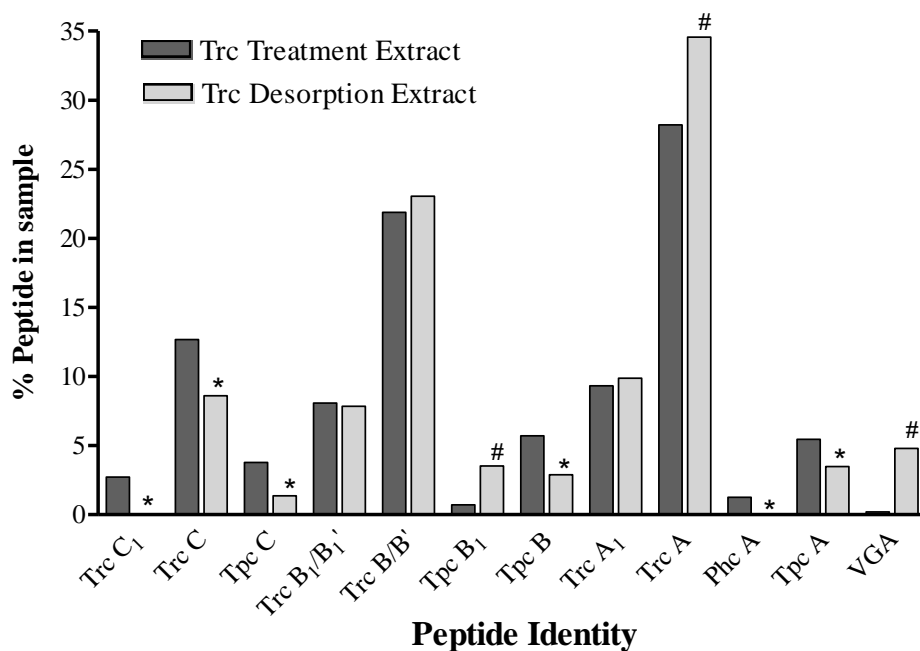


Figure 3.6: Comparison of all the Trcs and Trcs analogues detected with UPLC-MS analysis and presented as a percentage contribution to the total Trc present with in the Trc Extract and the extract obtained from the desorption step. * = lower contribution and # = higher contribution of the desorption extract than the treatment extract.

The contribution of the Trcs and analogues occurring in the original peptide extract used to treat the CL filters were compared to that of the desorption extract (Fig. 3.6). It was observed that the desorbed sample contained slightly less TrcC₁, TrcC, TpcC, TpcB, PhcA and TpcA than the original treatment extract. These peptides are predominantly Trp containing peptides (except PhcA) which can indicate that these peptides remained absorbed to the filter due to aggregation or the Maillard reaction. The less polar Trp and Phe containing Trcs (PhcA, TpcA and TrcA) are prone to aggregation, especially in polar solvents. The filters were treated with a Trc extract in water which could have caused the initial aggregation of the peptides on the filters. The Maillard reaction can also be the cause of the decrease in peptide contribution of the desorption sample since it involves amino acids Orn, Lys, Trp, Tyr [18-20]. Based on this knowledge and that the main component of the CL contains a sugar moiety, it can be hypothesized that the loss of peptide can be attributed to the Maillard

reaction occurring between the sugar moiety and that of key amino acids residues [18-20]. The first step of the Maillard reaction entails the reversible binding between an amino group of a protein and a reducing sugar forming a Schiff base [18-20]. Due to the unstable nature of the Schiff base it is rearranged to a more stable compound, the Amadori product. The Amadori product can undergo more rearrangements over time to finally form advanced glycation end products or Maillard products. This reaction thus entails the covalent bondage of amino groups to glucose which in the case of Trcs could render the peptide possibly ineffective [18, 19] and bound to the filter. It was also observed that the desorbed sample contained more (as a percentage present in the sample) TpcB₁, TrcA and VGA. This is possibly due the CL providing favourable conditions of attachment in a polar solvent (incubation occurred in water), but that when more hydrophobic peptides are exposed to a non-polar solvent, such as the 50% (v/v) ACN used for desorption, these peptides readily dissociates from the filter since they are only soluble in a partially non-polar solvent. The results obtained from the desorption analysis correlated with the MALDI-MS results in that it confirms the presence of Trcs on the CL filters. The MALDI-MS analysis, however, only detected TrcC whereas the desorption analysis detected all the Trcs and analogues found in the Trc extract used to treat the solid surface. It can thus be concluded that, should the filter characteristic permit it, that the desorption analysis is comprehensive in detecting the identity of the Trcs and Trc analogues adsorbed to the various solid surfaces.

3.4.2 Wettability of peptide treated filters

The wettability, an indicator of hydrophilic/hydrophobic character, could possibly be altered by the adsorption of peptide, and this change in character would be an indication of the orientation in which in the peptide adsorbed to the various filters. The wettability of untreated and treated (Trc extract and gramicidin S) filters were determined to ascertain the original

hydrophobicity/hydrophilicity of the filters and the effect the peptide had on the character of the filters (Table 3.4).

The untreated filters can be organised according to hydrophobicity based on the time it took for the water droplet to completely absorb into the filter. The CL filters were observed to be very hydrophilic (absorbed in less than 10s), followed by HAWP, GSWP, CA, HVLP and GVHP. HDC and PC which were observed to be hydrophobic as the water droplet was not absorbed within 30 minutes.

The effect the peptides had on the filter character could be seen with the time it took for the water droplet to completely absorb into the filter as well as change in the diameter of the water adsorbed into the filter. Gramicidin S and Trc extract treated PC, HDC and CL filters showed no visible change in hydrophobic/hydrophilic nature. The changes after treatment was more pronounced in the other filters tested compared to the results obtained for the mixed CL filters. Trcs made the HAWP filters more hydrophobic whereas the gramicidin S made the filters more hydrophilic. The hydrophobicity of GSWP filters increased when treated with Trc extract and gramicidin S. HVLP filters increased in hydrophobicity when treated with Trcs and gramicidin S, as did GVHP filters. CA showed a marked increase in hydrophilic character when treated with Trc extract and less so with gramicidin S. The chromatographic separation of the food colouring (E133), from the water, remained relatively the same regardless of the adsorbed peptide for all the filters with the exception of the polyvinylidene fluoride filters. A separation of the water and food colouring was observed for both filters (HVLP & GVHP) treated with gramicidin S, possibly linked to the change in hydrophobic character compared to the untreated counterparts.

Table 3.4: Summary of the wettability of untreated, Trc and Gramicidin S treated filters with regards to water drop diameter, the time that elapsed till it was completely absorbed, comparison of the water diameter (cm) of the untreated vs peptide treated filters and the chromatographic separation of the water from the food colouring.




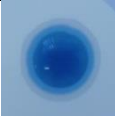


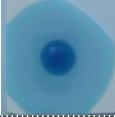





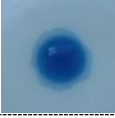

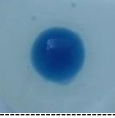


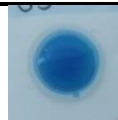
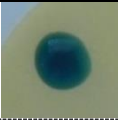
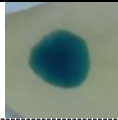
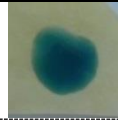


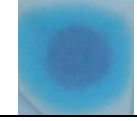
	Untreated	Trc	GS
HAWP (0.45 μm)			
Time until absorbed (min)	2.25	2.35	2.20
Drop Diameter (cm)	0.88	0.72	0.96
Peptide/Untreated	-	0.78	1.08
R_f Water vs Dye	0.85	0.78	0.8
GSWP (0.22 μm)			
Time until absorbed (min)	3.25	3.47	3.55
Drop Diameter (cm)	0.81	0.89	0.81
Peptide/Untreated	-	1.06	0.95
R_f Water vs Dye	0.84	0.80	0.89
HVLP (0.45 μm)			
Time until absorbed (min)	7.26	10.34	9.20
Drop Diameter (cm)	0.64	0.57	0.72
Peptide/Untreated	-	0.61	0.89
R_f Water vs Dye	1	0.94	0.74
GVHP (0.22 μm)			
Time until absorbed (min)	11.00	16.40	17.25
Drop Diameter (cm)	1.00	0.73	1.00
Peptide/Untreated	-	0.74	1
R_f Water vs Dye	0.75	1	0.77
PC (0.4 μm)			
Time until absorbed (min)	>30	>30	>30
Drop Diameter (cm)	0.73	0.73	0.73
Peptide/Untreated	-	1.10	-
R_f Water vs Dye	1	1	-

Table 3.4: continued

CA (0.45 μm)			
Time until absorbed (min)	6.15	1.49	5.01
Drop Diameter (cm)	0.85	1.01	0.85
Peptide/Untreated	-	0.87	0.87
R_f Water vs Dye	0.80	0.84	0.77
HDC			
Time until absorbed (min)	>30	>30	>30
Drop Diameter (cm)	0.80	0.80	0.94
Peptide/Untreated	-	-	-
R_f Water vs Dye	-	-	-
CL			
Time until absorbed	<10s	<10s	<10s
Drop Diameter (cm)	-	-	-
Peptide/Untreated	-	1.14	1.04
R_f Water vs Dye	1	1	1

An observed trend was that it is possible that Trcs prefer a slightly hydrophobic surface (e.g. HVLP) to adsorb opposed to a hydrophilic surface (e.g. HAWP). Based on the amphipathic nature of Trcs it can be assumed that an increase in hydrophobicity translates to the peptide binding to the surface with its hydrophilic sequence (Asn-Gln-Tyr-Val-Orn/Lys), leaving the hydrophobic sequence (Leu-Phe-Pro-Phe/Trp-Phe/Trp) to be exposed to the water and *vice versa*.

For gramicidin S, the hydrophilic association was most probably via the cationic Orn residues, while hydrophobic association probably took place via the rest of the residues. The extent of the change observed in hydrophobic/hydrophilic character would depend on how much of the peptide is a certain orientation. The change in hydrophobicity of the solid surfaces could play a role in the biological activity discussed in later chapters.

3.4.3 Visualisation with scanning electron microscopy

The peptide treated solid surfaces/matrices are membrane filters, thus it was important to determine whether the adsorption of Trcs to the filters changed the structure of the filter in any way that would hamper its effectiveness. Therefore the treated filters were compared against their untreated counterparts for any changes in structure by using scanning electron microscopy (Fig. 3.7). The general outcome of the SEM analysis was that there was no visual difference between the Trc treated and untreated filters studied (GSWP, CA and CL), other than a general thickening of the Trc treated filter structure. In all cases the filter pore structure and structural integrity seemed unchanged.

The bulking of the filter structure is possibly indicative of the water retaining ability observed for Trcs. GSWP (Fig 3.7: A, B), mixed CL ester filter, is characteristically known to have a high protein binding capacity, thus the attachment of Trcs to its structure was to be expected. Based on the results obtained during the wettability assay (Table 3.4) the Trcs adsorb to the hydrophilic filter with the hydrophilic sequence of the peptide (Asn⁵-Gln⁶-Tyr⁷-Val¹⁰-Orn⁹) leaving the hydrophobic side exposed as seen with the increase in hydrophobicity of the filter. It is clear from the more dense and bulky structure that the Trcs bound to the CA filter (Fig. 3.7: C), when comparing it to its untreated counterpart (Fig. 3.7: D).

However, regardless of low protein binding capacity of CA [14-16] the adsorption of Trcs to the CA filter caused a change in filter structure and the greatest change in hydrophobicity compared to the other filters treated (Table 3.4). Based on the increase in hydrophilic character, it can be concluded that the hydrophobic sequence of the peptide (Leu¹⁰-D-Phe¹-Pro²-Phe³-D-Phe⁴) adsorbed to the filter leaving the hydrophilic region exposed.

The CL filters (Fig. 3.7: E, F) have a very low binding capacity however it creates an environment that favours the binding of Trcs as was seen with the desorption analysis. When

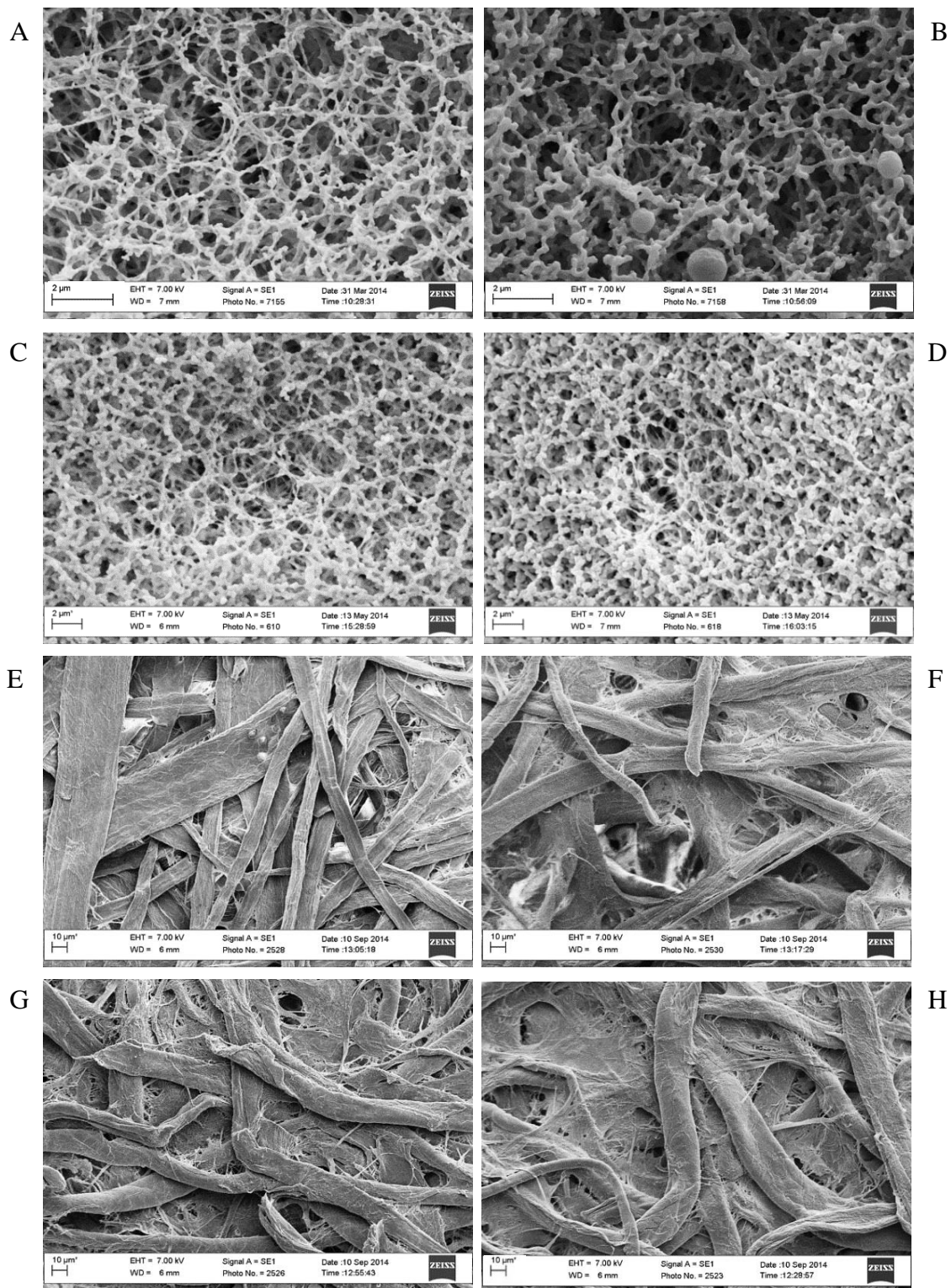


Figure 3.7: Scanning electron microscopy images obtained of the **A** PVDF GSWP filter, 10 000× magnification **B** PVDF GSWP Trc treated filter, 10 000× magnification **C** CA filter, 10 000× magnification **D** CA Trc treated filter, 10 000× magnification **E** CL filter, 1 000x magnification **F** CL Trc treated filter, 1 000× magnification **G** Trc treated CL filters plus *M. luteus*, 1 000× magnification **H** Trc treated CL filters plus erythrocytes, 1 000× magnification.

one compares the peptide treated CL filter (Fig 3.7: F) to the peptide treated filters further treated with target cells (Fig 3.7: G, H) one can see an increase of debris formation on the surface of these filters, but no intact cells, which indicates that cells are lysed. This suggests that the activity of the filter lies therein that the filter could entrap the target cells, where the bound Trcs can interact and lyse target cells.

Due to the hydrophilic nature of the filter, the Trcs are most likely adsorbed to the filter via the hydrophilic residues leaving the hydrophobic side of the peptide exposed to interact with the target cells and cause the observed lysis. Alternatively, it could be that aggregated Trcs are released from the filter structure, in particular dimeric Trcs which is proposed to be the active structure [21], as target cells associate with the Trc-coated surface. However, further investigation is needed to prove the exact mode of action of the Trcs adsorbed on the filters.

3.5 Conclusion

Antimicrobial resistance of pathogens to known antimicrobial agents due to the formation of biofilms has placed the focus on the development of antimicrobial surfaces to counter the formation of biofilms by preventing the initial attachment of the pathogens to various surfaces. Trcs have a broad spectrum of activity and resistance is less likely to develop due to its membranolytic mode of action. However, little is known about the antimicrobial activity of the Trcs when adsorbed to solid surfaces. In the light of the possible development of Trc antimicrobial surfaces it was crucial to compile quality control and quantification methods. Detection methods included change of absorbance of the incubation solution, ninhydrin staining, Sanger and Kaiser tests, amino acid analysis, wettability assay, FTIR, MALDI-MS Ionization, desorption of the peptide and SEM.

Results obtained from all the detection methods are summarised in Table 3.5. The change in absorbance showed that the mixed cellulose esters adsorbed the most peptide, followed by the

polyvinylidene fluoride and polycarbonate. This method did not produce any results for the CA filters and cellulose (paper and high density packing cardboard) due to an increase in absorbance of the incubation solution after incubation.

Table 3.5: Summary of the results obtained for all the methods utilized to quantify the peptide adsorbed to the different filters treated with Trc extract.

Method of quantification	Positive Control	Mixed CL esters HA WP (0.45 μm)	Mixed CL esters GSWP (0.22 μm)	PVDF HVLP (0.45 μm)	PVDF GVHP (0.22 μm)	Polycarbonate	Cellulose Acetate	HDC ^b	Cellulose (Paper)
Absorbance (A ₂₃₀) $\mu\text{g}/\text{cm}^2$	50 ^a	3.27 ± 0.65	3.40 ± 2.42	1.78 ± 1.00	1.55 ± 0.82	0.88 ± 0.47	nd	nd	nd
Ninhydrin	Purple	nd	nd	nd	nd	nd	nd	nd	Purple
Sanger's Test	A ₃₈₁ = 0.154	nd	nd	nd	nd	nd	nd	nd	nd
Kaiser Test	Purple	Light Green	Light Green	Light Green	Light Green	Light Green	Light Green	Light Green	Light Green
Amino acid analysis	Trc Peptide Standard	nd	nd	nd	nd	nd	nd	nd	nd
FTIR	-	-	-	-	-	-	-	-	?
MALDI	-	-	-	-	-	-	-	-	Peptide detected (Trc C)
Desorption ^b $\mu\text{g}/\text{cm}^2$	-	-	-	-	-	-	-	-	3.77

^a Trc concentration in $\mu\text{g}/\text{mL}$ in incubation solution

^b Desorption was achieved by incubating cellulose filters in 50% (v/v) acetonitrile for 5 days, analysed with UPLC-MS

nd – not determined or detected

(-) – not analysed.

? inconclusive

Ninhydrin staining only showed a purple change of colour for the CL filter and showed full coverage but uneven spread of the peptide on the filter which could possibly affect biological studies. The Sanger's test, Kaiser test, amino acid analysis and FTIR showed no overt

difference in results compared to the untreated counterpart. This is possibly due to the amount of peptide that is below the detection limit of the applied method or technique. MALDI-MS confirmed the presence of TrcC on the CL filters and it can be assumed that the other Trcs and Trc analogues were also adsorbed to the filter. Desorption of the peptide from treated CL filters showed that at least $3.77 \mu\text{g}/\text{cm}^2$ peptide are adsorbed to the filter and that all of the Trcs and Trc analogues adsorb and desorb to the filter to varying degree. This was especially seen for VGA which was present on the filter at a higher sample percentage compared to the percentage present in the Trc extract used for treatment of the solid surfaces.

The wettability assay was performed to determine the hydrophobic/hydrophilic nature of the filters before and after treatment with peptide, Trc extract and gramicidin S. It was found that CL was very hydrophilic, followed by HAWP, GSWP, CA, HVLP and GVHP. HDC and PC filters were conclusively hydrophobic. After treatment with Trc extract and gramicidin S some of the filters showed an increase in hydrophobic character which is important for cell adhesion, which in turn will aid the antimicrobial activity of the solid surfaces [22-24]. CA peptide treated filters showed an increase in hydrophilic nature especially with the treatment of Trcs. No clear conclusions could be made regarding a change in hydrophobic/hydrophilic nature for HDC, CL and PC after peptide treatment.

Scanning electron microscopy was performed to determine the effect the adsorption had on the structure integrity of the filters. It was found that the adsorption of Trcs to the filters does not change its structure and the filters will still be able to perform its function after treatment, which is important for possible industrial application of Trc impregnated filters. Some debris that collected on the CL filters after target cell exposure (erythrocytes and *M. luteus*) indicates that the cells come in contact with the peptide while the peptide is still adsorbed to the filter after which the cells are lysed.

It can be concluded that Trcs adsorb to the selected solid surfaces without disrupting the structure integrity. It does however highlight the need for a high throughput method to confirm the presence of the peptide and the development of a quality control method set to quantify the amount of peptide adsorbed to the filters that is suitable for all the solid surface types. This confirmation and quantification of peptide adsorbed would aid in predicting the biological activity of the treated solid surfaces and selecting the appropriate applications thereof.

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Chapter 4

Determination of the activity, stability and application of antimicrobial peptide treated solid surfaces

4.1 Introduction

It has been observed that the tyrocidines (Trcs), analogues and gramicidin S easily adsorb to different solid matrixes (Chapter 3). However, since these peptides are not covalently bound to the surfaces it is crucial to determine whether biological activity can be maintained and under which conditions these surfaces lose their antimicrobial activity. These studies will give some insight into which applications these antimicrobial materials can be used for or be further developed for a specific application.

Trcs, AMPs produced by *Bacillus aneurinolyticus*, have activity against Gram-positive bacteria such as *Micrococcus luteus* and *Listeria monocytogenes* [1, 2], antifungal activity against *Fusarium verticillioides*, *Fusarium solani*, *Botrytis cinerea* [3] and *Candida albicans* [4], as well as activity against the human malaria parasite *Plasmodium falciparum* [5]. It was originally used as a topical antibiotic in medicine [6], but regardless of its broad spectrum of activity no research has been reported on the surface activity of Trc impregnated matrices.

Trcs are amphipathic in their dimeric form [7, 8] have been shown to be relatively heat stable depending on the concentration [9], yet very little information exists on its pH stability. During culturing and purification, extracts are exposed to pH changes between pH 3 to pH 9 and is thus expected to remain stable between these pH ranges [10, 11]. Composition studies have shown a dissociation occurring within the structure at pH levels higher than pH 10 [12]. This in turn points to the dissociation of Orn⁹/Lys⁹ and Tyr⁷ with pK_a values of 10.76, 10.53 and 10.07, respectively [13].

Therefore, the antimicrobial activity of a range of different types of solid surfaces treated with Trcs and gramicidin S, as control, against the Gram-positive model organism *M. luteus* was determined. The amount of peptide absorbed to the different filters, which retained haemolytic activity, was quantified with a haemolytic assay developed for solid surfaces. Cellulose filters retained the highest antimicrobial activity and was therefore selected to determine the stability of the adsorbed Trcs by subjecting the filters to multiple washes, as well as a range of pH values and temperatures. These Trc-treated cellulose filters were also used in a plant culturing pilot study to determine the possible application of Trc treated antimicrobial materials in plant culturing.

4.2 Materials

The National Collection of Type Cultures (Porton Down, Salisbury, United Kingdom) provided the *Micrococcus luteus* NCTC 8340 cultures used in all antibacterial assays. Agar, sodium chloride, hydrochloric acid, tryptone, yeast extract were obtained from Merck (Darmstadt, Germany). Petri dishes were obtained from Greiner bio-one (Frickenhausen, Germany) and Falcon ® tubes were obtained from Becton Dickson Labware (Lincoln Park, USA). Acetonitrile (ACN, HPLC-grade, far UV cut-off) was provided by Romil Ltd. (Cambridge, UK). Analytical grade water (HP water) was used for all solvents and wash steps and was obtained utilising a Millipore Milli-Q water purification system (Milford, USA) and water filtered from a reverse osmosis water purification plant. Nitrocellulose filters (HAWP, GSWP), polyvinylidene fluoride filters (HVLP, GSWP) and 0.22 µm polycarbonate syringe filters was supplied by Waters-Millipore (Milford, USA). Polycarbonate filters were supplied by Nuclepore Corp (Pleasanton, CA, USA). Cellulose acetate and high density cellulose filters were provided by Sartorius (Gottingen, Germany). Cellulose filters (paper filter, MN 615/No 1) were obtained from Macherey-Nagel (Düren, Germany). RPMI 1640 medium, glucose, HEPES, hypoxanthine, NaOH, gentamycin, sodium bicarbonate,

polypropylene plates, tyrothricin (extract from *Bacillus aneurinolyticus*), gramicidin S (from *Aneurinibacillus migulanus*), Resazurin sodium salt, triethylamine, trifluoroacetic acid and Corning Incorporated® polystyrene plates were supplied by Sigma-Aldrich (St. Louis, MA, USA). NaCl was supplied by Merck (Wadeville, Gauteng).

4.3 Methods

4.3.1 Preparation of Filters

Filters were prepared, as described in Chapter 3, in a 50 µg/mL peptide solution (Trc extract or gramicidin S), rinsed with analytical grade water and dried for ±16 hours in a low temperature oven. Filters (diameter 47 mm) used in this study were composed of mixed cellulose esters (HAWP, GSWP), polyvinylidene fluoride (HVLFP, GSWP), polycarbonate (PC), cellulose acetate (CA), high density cellulose (HDC, used as an insert between filter packaging) and cellulose (CL, paper) filters. Filters were pre-sterilized using either 50% acetonitrile (ACN) or autoclaved based on the characteristics of the filters and which method least affected the filter integrity. Filters (47 mm diameter) were either cut in half for direct surface contamination with *M. luteus* to determine survival of colony forming units (CFUs) or made into small punch disks (5 mm diameter) to fit into the wells of a 96-well plate for the haemolytic assays and adapted resazurin cell vitality assay (also known as Alamar Blue® or CellTiter-Blue® assay).

4.3.2 Biological activity of peptide treated filters

Loss of activity is a possible consequence of the attachment of antimicrobial peptides to solid surfaces albeit through covalent bonds or adsorption [14, 15], due to the interaction between peptide and solid surface. Thus it is of great importance to confirm biological activity of the peptide once adhered to the surface especially in the light of the development of antimicrobial surfaces and application thereof. Therefore the haemolytic activity and antimicrobial activity

against *Micrococcus luteus*, a model Gram-positive organism, of the peptide treated filters were determined.

4.3.2.1 Solid phase of haemolytic activity assay

The erythrocytes were prepared according to a previously developed methodology [5]. Blood (from an anonymous A+ donor), containing 300 mL erythrocyte fraction enriched with 63.0 mL citrate phosphate dextrose anticoagulant and 100 mL saline-adenine-glucose-mannitol red blood cell preservation solution, was obtained from either the National Health Laboratory Services or the Western Cape Blood services in South Africa adhering to the relevant ethics and legislation related to the experiments done during this study. The blood was transferred to a sterile Falcon[®] tube, filled with RPMI media and centrifuged for 3 minutes at 1200×g in the first washing step. The supernatant was removed, refilled with RPMI media and centrifuged again in the second washing step. The supernatant was removed and the remaining blood cells were used for further assays.

The RPMI media contained RPMI-1640 (10.4 g/L), hypoxanthine (0.4 g/L, dissolved in 1.0 mL 1.0 M NaOH), gentamycin (50 mg/mL), HEPES (6 g/L), glucose (4 g/L), sodium bicarbonate (2.1 g/L). Analytical grade water was used, the pH adjusted to 7.2 -7.3 and filtered through a 0.22 µm filter to obtain sterility [16, 17].

The haemolytic assay was adapted and optimised for solid surface haemolytic activity detection from an established methodology [5]. The assay was done in a 96-well flat bottom polystyrene plate and the layout was as follows: the first column contained 10 µL analytical grade water (blank) in all the wells, in the second column the first four wells contained 10 µL 100 µg/mL gramicidin S to obtain 100% haemolysis and the last four wells contained 10 µL 15% ethanol. The next three columns contained 10 µL of a doubling dilution of Trc standard starting at 500 µg/mL. The Trc standard was first dissolved in 15% v/v ethanol (2 mg/mL

stock) and was then diluted in doubling dilutions in 15% v/v ethanol in a separate polypropylene plate to create a standard curve. Triplicate punched 5 mm disks of the treated and untreated filters were placed in the wells of the following columns and dampened with 10 µL analytical grade water. A 90 µL 2% haematocrit solution in Dulbecco's phosphate buffered saline (PBS: 0.8% w/v NaCl, 0.04% w/v KCl, 0.144% w/v Na₂HPO₄, 0.02% w/v KH₂PO; pH 7.4) was then added to all the wells.

The 96-well plates with filters were incubated at 37°C for 2 hours after which the plates were centrifuged at 900×g for 10 minutes. PBS (90 µL) and 10 µL of the supernatant of each of the wells were added to a new 96-well polystyrene plate and the absorbance measured at 415 nm using a BioRad Model 680 microplate reader. The haemolytic activity was calculated using the following equation:

$$\% \text{ Haemolytic activity} = 100 \times \frac{\text{Abs at 415 nm of well} - \text{Avg Abs. of background}}{\text{Avg Abs Fully lysed} - \text{Avg Abs of background}}$$

The induced haemolysis of each of the treated filters was normalised by subtracting the percentage haemolytic activity of the untreated filter from its treated counterpart. Identical assays were done for gramicidin S, except that for gramicidin S the stock solution was dissolved in analytical grade water and doubling dilutions was also done in analytical grade water.

4.3.2.2 Determination of antimicrobial activity of filters against *M. luteus*

4.3.2.2.1 Culturing of target organisms

M. luteus NCTC 8340 was streaked out from a freezer stock onto Luria Bertani (LB) agar plates (LB: 1% w/v NaCl, 1% w/v tryptone, 0.5% w/v yeast extract, 1.5% w/v agar) and incubated for ±48 hours at 37°C. Fresh LB media (20 mL) was inoculated with three to five

single colonies and incubated at 37°C on a shaker at 150 rpm until an A_{620} of 0.8-1.2 was reached (± 16 hours).

4.3.2.2.2 CFU agar based growth assay

In order to assess if the filters can prevent colonisation of microorganisms in a high nutrient environment an assay was developed using nutrient agar as nutrient matrix. Half a filter (± 24 mm diameter), treated and untreated with peptide, was transferred onto an LB agar plate. A mid-log phase *M. luteus* culture was diluted to OD_{620} of 0.20 and subsequently diluted 10^3 , 10^4 and 3×10^4 times. After which the following amounts were pipetted onto the treated and untreated filters: 10 μ L and 20 μ L of the $\times 10^3$ dilution, 100 μ L of the $\times 10^4$ dilution and 100 μ L of the 3×10^4 dilution. It was then incubated at 37°C for 48 hours and inspected for single colonies forming units (CFU).

The antimicrobial activity of the stability testing filters were treated similarly, with the exception that 10 μ L of a OD_{620} of 0.20 *M. luteus* culture was pipetted onto the filters.

4.3.2.2.3 Cell viability assay for determination of direct sterilisation

In order to assess if the filters can directly kill microorganisms in a low nutrient environment an assay was developed using resorufin as indicator of cell metabolism and cell vitality [18]. Resazurin (blue dye compound) is reduced to resorufin (red/pink dye compound with fluorescent properties) by actively respiring cells [18]. Black 96-well microtiter plates were used with triplicate samples of untreated, Trc treated and gramicidin S treated filters. A mid-log phase *M. luteus* culture was diluted to an OD_{620} of 0.20 (1.3×10^7 CFU/mL) of which 5 μ L (or 5 μ L of a 2-fold dilution of the culture) was transferred to each of the filters. The plate was incubated at 37°C for 1 hour after which 90 μ L PBS and 10 μ L of resazurin was added to each well. Triplicate samples of untreated filters without cells were also incubated with 90 μ L PBS and 10 μ L resazurin solution to determine the baseline fluorescence of the filters. The

plate was again incubated for 2 hours at 37°C. After the incubation the fluorescence (F) was determined at an excitation wavelength of 530 nm and emission wavelength of 590 nm. Cell viability was calculated with the equation:

$$\% \text{ Cell viability} = 100 \times \frac{F \text{ of well} - \text{Average } F \text{ of no growth}}{\text{Av. } F \text{ of growth control} - \text{Av. } F \text{ of no growth}}$$

Fluorescence reading was taken with a Varioskan™ Multimode reader from Thermo Scientific™ controlled by SkanIt Software 2.4.1 from Thermo Electron. Each plate was shaken for 5 seconds before readings were taken at 25°C.

4.3.3 Determination of stability of antimicrobial activity on filters

CL filters (47 mm diameter), with or without 50 µg/mL Trc treatment, were challenged with multiple wash steps. CL filters were placed in 100 mL of HP water and washed for 1 minute, after which they were transferred to fresh HP water and washed again. This procedure was repeated 12 times.

Solvent-challenges were performed on CL filters (47 mm) when placed in 100 mL 100% ACN or HP water containing either 2% NaCl, 1% triethylamine (TEA), 1% trifluoroacetic acid (TFA) or 50% ACN and washed for 1 minute. The temperature challenges were performed on CL filters for 1 minute with 100 mL HP water heated to 25°C, 40°C, 60°C, 80°C and 100°C. The pH stability of the Trc treated CL filters was determined by exposing the filters to a range of pH values. The pH of wash solvent (100 mL HP water) was adjusted with HCl for pH 1, pH 3 and pH 5 and HCl/NaOH to obtain pH 7 and NaOH for pH 9, pH 11 and pH 13.

Both peptide-treated and untreated filters were subjected to the wash treatments. After treatment, the filters were washed (one wash with 100 mL HP water) and dried overnight (16±1 hours) in a low temperature oven (55±5°C). The change in retained activity was

studied with the haemolytic assay and the CFU antimicrobial growth assay or vitality assay as previously described.

4.3.4 Tyrocidine treated cellulose filters in plant culture applications

Plant tissue culture is used in various ways to grow and study plants. In some cases seeds are first allowed to germinate prior to tissue culture steps. This germination occurs under sterile conditions in deep petri dishes lined with two damp CL filters to maintain the moisture needed for growth. It is also during this time that the seedlings are most susceptible to infection. The Trc treated CL filters were used to replace one of the two CL filters in the hope of positively affecting germination and prevent opportunistic bacterial and fungal infections.

CL filters were treated with a 5 µg/mL, 25 µg/mL or 50 µg/mL solution of a Trc extract as previously described and used to study the effect it has on the germination of tomato seeds over an 8 day period. Deep petri dishes were lined with an untreated cellulose filter followed by a Trc treated filter. Sterile analytical grade water (10 mL) was added to provide the needed moisture for germination. The tomato seeds (Money Maker, Starke Ayres) were washed in 70% ethanol for seven minutes followed by a decontamination step in 0.35% NaOCl for 15 minutes. It was lastly washed five times in sterile analytical grade water and placed on the prepared petri dishes (25 seeds per dish).

4.3.5 Data analyses

Data presentation and all statistical analysis were done using GraphPad Prism ® 4.03 (GraphPad Software, San Diego, USA). Statistical analysis included 95% confidence levels, standard error of the mean, absolute sum of squares and One-way Anova using Bonferroni's post-test.

4.4 Results and Discussion

4.4.1 *Haemolytic activity of peptide treated filters*

A haemolytic assay was developed and performed in an attempt to validate the presence of Trcs on the filters, especially for filters for which the absorbance method did not produce any results (Chapter 3). This assay works on the principle that both Trc and gramicidin S have haemolytic activity [19] and that some correlation between amount of peptide present and haemolytic activity is to be expected (Fig. 4.1 A).

The haemolytic activity assay of the Trc treated filters confirmed the presence of peptide that retained haemolytic activity on the filters, which is especially important for the HDC, CL and CA filters for which could not be confirmed that peptide was present with the absorbance method (refer to Chapter 3) (Table 4.1). For the other filters the amount of haemolytic active peptide adsorbed was found to be much lower compared to the amount of peptide retained based on change in absorbance (refer to Chapter 3) (Table 4.1). The difference can be attributed to the difference in activity when absorbed to the filter and activity in solution of Trcs. When target cells come into direct contact with the Trcs absorbed on the solid surfaces, the Trcs can elicit only a lytic response upon contact if the peptides are correctly orientated for membrane interaction. If the peptide molecules are all oriented to have a productive and lytic interaction with the target cell membrane the activity would closely relate to the amount of peptide retained as calculated with the absorbance method. The haemolytic assay is performed in 100 μ L which requires either that the erythrocytes sediment to the solid surface for lytic interaction or that all the peptide to dissociate from the solid surface in order to elicit the lytic response. It is therefore probable that the direct surface interaction did not lead to productive and lytic interaction with the erythrocytes or that very little peptide was liberated from the solid matrix. Thus the haemolytic activity did not correlate to the amount of retained peptide as determined with the absorbance method.

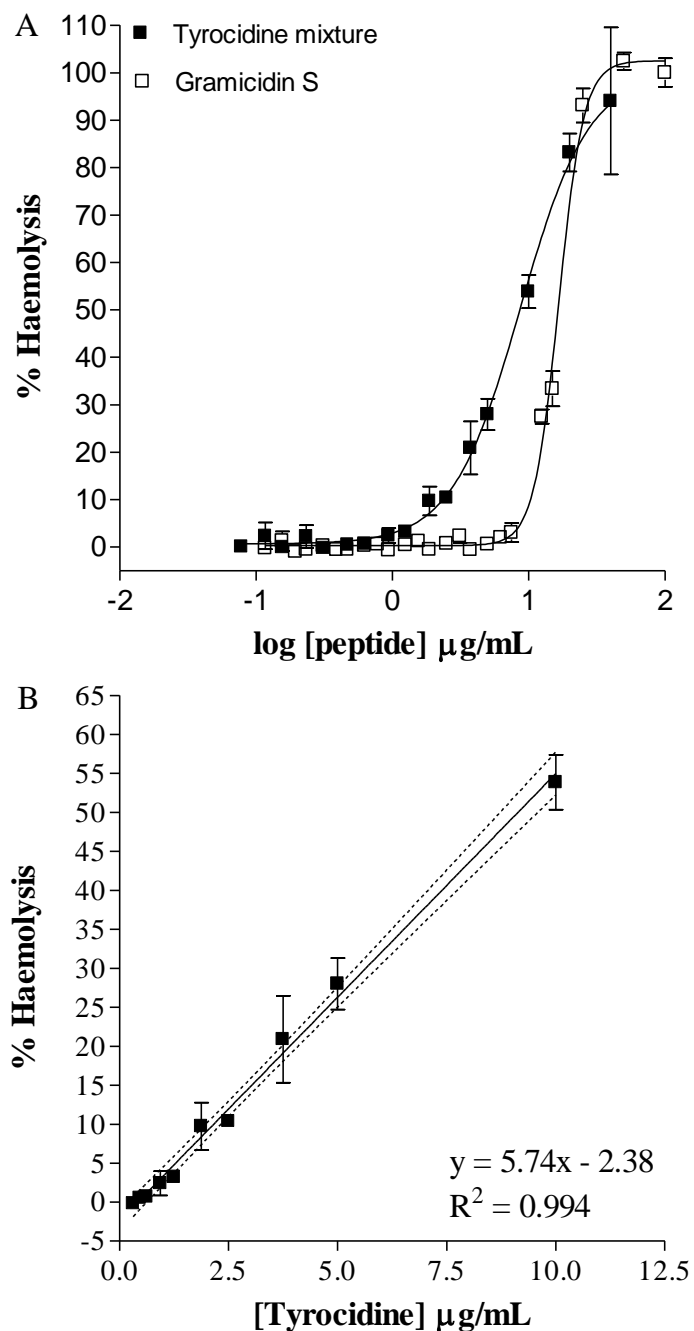


Figure 4.1: **A.** Haemolytic activity dose response curves of Trc mixture and gramicidin S. **B** Standard curve as compiled from the haemolytic activity of the Trc mixture used to determine the amount of peptide retained on the solid surfaces. The dotted lines show the 95% confidence interval of the standard curve. Data point in graphs A and B are the mean of 3-16 determinations with standard error of the mean.

Good correlation, however, was found between the $3.77 \mu\text{g}/\text{cm}^2$ peptide desorbed from CL of (Chapter 3) and $3.63 \mu\text{g}/\text{cm}^2$ peptide determined with the Trc haemolytic activity standard

curve (Table 4.1, Fig. 4.1B). Although such good correlations were not found for all the filters, the haemolytic activity assay did confirm the presence of Trcs on the solid matrixes, but also stressed the need for the future development of a peptide quantification method set, as discussed in Chapter 3.

The amount of gramicidin S adsorbed to the filters based on haemolytic activity could not be calculated since gramicidin S only elicit haemolytic activity above concentrations of 10 µg/mL (30% haemolysis), which is well above what is predicted to be retained on the filters (Fig 4.1A). The amount of GS retained based on the absorbance method was determined, (Chapter 3), for GVHP ($5.3 \pm 1.1 \mu\text{g}/\text{cm}^2$), HVLP ($2.8 \pm 0.6 \mu\text{g}/\text{cm}^2$) and polycarbonate ($1.2 \pm 2.2 \mu\text{g}/\text{cm}^2$). It was not possible to determine the peptide absorption for the other filters since the absorbance value of the incubation solution increased after incubation due to the release of an absorbing component from the filters (refer to Chapter 3).

Table 4.1: Amount of Trc retained as determined by the absorbance method (Chapter 3) and using the Trc haemolytic activity standard curve (Fig 4.1B) for the various solid surfaces with (*n*) number of repeats.

Solid surface	Peptide retained ($\mu\text{g}/\text{cm}^2$) as determined by	
	Absorbance (<i>n</i>)	Haemolysis (<i>n</i>)
Mixed cellulose esters (HAWP 0.45 µm)	3.27 ± 0.65 (7)	0.34 ± 0.25 (8)
Mixed cellulose esters (GSWP 0.22 µm)	3.39 ± 2.41 (8)	0.25 ± 0.12 (8)
Polyvinylidene fluoride (HVLP 0.45 µm)	1.77 ± 1.00 (8)	1.06 ± 1.09 (8)
Polyvinylidene fluoride (GVHP 0.22 µm)	1.54 ± 0.82 (3)	0.92 ± 0.70 (8)
Polycarbonate (0.40 µm)	0.88 ± 0.47 (4)	0.22 ± 0.13 (8)
Cellulose Acetate (0.45 µm)	-	0.13 ± 0.06 (8)
High density cellulose	-	2.17 ± 1.26 (8)
Cellulose	-	3.63 ± 1.56 (11)

When the haemolytic activity was compared between the Trc treated filters, it was found that for the Trc-treated filters, that CL elicited the highest haemolytic activity for a single 5 mm filter punch challenged with 100 μ L of 2% haematocrit, followed by HDC, GVHP, HVLP, HAWP, GSWP, PC and last CA (Fig. 4.1).

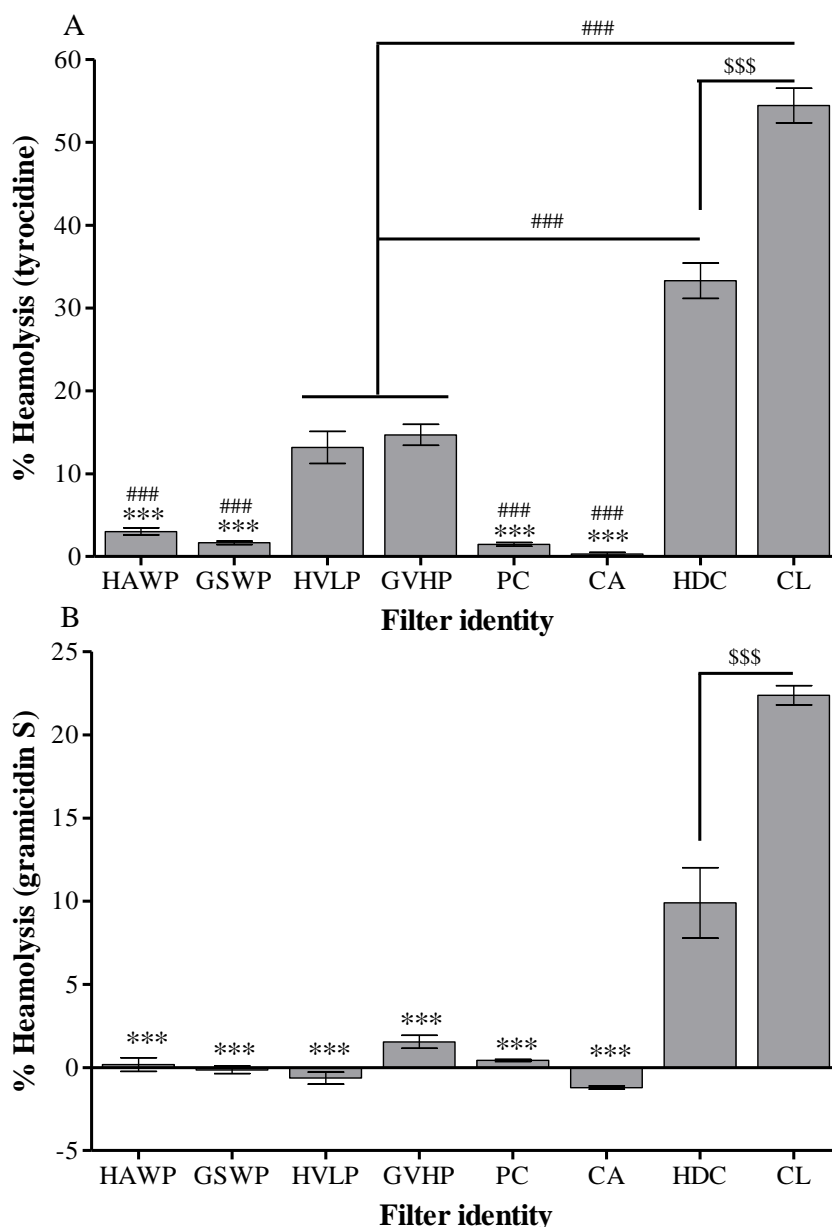


Figure 4.2: Haemolytic activity observed for filters treated with **A** Trc extract and **B** gramicidin S. Refer to Chapter 3 for details on each of the filters. Statistical differences as determined with One-way Anova (Bonferroni's Multiple Comparison Test) for haemolytic activity of HDC versus CL $^{$$$}$ $P < 0.0001$ in A, B; HDC&CL versus other filters *** $P < 0.001$ in A, B and HVLP&GVHP versus other filters $^{###}$ $P < 0.001$ in A. Each bar depicts the average of at least 9 repeats with SEM.

The same haemolysis assay was done for GS treated filters (Fig. 4.2B) and it was found that CL elicited the highest haemolytic activity at 22% haemolysis, followed by HDC (10%), while GVHP, PC and HVLP showed less than 2% haemolysis. GSWP, HAWP and CA did not elicit any haemolytic response. From this it was also clear that gramicidin S elicited much less haemolytic activity on HDC and CL at 10% and 22% respectively than the Trc treated filters at 33% and 54% respectively (Fig 4.2).

4.4.2 Antimicrobial activity of peptide treated filters

4.4.2.1 CFU agar growth assay against *M. luteus*

A CFU agar assay was developed to assess if the peptide treated filters can prevent colonisation of microorganisms in a high nutrient environment. Using nutrient agar as the high nutrient matrix, it was found that of the eight different polymer filters treated with the Trc extract, seven filters showed antimicrobial activity against the Gram-positive model organism, *M. luteus* (Table 4.2). The CL filter had the highest observed antimicrobial activity followed by the HDC, GVHP, PC and HVLP filters. CA filters in this assay showed no observed antimicrobial activity.

The same assay was performed with gramicidin S treated filters. Gramicidin S has 50% sequence identity with the Trcs and share the VOLfP peptide sequence. Gramicidin S is commonly used by our group as a positive control in haemolytic and antimicrobial activity assays to ascertain the activity of Trcs and analogues. Challenges at very low CFUs revealed that only HVLP, HDC and CL showed antimicrobial activity. We did not investigate higher CFUs for these filters and because the CFU number were not comparable to that used in assays on the Trc treated filters, no clear conclusions could be made as to which of the Trc or gramicidin S treated filters have the most potent antimicrobial activity in a high nutrient environment.

Table 4.2: Summary of the growth inhibitory antimicrobial activity of Trc extract treated filters against the model organism, *M. luteus* as determined by the CFU agar growth assay.








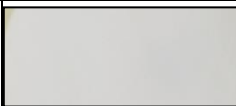





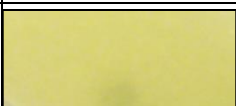

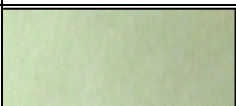





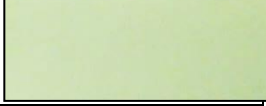


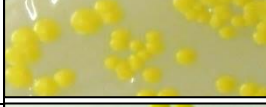






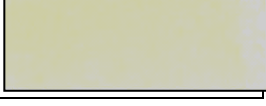
Solid Matrix	CFU/cm ²		Untreated	Treated
	Untreated	Treated		
Mixed CL esters (HAWP 0.45µm)	28±1	1±1		
Mixed CL esters (GSWP 0.22 µm)	29±2	2±2		
Polyvinylidene fluoride (HVLP 0.45 µm)	27±4	0		
Polyvinylidene fluoride (GVHP 0.22 µm)	34±1	0		
PC (0.4 µm)	32±3	0		
CA (0.45 µm)	8±2	8±2		
HDC	43±4	0		
CL (No 1 paper filter)	45±2	0		

Table 4.3: Summary of the growth inhibitory antimicrobial activity of gramicidin S extract treated filters against the model organism, *M. luteus* as determined by the CFU agar growth assay.

Solid Matrix	CFU/cm ²		Untreated	Treated
	Untreated	Treated		
Mixed CL esters (HAWP 0.45µm)	4±1	4±1		
Mixed CL esters (GSWP 0.22µm)	3±1	3±1		
Polyvinylidene fluoride (HVLP 0.45 µm)	3±1	0		
Polyvinylidene fluoride (GVHP 0.22 µm)	3±1	3±1		
PC (0.45 µm)	3±1	4±1		
CA (0.45 µm)	3±1	3±1		
HDC	4±1	0		
CL (No 1 paper filter)	3±1	0		

4.4.2.2 Cell viability assay for determination of direct sterilisation

The first step to prevent colonisation of surfaces by microorganisms is sterilisation and the peptide treated filters must be able to act as solid phase sterilisation matrices in a low nutrient environment. An assay, based on the Alamar Blue® assay also known as the CellTiter-Blue® assay, was developed using resorufin as indicator of cell metabolism and cell vitality [18]. The cell viability assay was performed with very high cell numbers to clearly distinguish between the type of filters and peptides used in terms of sterilising or microbiocidal antimicrobial activity. Two cell counts were used namely $3.4 \times 10^5/\text{cm}^2$ and a doubling

dilution $1.7 \times 10^5/\text{cm}^2$, to assess if the filter activity is maintained when challenged with higher cell counts (Fig 4.3).

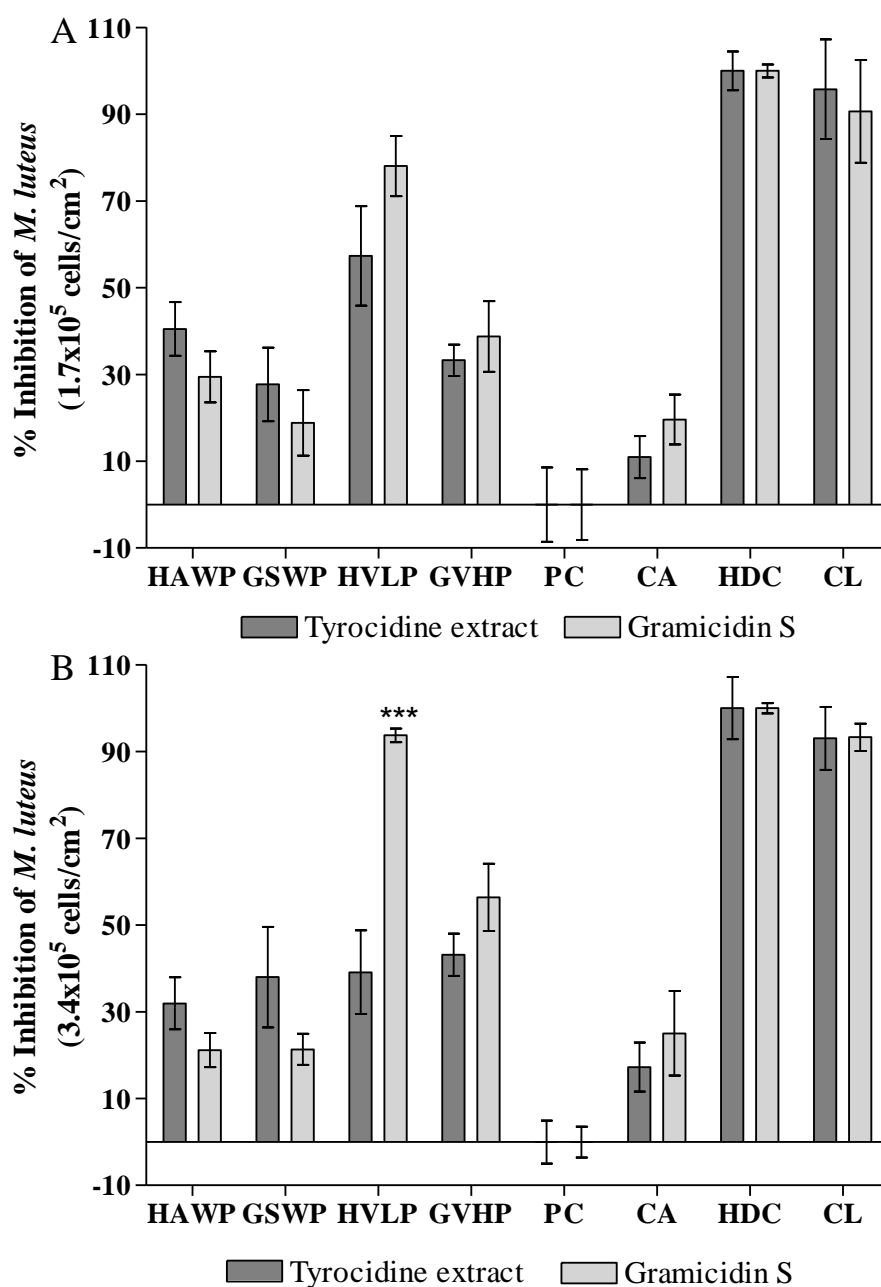


Figure 4.3: Sterilisation activity as determined with the viability assay of gramicidin S and Trc treated solid surfaces when challenged with **A** 1.7×10^5 cells/cm² and **B** 3.4×10^5 cells/cm² of the model target, *M. luteus*. Each bar depicts the average of 6 repeats with error bars representing the standard error of the mean. Statistical differences between Trc and gramicidin S treated filters was determined with the Student t-test with *** $P < 0.0001$.

Full to moderate sterilisation of *M. luteus* at 1.7×10^5 cells/cm² was induced by all the peptide treated filters except PC (Fig 4.3: A). For the sterilisation of 3.4×10^5 cells /cm² it was found that there was again no difference in activity between Trc and gramicidin S treated filters except for HVLP filters which gramicidin S treatment was significantly better than the Trc filter and remained potent with close to 100% sterilisation (Fig 4.3: B).

The best activity of the Trc treated filters were again found for HDC, CL>GVHP, HVLP, GSWP, HAWP>CA. No activity was observed for PC filters at either cell concentrations (Fig. 4.3 and Table 4.4). The best activity observed for the gramicidin S treated filters was that of HDC, CL, HVLP>GVHP, HAWP>GSWP, CA with no significant sterilisation activity for PC. Statistical analyses of the comparison in terms of the sterilisation of the filters are given in Table 4.4.

Correlations between the CFU agar growth assay and the cell viability assay is that for both the gramicidin S and Trc treated filters the HDC and CL filters have the highest antimicrobial activity. However, Trc did not prove to be as effective in direct sterilisation, particularly for the more hydrophobic filters (e.g. PC) and filters where it led to increased hydrophobicity of the filters (HAWP, GSWP, HVLP and GSWP) (refer to chapter 3 and Fig. 4.3). This could be due to the limited spreading of the 5 µL culture on the PC filter, limiting the contact of the bacteria with the rest of the peptide-coated filter, and thus this assay could have underestimated the overall filter activity. The opposite was true for CA, where both the peptide treatments increased the hydrophilicity dramatically and led to an increased sterilisation as detected with the vitality assay (Refer to Chapter 3 and Fig. 4.3). Growth inhibitory activity of peptide treated filters could not be observed with the CFU agar assay. Thus the cell viability assay is more sensitive in detecting sterilising antimicrobial activity for

the selection of optimal peptide-solid surface combinations for activity against a specific target organism.

Table 4.4: Summary of the P-values from the One-way Anova using Bonferoni's multiple comparison test. The precise P-values are less than the limit value shown in table. "ns" denotes a P-value > 0.05. (-) Indicate overlapping data. In each cell, the first value corresponds to the maximum P-value for Trc treated filters and the second value corresponds to that of gramicidin S treated filters.

Filter	Cell count/cm ²	HAWP	GSWP	HVLP	GVHP	PC	CA	HDC	CL
HAWP	3.4x10 ⁵	-	ns, ns	ns, 0.001	ns, 0.05	0.05, ns	ns, ns	0.001, 0.001	0.001, 0.001
	1.7x10 ⁵	-	ns, ns	ns, 0.01	ns, ns	ns, ns	ns, ns	0.001, 0.001	0.01, 0.01
GSWP	3.4x10 ⁵	-	-	ns, 0.001	ns, 0.05	0.01, ns	ns, ns	0.001, 0.001	0.001, 0.001
	1.7x10 ⁵	-	-	ns, 0.001	ns, ns	ns, ns	ns, ns	0.001, 0.001	0.001, 0.001
HVLP	3.4x10 ⁵	-	-	-	ns, 0.05	0.01, 0.001	ns, 0.001	0.001, ns	0.001, ns
	1.7x10 ⁵	-	-	-	ns, ns	0.001, 0.001	0.05, 0.001	ns, ns	ns, ns
GVHP	3.4x10 ⁵	-	-	-	-	0.001, 0.001	ns, ns	0.001, 0.01	0.01, 0.05
	1.7x10 ⁵	-	-	-	-	ns, ns	ns, ns	0.001, 0.001	0.001, ns
PC	3.4x10 ⁵	-	-	-	-	-	ns, ns	0.001, 0.001	0.001, 0.001
	1.7x10 ⁵	-	-	-	-	-	ns, ns	0.001, 0.001	0.001, 0.001
CA	3.4x10 ⁵	-	-	-	-	-	-	0.001, 0.001	0.001, 0.001
	1.7x10 ⁵	-	-	-	-	-	-	0.001, 0.001	0.001, 0.001
HDC	3.4x10 ⁵	-	-	-	-	-	-	-	ns, ns
	1.7x10 ⁵	-	-	-	-	-	-	-	ns, ns

There are also some correlations observed between the percentage haemolysis and the sterilisation antimicrobial activity (compare Figs. 4.2 and 4.3). The highest percentage haemolysis and also the highest antibacterial activity for both Trc and gramicidin S treated

filters were observed for CL and HDC, indicating that haemolytic activity can predict antimicrobial activity to some extent. However, no haemolytic activity was observed for Trc treated CA and gramicidin S treated HAWP, GSWP, GVHP and CA. Yet these filters did prove to have antimicrobial activity. Thus the haemolysis assay can detect peptide present on some of the solid surfaces, but not for all the filters. This is a strong indication that the peptide is bound to the filters in a way to rather recognise bacterial cells than erythrocytes. Haemolytic activity is mostly dependent on hydrophobic interaction of antimicrobial peptides with the neutral erythrocyte membrane [20], while antibacterial activity depends on electrostatic interaction between the cationic peptide and negative cell wall and membrane of the target bacterium.

Based on the collective results, a hypothesis can be formulated on how the Trcs adsorbed to the various filters and how the peptides are still able to elicit it antimicrobial activity. Since the application of the peptide to the solid surfaces is limited to the adsorption of the peptide onto the filters, the only control in the orientation of the peptide is the character of the matrix, which does shed some light onto the mechanism of action or then at least important orientations/amino acid residues to the activity of the peptide against different target cells.

In the case of hydrophobic surfaces (PC, HDC) the hydrophobic residues, Val, Leu and aromatic amino acids probably associated with the filter, allowing the positively charged residue (Lys⁹/Orn⁹) to attach to the negatively charged microbial membrane and the hydrophilic sequence (N⁵Q⁶X⁷V⁸O⁹) to associate with the outside of the cell which in turn could cause desorption and disruption of the membrane integrity resulting in cell lysis.

In the case of hydrophilic filters the hydrophilic residues (Asn, Gln, Tyr and Orn/Lys) probably associated to the filter. This would leave the hydrophobic residues to either interact with these residues of a second Trc molecule, as these peptides tend to form dimers [7, 8] and

higher order oligomers [21]. In an absorbed dimeric structure the positively charged Lys or Orn residue of the second molecule would be available to attach to the negatively charged microbial cells after which the peptide or dimer could be desorbed and insert into the membrane to cause cell leakage [4, 8]. The CL and HDC hydrophilic filters also showed high haemolytic activity which could indicate that hydrophobic residues are available to interact with the neutral erythrocyte membrane, which in turn indicates monomeric absorption or aggregates in which hydrophobic residues are exposed. For gramicidin S, the hydrophilic association is most probably via the cationic Orn residues, while hydrophobic association probably took place via the rest of the residues. However, it remains to be determined whether the peptides associate to the filters as monomers, as higher order assemblies [7, 8] or peptide aggregates [21] which would play a role in the antimicrobial activity [9].

4.4.3 Stability of antimicrobial activity of tyrocidine treated CL

Trc extract treated CL filters were chosen for the initial stability testing due to its high antimicrobial activity. CL is commonly used in low non-specific binding application such as biological sample filtration, tissue culture or protein recovery applications [22, 23]. These hydrophilic filters are also very stable having resistance against weak acids, alcohols, aliphatic and aromatics amines, esters, hydrocarbons and ketone solvents [22-25]. It has limited resistance against aldehydes and bases and no compatibility with strong acids [22, 23]. These filters remain stable in aqueous samples with pH ranging from pH 3 to pH 12, under wet temperatures of 320°C/25MPa and [26] and up to 340°C dry temperature [22-24, 27]. It does, however, have a low resistance to solvents with $\text{pH} \geq 12$ [22, 23]. These stability treatments included multiple water wash steps, changes in pH and temperature changes of the wash solution.

If was found that after the 12 water wash steps that there was no statistical difference observed with the haemolytic activity of the Trc treated CL filters (Fig. 4.4). Based on the percentage haemolysis observed, an estimation of the amount of peptide present on the filters was calculated by comparing the haemolysis of the filter with a dilution of Trc mixture. It was found that the water washed filters contained on average $1.5 - 2.2 \mu\text{g}/\text{cm}^2$ of peptide.

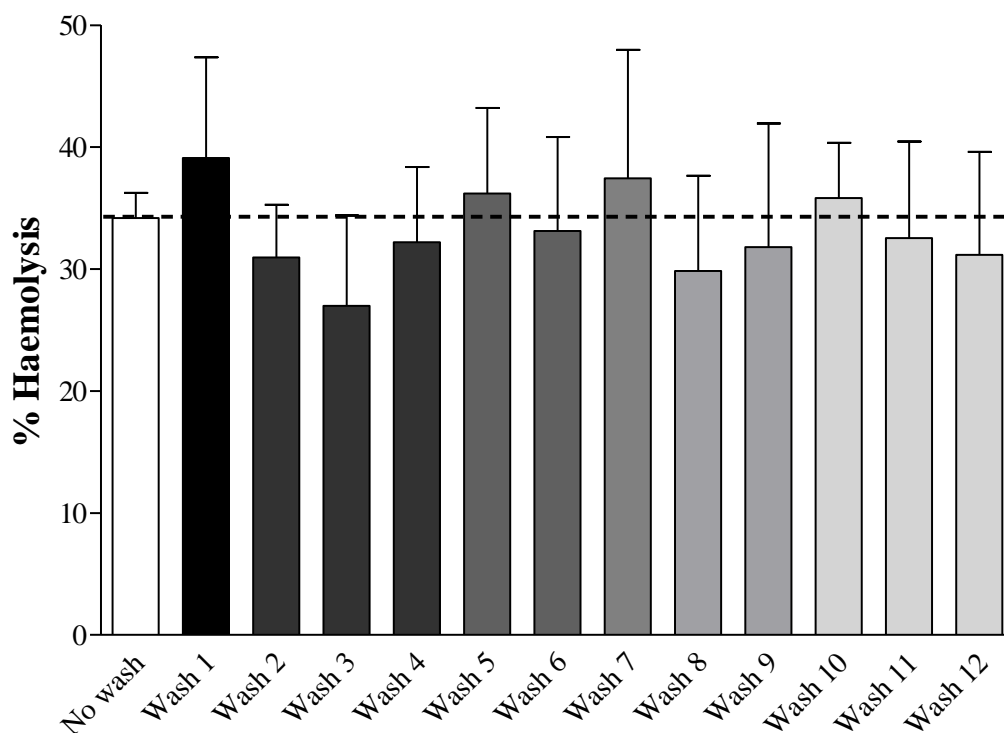


Figure 4.4: Comparison of the haemolytic activity observed for the peptide treated CL filters subjected to water wash steps. A $50 \mu\text{g}/\text{mL}$ treated cellulose filter as control for the initial haemolytic activity present on the filter and the negative control is untreated cellulose filters. Each bar depicts the average of at least 9 repeats with error bars representing SEM.

The antimicrobial activity remained relatively unchanged showing < 10 CFUs of *M. luteus*, surviving from a possible 1.3×10^5 CFUs per filter, on only one of the three repeats for water washes 1, 4 and 6-12 (Table 4.5). It was also observed that by wash 12 the cellulose filter started showing signs of disintegration, while antimicrobial activity was intact. This leads to the conclusion that the filter would disintegrate before the peptide would be washed off the

filter. This was expected because Trcs are not very soluble in water [28] and would not readily dissociate from the filter in the presence of water.

Table 4.5: Summary of wash treatments of CL filters treated with 50µg/mL Trc extract based on the change in calculated Trcs on the filter (SEM) and antimicrobial activity for 3 repeats of each treatment. Key: +++ Overgrown; + CFU \leq 10

Treatment	Tyrocydine on filter ($\mu\text{g}/\text{cm}^2$)	CFU observed for repeats		
		1	2	3
Control - untreated	-	+++	+++	+++
Control - treated	1.9 \pm 0.6	-	-	-
Wash 1	2.2 \pm 1.1	-	-	-
Wash 2	1.4 \pm 0.5	-	-	-
Wash 3	1.3 \pm 0.7	-	-	-
Wash 4	1.6 \pm 0.7	+	-	-
Wash 5	2.0 \pm 1.0	-	-	-
Wash 6	1.8 \pm 1.0	-	+	-
Wash 7	2.2 \pm 1.4	-	-	+
Wash 8	1.5 \pm 0.8	-	-	+
Wash 9	1.8 \pm 1.2	+	-	-
Wash 10	1.8 \pm 0.9	-	+	-
Wash 11	1.7 \pm 0.9	+	-	-
Wash 12	1.6 \pm 1.0	-	-	+

The Trc treated CL filters were also challenged with washes at different temperatures (40°C to 100°C) and no statistical change in haemolytic activity was observed compared to that of the control filters (25°C) (Fig. 4.5:A). The average amount of peptide on the filters calculated based on the haemolytic activity was 1.5 – 1.8 $\mu\text{g}/\text{cm}^2$. There was also no change in antimicrobial activity observed between the treated and control filter (Table 4.6). This was to be expected since it has been shown that Trcs are heat stable [9], but also that CL filters remains stable up to 340°C at dry temperatures [27]. This shows that the peptide and filter remains adsorbed to each other even with temperature challenged washes.

The washes at different pH values showed only a statistically significant decrease in haemolytic activity for the filter washed in the pH 13 solution (Fig. 4.5: B). It was calculated from the observed haemolytic activity that the filters treated with pH 1 – pH 11 contained between 1.4 – 1.9 $\mu\text{g}/\text{cm}^2$ and the pH 13 filter containing, on average, 0.21 $\mu\text{g}/\text{cm}^2$ peptide.

The antimicrobial activity was mildly affected between pH 9 – pH 13, showing <10 CFUs of *M. luteus* per filter in one of the triplicate repeats (Table 4.7).

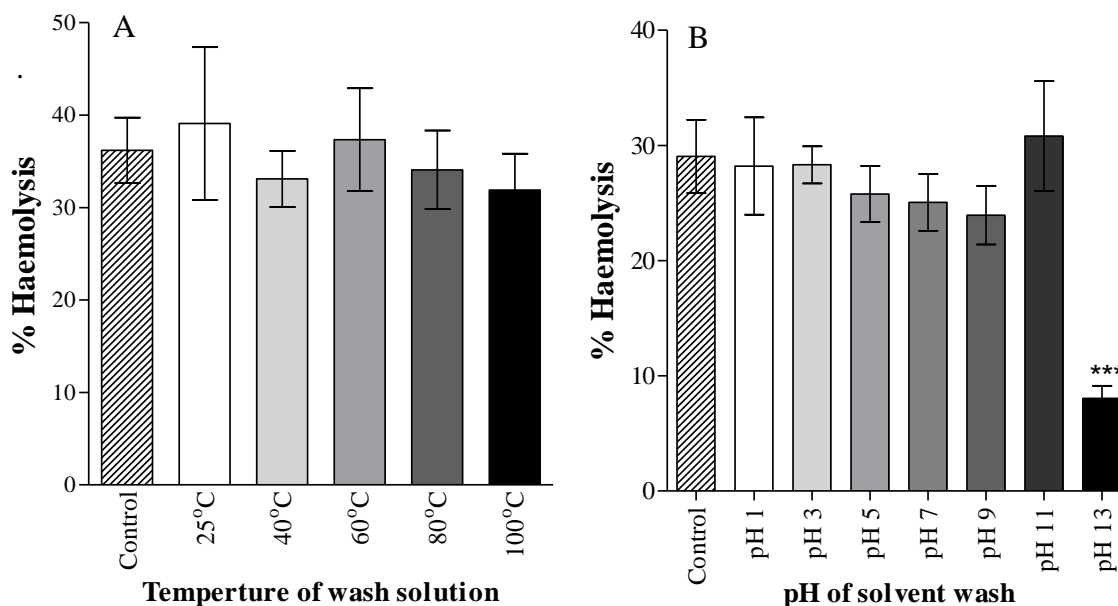


Figure 4.5: Comparative changes in haemolytic activity observed for the peptide treated cellulose filters subjected to **A** water washes at different temperatures and **B** different pH values. A 50 µg/mL treated cellulose filter as control for the initial haemolytic activity present on the filter also the peptide stability at 25°C. Each bar depicts the average of at least 9 repeats with error bars representing the standard error of the mean. Statistical differences as determined with One way Anova (Bonferroni's Multiple Comparison Test) for antimicrobial activity of pH 13 versus pH1, pH 3, pH 5, pH 11 ***P<0.001

The change in antimicrobial activity seen between the treatments of pH 9 to pH 13 is minor, and compared well with the water washes. However, the drop in haemolytic activity at pH 13 does differ significantly with the other pH treated filters. This could indicate that at pH 13 there is a loss of peptide from the filter that is associated in a way to lead to productive lytic interaction with erythrocytes. CL filters have decreased chemical resistance to solvents with a pH higher than pH 12, thus the drop and haemolytic activity could also be possibly due to damage to the cellulose structure resulting in the desorption some Trc molecules from the filter during the wash step. The decrease in amount of peptide retained did, however, have very little effect on the antimicrobial activity against *M. luteus*. Therefore it seems that the local concentration of Trcs on the filter is very high compared to what is needed to kill the

maximum number of target cells that can grow per cm², as activity was maintained after an almost 3-fold drop in amount of retained peptide (Table 4.7).

Table 4.6: Comparison of temperature treatment of CL filters (untreated and treated with 50 µg/mL Trc extract) in terms of haemolytic Trc concentrations on the filter and antimicrobial activity for triplicate repeats of each treatment. Key: +++ Overgrown; + 10_≥CFU

Treatment	Tyrocydine on filter (µg/cm ²)	CFU observed		
		1	2	3
Control - untreated	-	+++	+++	+++
Control (treated, 25°C)	1.8 ± 0.6	-	-	-
40°C	1.6 ± 0.5	-	-	-
60°C	1.8 ± 0.4	-	-	-
80°C	1.6 ± 0.4	-	-	-
100°C	1.5 ± 0.3	-	-	-

Table 4.7: Comparison of pH treatments of CL filters (untreated and treated with 50 µg/mL Trc extract) in terms of haemolytic Trc concentrations on the filter (SEM) and antimicrobial activity for triplicate repeats of each treatment. Key: +++ Overgrown; + 10_≥CFU

Treatment	Tyrocydine on filter (µg/cm ²)	CFU observed		
		1	2	3
Control - untreated	-	+++	+++	+++
Control – treated	1.7 ± 0.6	-	-	-
pH 1	1.7 ± 0.8	-	-	-
pH 3	1.7 ± 0.3	-	-	-
pH 5	1.5 ± 0.4	-	-	-
pH 7	1.8 ± 0.6	-	-	-
pH 9	1.4 ± 0.6	+	-	-
pH 11	1.9 ± 0.8	+	-	-
pH 13	0.2 ± 0.2	-	-	+

4.4.4 Tyrocidine treated CL filters and application in plant culturing

During the antimicrobial activity determinations the Trc treated CL filter was found to have the highest and robust antimicrobial activity and as a result was selected to be used in the first pilot application study. As part of plant tissue culture methodologies, seeds are first allowed

to germinate on moist CL filters before the seedlings are transferred to tissue culture for further studies. It is during germination in the highly humid environment the seeds are vulnerable to infection and the process could benefit from a decrease in the risk of infection. As CL filters are already part of the culturing process as a method to retain moisture in culture dishes, the CL filters were treated with 5 $\mu\text{g/mL}$, 25 $\mu\text{g/mL}$ or 50 $\mu\text{g/mL}$ solutions of a Trc extract, incubated with tomato seeds prepared according to the plant culture methodology. The effect of the treated CL filter on germination and infection was studied over a period of eight days. The Trc treated filters had no effect on germination compared to the untreated filters and neither did the change in Trc concentration (Fig. 4.6).

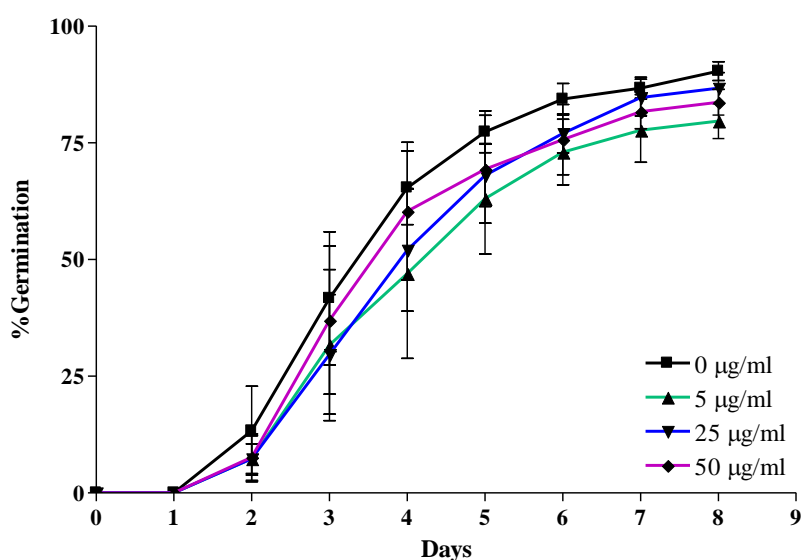


Figure 4.6: Germination of tomato seed over eight days while subject to Trc treated CL filters (0, 5, 25, 50 $\mu\text{g/mL}$). Germination of total 25 seeds with 9 repeats of each treatment concentration is shown.

The Trc treated filters failed to fully maintain the sterility during the germination on some of the treated filters. No bacterial infections were observed on the treated filters, however, a limited number of fungal spores did germinate on some of the filters. Concerning the tomato seedling growth, the Trcs did have an effect on shoot length, weight and root length. The 5 $\mu\text{g/mL}$ treated filter promoted root length whereas the 50 $\mu\text{g/mL}$ treated filters had a slightly negative effect (Fig. 4.7:A).

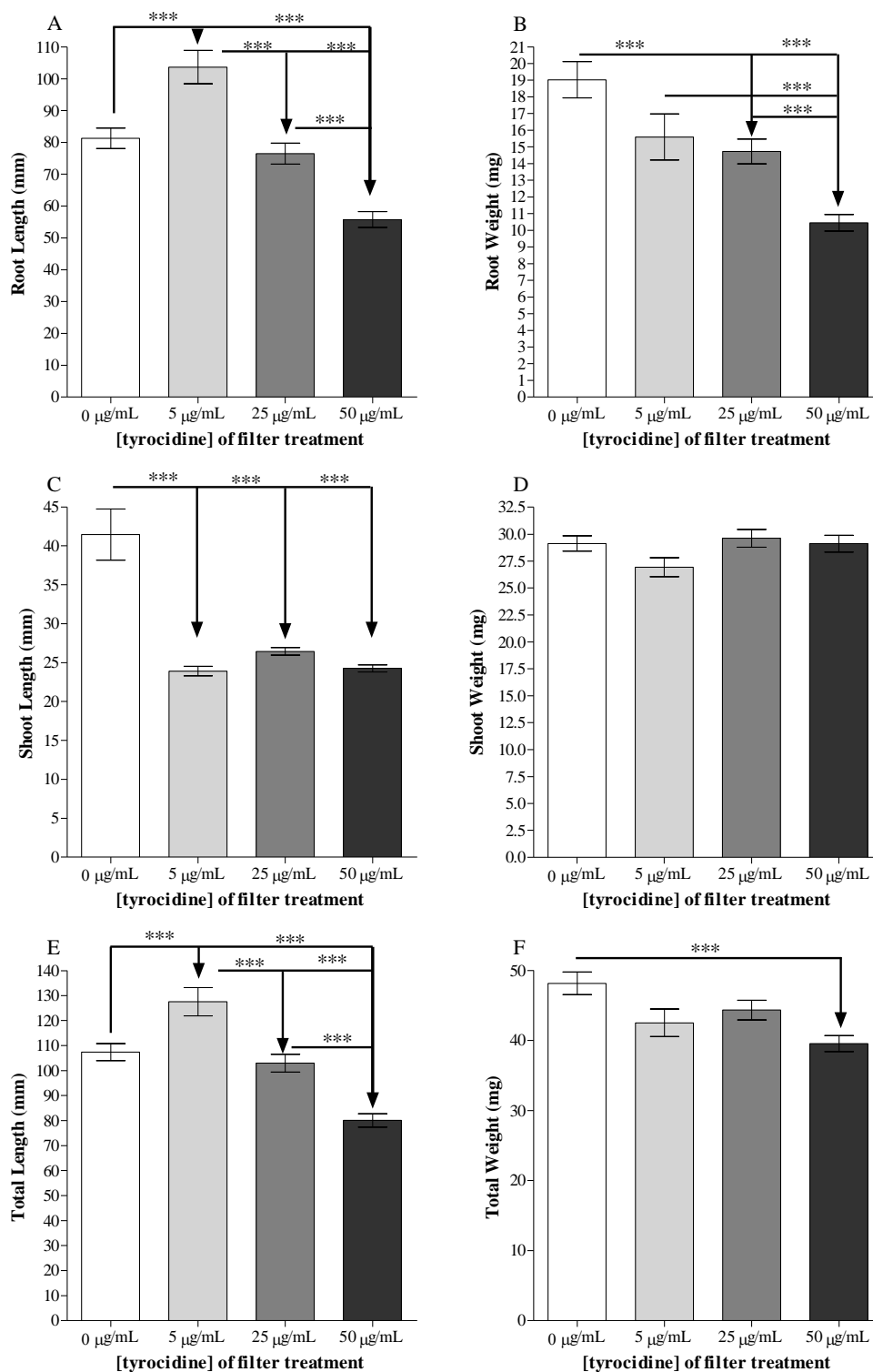


Figure 4.7: The effect of Trc treated filters at varying concentrations (0, 5, 25 and 50µg/mL) on **A** root length, **B** root weight, **C** shoot length, **D** shoot weight, **E** total length, **F** total weight of tomato seeds after germination. For each filter treatment concentration the germinated plans of 75 seed were analysed with no repeats with error bars representing the standard error of the mean. Statistical differences as determined with One way Anova (Bonferroni's Multiple Comparison Test) ***P<0.001

The filters treated with the two higher concentrations had a significant negative effect on the root weight (Fig. 4.7: B), while all the treated filters led to a shorter shoot length (Fig. 4.7: C), but the shoot biomass remained unchanged in the germinated seedlings (Fig. 4.7: D). When considering total biomass (Fig. 4.7: F), the seedlings were unaffected by the Trc treatment of filters with the exception of the 50 $\mu\text{g/mL}$ filter that caused a slight loss of biomass, indicating some growth inhibition. Overall the 5 $\mu\text{g/mL}$ treated filters caused for longer seedlings compared to the control and other treatment concentrations (Fig. 4.7: E). Therefore it can be concluded that the Trcs in general did not have a major phytotoxic effect on the germination or growth of the seedlings. In fact it may have a positive effect at low concentrations such as 5 $\mu\text{g/mL}$ promoted root length which is preferred when transferring seedlings from germination to plant tissue culture.

4.5 Conclusions

A range of different polymer filters were treated with a Trc extract, after which the biological activity and stability were determined. For both filters treated with the Trc extract and filters treated with gramicidin S the haemolytic assay was successful in confirming the presence of peptide on the different treated filters. However, for some filters, such as the Trc treated GSWP, PC and CA, the level of haemolysis was too low to quantify the amount of retained peptide. The comparison between the amounts retained as calculated by the absorbance method *versus* the haemolytic method showed that the haemolytic assay only detected a fraction of the available peptide. This further highlighted the need for a peptide quantification method set as discussed previously (Chapter 3).

It was observed that even though adsorbed to a solid matrix, Trcs maintained activity and that high haemolytic activity correlated with potent antimicrobial activity. HDC and CL filters had the highest observed antimicrobial activity when tested with the CFU agar assay. The PC

filter had the highest antimicrobial activity per amount of peptide detected. This point to the importance of the filter characteristics (hydrophobic/hydrophilic) that would influence peptide adsorption and orientation which in turn would affect both haemolytic and antimicrobial activity. This was observed for gramicidin S treated GVHP and PC filters which both had haemolytic activity, but displayed no observed antimicrobial activity even at low CFU loading. Of the other gramicidin S treated filters only CL, HDC and HVLP had both haemolytic activity and antimicrobial activity. It can thus be concluded that Trcs are better suited to be adsorbed to various solid surfaces compared to GS based their antimicrobial activity and inhibition bacterial colonisation in a high nutrient environment, as determined by the *M. luteus* based CFU growth assay. However, the CFU growth assay is limited in true quantification antimicrobial activity as it depends on manual counting of colonies, which is complicated when colonies do not have a discernible colour different from the filters or are growing into a very dense lawn. Therefore a cell viability assay was developed to determine direct killing/biocidal or sterilisation in a low nutrient environment. To maximally challenge the peptide treated filters, we determined the sterilisation in the presence of higher cell counts ($>10^5$ cells/cm²). For the most part the result correlated with the results obtained for the CFU agar assay. Both gramicidin S and Trc treated HDC and CL had the highest sterilisation or direct biocidal activity. Polycarbonate filters, GS and Trc treated, showed to have very little activity at the higher cell counts used in the cell viability assays compared to what was seen for the CFU assay. There was no difference inactivity between gramicidin S and Trc treated filters with the exception of gramicidin S treated HVLP, which significantly outperformed the Trc treated counterpart at 3.7×10^5 *M. luteus* cells per cm². In this sterilisation assay, treated CA was found to have some sterilisation activity and all the gramicidin S treated filters showed activity which was not previously detected in the CFU agar assay. Thus it can be concluded that although the haemolytic assay

can detect the presence of peptides for the most part, that it is greatly dependent on the peptide orientation on the filter. A preliminary study not reported here indicated that peptide treated CL filters had no activity against Gram-negative *Escherichia coli* and *Pseudomonas fluorescens* which highlight the need of peptide combinations to create solid surfaces active against both Gram-negative and Gram-positive organisms.

The future application of Trc treated solid surfaces is dependent on the stability of the peptide on the filter when it is subjected to various environmental changes. It was observed that Trcs remains stable on CL filters and maintains antimicrobial activity against *M. luteus* when exposed to multiple wash steps, extreme temperatures and pH changes. The Trc treated CL filters exposed to pH 13 showed a decrease in haemolytic activity linked to the damage of the cellulose filter at that pH, which caused the release of peptide from the filter. Based on the CFU agar assay this release of peptide did not have a noticeable effect on the antimicrobial activity compared to the control filters, indicating a high concentration of peptide available for antimicrobial activity. This study showed the potential of the treatment of a variety of materials with Trcs and gramicidin S to combat colonisation and possibly the formation of biofilms by Gram-positive bacteria. In an application of CL filters to test this potential it was observed that Trc treated filters had no bacterial infections and generally it did not affect the germination of tomato seeds. The study showed that a 5µg/mL Trc treatment of the filters resulted in longer root length and overall positive effect on the seedling. It can thus be possibly implemented as an aid to ensure better roots in preparation of subsequent transfer into plant tissue cultures.

4.6 References

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Chapter 5

Conclusions and recommendations for future studies

5.1 Introduction

The main goal of this study was to determine whether tyrocidines (Trcs) maintained antimicrobial activity when adsorbed to a range of solid surfaces. In order to realise this goal tyrocidines and tyrocidines analogues had to be produced and purified from normal and amino acid supplemented media; a range of solid surfaces had to be treated with a selected tyrocidine extract; the amount of peptide adsorbed to the solid surfaces had to be quantified; the antimicrobial activity against model organisms *Micrococcus luteus* and haemolytic activity had to be determined, the stability of the peptide and biological activity had to be determined after the treated surface were exposed to a range of conditions and the effect of tyrocidine treated cellulose filters had on plant germination, as a possible application had to be determined.

This study gives a detailed report on the biological activity of solid surfaces (Chapter 4) treated with a purified tyrocidine extract (Chapter 2) for which the amount of peptide retained was quantified (Chapter 3). The stability and possible application of these solid surface were also determined (Chapter 4).

5.2 Experimental Conclusions

5.2.1 Production and purification of tyrocidines and tyrocidine analogues

In order to determine the activity of antimicrobial peptide treated solid surfaces and the stability thereof, with the prospect of developing these surfaces for a specific application, sufficient amounts of peptide needed to be obtained. A tyrocidine standard was obtained by separating the tyrocidines from linear gramicidins as found in a commercially available tyrothricin mix with an established protocol. Tyrocidines were also isolated from a culture

broth of *Bacillus aneurinolyticus* in amino acid supplemented and normal media. The same shift in peptide profile with the addition of either Trp or Phe was observed compared to previous studies [1]. The supplementation of Trp caused the production of predominantly tryptocidines whereas the supplementation of Phe caused the predominant production of Trc A analogues (TrcA, TrcA₁, PhcA and PheA₁). The tyrocidine extract obtained from the unsupplemented culture was purified with a non-polar column separation step to yield fraction of >80% purity, based on analytical reverse phase HPLC analysis, and good antimicrobial activity against model organism *M. luteus*. This fraction was used for the treatment of solid surfaces to determine its antimicrobial activity. The identity of all peptide extracts and fractions were determined with MS.

5.2.2 Quantification of peptide adsorbed to filters

Commercial solid surfaces selected for the pilot study included nitrocellulose ester filters (HAWP, GSWP), polyvinylidene fluoride filters (HVLP, GVHP), polycarbonate (PC), cellulose acetate (CA), high density cellulose (HDC) and cellulose (CL, paper). Surfaces were incubated in a 50 µg/mL peptide solution and the amount of peptide adsorbed quantified.

The amount of peptide adsorbed to the filters were determined with change of absorbance (detecting the decreased of peptide in the incubation solution), ninhydrin staining (detecting primary amines), Sanger's test (detecting primary and secondary amines), Kaiser ninhydrin test (detecting primary amines), amino acid analysis (quantification of the amino acids present), Fourier transform infrared spectroscopy (FTIR) (detecting compounds present based on an increase of absorption at different infrared wavelengths), scanning electron microscopy (detecting structural changes), matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) (verifying the presence of peptide on the filter) and electrospray mass

spectrometry (MS) for analysis of peptide after desorption from the filter. The change of absorbance method indicated that the mixed CL ester filters adsorbed more peptide than polyvinylidene fluoride and PC filters. This method did not work for CA, HDC and CL filters since the absorbance of the incubation solution increased after incubation, due to the desorption of a pigment from the filters. Ninhydrin staining only showed results with the treated cellulose filter. The Sanger's test, the Kaiser test, amino acid analysis and FTIR proved to be insensitive for detection of the low amount of peptide per square centimetre of the filters. Scanning electron microscopy showed that no overt structural changes of the filter can be seen other than a bulking which is possibly due to the water retention ability of tyrocidines. MALDI-MS of CL fibres and MS analysis of compounds desorbed from the CL filters both showed that tyrocidines are present on the treated CL filters and subsequently responsible for the antimicrobial activity. The wettability assay was performed on untreated, tyrocidine treated and gramicidin S treated filters. It was found that cellulose filters were the most hydrophilic, followed by HAWP, GSWP, CA, HVLP and GVHP. PC and HDC were found to be hydrophobic. After treatment with gramicidin S and tyrocidine the filters were found to be more hydrophobic with the exception of CA which was found to be more hydrophilic. No clear conclusions could be made for CL and PC filters.

5.2.2 Biological activity of peptide treated filters

The possible development and application of Trc treated solid surface lies with Trc maintaining its antimicrobial activity and the amount of peptide retained that can be determined in the light of possible toxicity. Since the methods used to quantify the amount of peptide adsorbed to the filters was unsuccessful, mostly due to the low level of peptide to be detected, a haemolytic assay was developed to confirm the presence of tyrocidines on the filters. Haemolytic activity (Table 5.1) and thus the presence of tyrocidines were detected on most of the filters with the exception of the cellulose acetate filter which showed very little to no

haemolytic activity. The presence of tyrocidines on the CL and HDC filters were confirmed, which could not be done with the change in absorbance readings. According to the measured haemolytic activity, cellulose and HDC filters absorbed the most tyrocidines, followed by the polyvinylidene fluoride filters (HVLV, GVHP), the mixed cellulose filters (HAWP, GSWP) and then last the PC filters. Although there was some dissimilarity between the amounts of peptide adsorbed according to change of absorbance compared to the detected haemolytic peptide, the haemolytic assay is currently the best method to confirm the presence of tyrocidines on the treated filters.

The filters were also treated with the analogous cyclodecapeptide, gramicidin S, as a control and the amount of peptide adsorbed was calculated with both the change in absorbance and the haemolytic assay. The results between the two determinations were inconsistent. In the change of absorbance test the total peptide adsorbed could only be determined for HVLV, GVHP, and PC filters. The haemolytic assay showed the presence of peptide for the GVHP, PC, HDC and CL filters. The latter could not be detected in the change of absorbance test, as was seen with tyrocidine treated filters. This data only emphasize the need for the development of chemical quantification tests as described in the aforementioned future studies. Standardised tests would give comparable results to be used in assessing peptide mixtures and solid surface combinations.

The antimicrobial activity of both the gramicidin S and tyrocidine extract treated filters against the model organism, *M. luteus*, were determined by comparing the CFU/cm² on the untreated filter with its treated counterpart, using an agar-based assay (Table 5.1). It was found that for the tyrocidine treated filters that the HDC and CL filters had the highest antimicrobial activity killing 43 CFU/cm² and 45 CFU/cm² respectively, when compared to the control filters, leading to no visible growth on the treated filters. It was followed by the

polyvinylidene and PC filters. The mixed cellulose filters had very low activity and the CA filters had no detectable antimicrobial activity. The PC filter had the highest antimicrobial activity per amount of peptide detected. Only three of the gramicidin S treated filters showed activity of which the cellulose filter showed the most activity. This was followed by the HDC filter and a polyvinylidene fluoride filter (HVLP). None of the other gramicidin S-treated filters showed detectable antimicrobial activity. These results partially correlated with the amount of peptide adsorbed as calculated from the haemolytic assay.

No clear conclusion could be made as to which peptide-solid surface combination showed the highest antimicrobial activity which resulted in the development of a cell viability assay that could test higher cell counts of *M. luteus* (Table 5.1). It was observed that HDC and CL, GS and Trc treated, still had the highest antimicrobial activity. Both GS and Trc treated PC filters showed to have very little activity at the increased cell density. No difference in antimicrobial activity was observed between GS and Trc treated filter with the exception of GS treated HVLP, which had a higher activity than its Trc treated counterpart at $3.7 \times 10^5/\text{cm}^2$ *M. luteus* cells. CA, GS and Trc treated, which had no activity with the CFU agar assay proved to have an antimicrobial activity between 20-30% inhibition of *M. luteus*. All the GS treated filters also proved to have antimicrobial activity.

Trcs can be divided into a hydrophilic region (Asn⁵, Gln⁶, Tyr⁷) and hydrophobic region (Val⁸, Leu¹⁰, D-Phe¹, Pro², Phe³, D-Phe⁴) were the positive charge on the Orn/Lys⁹ is important for the initial association with bacterial membranes and Phe/Trp^{3,4} is important for the peptide to elicit its membranolytic action [2,3]. The hydrophobic region associates with the neutral erythrocyte membrane to elicit its haemolytic action [4]. Some conclusions can be made regarding the peptide orientation and proposed mode of action when comparing the results obtained for the wettability assay, the haemolytic assay and the cell viability assay.

Based on the change in hydrophilic character, as observed in the wettability assay, the filters can be grouped on the hypothesised method of adsorption. The first is where the filters were shown to be hydrophilic in character, but after treatment with Trcs presented a hydrophobic character. This was seen for mixed CL filters (HAWP & GSWP) and polyvinylidene fluoride filters (HVLV & GVHP). The data is not conclusive but based on the hydrophilic nature of the filter it can be assumed that the peptide binds to the CL filter in a similar way and can be included in this group. It is hypothesized that the hydrophilic region of the peptide associates to the hydrophilic filter leaving the hydrophobic region exposed, giving rise to the change in filter character from hydrophilic to hydrophobic. The opposite is hypothesized for the second group, where the filters had a hydrophobic character, but after Trcs treatment presented a hydrophilic character. This can be assumed for both HDC and PC, as both are hydrophobic filters. The last group is where the filter had a hydrophilic character that was enhanced after treatment with Trcs, which was observed for CA filters. The hypothesis is that multi-peptide complexes and aggregates form on this filter. The hydrophilic region of Trcs associates with the filter after which a second Trc molecule associates with the first via the exposed hydrophobic regions, on the first Trc molecule, thus leaving the hydrophilic region, on the second Trc molecule, exposed giving rise to the enhanced hydrophobic character of the filter.

Based on the previously hypothesized association of the peptide to the filters it is expected that the hydrophobic filters (after Trcs treatment) have better haemolytic and antimicrobial activity compared to the hydrophilic filters (after Trcs treatment) since the hydrophobic region of Trcs are exposed to associate with neutral erythrocytes, but also contains the positive Orn/Lys⁹ and dipeptide unit Phe/Trp^{3,4} important for antimicrobial action. This was observed when comparing mixed cellulose, polyvinylidene fluoride and CL filters to PC and CA filters. The hydrophobic filters (after Trcs treatment) had on average a haemolytic activity between 1.7-14.7% and antimicrobial activity of 40.6-71.9% inhibition compared to

the hydrophilic filters (after Trcs treatment) that had a haemolytic activity between 0.3-1.5% and antimicrobial activity of 11.4-22.9%. This correlated with the expected results based on the hypothesized peptide association with the filters. The difference in haemolytic and antimicrobial activity between these filters e.g mixed cellulose filters HAWP and GSWP can be attributed to the amount of peptide bound to the solid surface. Both HDC and CL had the highest observed antimicrobial activity of all solid surfaces tested, however the same drop in haemolytic activity was observed for HDC compared to the CL filter as was observed between the hydrophobic and hydrophilic filters (after Trcs treatment). Thus even though Trcs have a preference to bind to cellulose and cellulose analogues, the better antimicrobial and haemolytic activity is observed for the hydrophilic solid surfaces where the hydrophobic region of the peptide is exposed after solid surface treatment.

The CL and HDC filters hold great promise as an application in packaging, but also indicates the potential use of Trcs as a protective wood coating to prevent brown rot spoilage, which targets cellulose and hemi-cellulose [5]. Trcs maintained its activity while adsorbed solid surfaces, thus the CL and HDC filters should have activity against *Listeria monocytogenes* and *Candida albicans* biofilms based on previous antimicrobial studies [6-8]. This is important since it has been reported that biofilms are more likely to form on surfaces that inherently serve as a carbon source such as cellulose and analogues [9]. The development and application of Trcs treated solid surfaces would ultimately depend on the spectrum of antimicrobial activity and the stability of the adsorbed peptide.

Table 5.1: Summary of the results obtained on the characteristic of the treated filters based on change in filter character regarding hydrophilic nature (Chapter 3), haemolytic /activity (Chapter 4), and antimicrobial activity as observed with the CFU and cell viability assays (Chapter 4). ND- not decisive, no clear decision could be made regarding the change in filter character.

Filter	Peptide	Change in filter character	Haemolytic activity	Antimicrobial Activity	
				CFU agar assay	Cell viability assay ($3.7 \times 10^5 / \text{cm}^2$)
HAWP (0.45 μm)	Trc	Hydrophobic	3.0 ± 1.3	27 ± 1	54.1 ± 6.5
	GS	<i>Hydrophilic</i>	-0.6 ± 1.1	0	41.2 ± 6.8
GSWP (0.22 μm)	Trc	Hydrophobic	1.7 ± 0.6	27 ± 2	40.6 ± 8.9
	GS	<i>Hydrophobic</i>	-0.1 ± 0.7	0	29.0 ± 8.7
HVLP (0.45 μm)	Trc	Hydrophobic	13.2 ± 5.8	27 ± 4	71.9 ± 12.1
	GS	<i>Hydrophobic</i>	0.2 ± 1.2	3 ± 1	97.5 ± 8.0
GVHP (0.22 μm)	Trc	Hydrophobic	14.7 ± 3.6	34 ± 1	46.5 ± 3.8
	GS	<i>Hydrophobic</i>	1.5 ± 1.2	0	52.0 ± 9.4
PC (0.4 μm)	Trc	Nd	1.5 ± 0.7	32 ± 3	11.4 ± 9.0
	GS	<i>Nd</i>	0.4 ± 0.2	0	7.17 ± 9.4
CA (0.45 μm)	Trc	Hydrophilic	0.3 ± 0.5	0	22.9 ± 5.1
	GS	<i>Hydrophilic</i>	-1.2 ± 0.3	0	29.9 ± 6.7
HDC	Trc	Nd	33.3 ± 6.4	43 ± 4	117.0 ± 4.7
	GS	<i>Nd</i>	9.9 ± 6.0	4 ± 1	122.9 ± 1.7
CL	Trc	Nd	54.5 ± 7.3	45 ± 2	112.5 ± 12.1
	GS	<i>Nd</i>	22.4 ± 1.8	3 ± 1	98.0 ± 18.0

5.2.4 Stability of the peptide treated filters

The stability of the adsorbed tyrocidines and analogues were tested with the CL filter because of the eight filters originally tested it showed the greatest antimicrobial activity. Stability testing was completed by exposing the filters to multiple wash steps, temperature change or pH changes.

No statistical difference in haemolysis was detected between the 12 wash steps and filters that were unwashed. Antimicrobial activity against *M. luteus* against remained relatively unaffected between wash steps. The stability testing conducted with temperature changes (25°C, 40°C, 60°C, 80°C, 100°C) and pH changes (pH 1, pH 3, pH 5, pH 7, pH 9, pH 11, pH 13) showed no statistical difference in haemolysis, compared to the unexposed filters, with the exception of pH 13 that showed lower haemolytic activity. Since the filters were rinsed after exposure to the change in pH the drop in haemolytic activity can only be attributed to a loss in peptide adsorbed to filter. The antimicrobial activity against *M. luteus* of the temperature exposed filter remained unaffected and the antimicrobial activity of the pH exposed filters remained unaffected with the exception of pH 9 to pH 13 which showed some growth after incubation with *M. luteus*.

The results obtained were expected since it correlated with the known characteristics of both the cellulose filters and Trcs. An incubation solution of predominantly water was selected to force the association of Trcs onto the selected solid surface since Trcs are not very soluble in water [10]. CL filters are very stable in water [11] and provide optimal conditions for the Trcs to bind, as was seen with the observed haemolytic and antimicrobial activity (Chapter 4). Thus it was expected for Trcs not to readily dissociate from the CL filter especially in the presence of a polar solvent such as water. CL are also heat stable till 340°C dry temperature [12] and wet temperatures of 320°C/25MPa [13]. Trcs are heat stable [14]

and the water wash had no effect on the amount of Trcs adsorbed. Thus the change in temperature wash was expected to have no effect on the haemolytic and antimicrobial activity as was observed. Trcs are exposed to pH levels between pH 3 to pH 9 during production and purification [15,16] and studies have shown a dissociation occurring within the structure at pH levels higher than pH 10 [17] which point to the dissociation of Orn²/Lys² and Tyr¹⁰ with pK_a values of 10.76, 10.53 and 10.07, respectively [18]. Orn and Lys residues are crucial to the antimicrobial activity of Trcs since the positive residues are indicated to be responsible for the initial attachment of the peptide to the negative Gram positive cell membrane [19]. Thus it is expected that Trcs would maintain activity from pH 3 to pH 10. The CL filters also have a low resistance to solvent with a pH ≥ 12 [11, 13, 20], hence a drop in activity was expected at pH 13 which was observed. However this drop in antimicrobial activity cannot be linked to inactivation of Trcs but rather to the loss of filter structure integrity of the CL filter since the treated filters were washed in water before it was challenged with *M. luteus* culture restoring the positive charge on the Orn/Lys. A decrease in haemolytic activity was observed which points to a release of peptide from the filter due to damage that occurred to the filter integrity after exposure to pH 13 water wash. Therefore the stability of the observed antimicrobial activity of the treated solid surfaces appears to be connected to the resistance of CL surface to the treatment than to the character and stability of Trcs.

5.2.4 Cellulose filters in the application of plant culturing

Plant tissue culture is used in various ways to grow and study plants. Prior to tissue culture steps, in some cases, seeds are first allowed to germinate. This germination occurs under sterile conditions in deep petri dishes lined with two damp cellulose filters to maintain the moisture needed for growth. It is also during this time that the seedlings are most susceptible to infection. Thus tyrocidine treated cellulose filters were suggested to replace one of the two

cellulose filters in the hope of positively affecting germination and preventing possible infection.

Cellulose filters treated with 5 µg/mL, 25 µg/mL or 50 µg/mL solutions of a tyrocidine extract was used to study the effect it had on the germination of tomato seeds over an 8 day period. There was no significant difference between the different peptide concentrations regarding the germination and the tyrocidine treated filters only offered partial sterilisation against bacterial infection, as some fungal contamination did occur. The tyrocidine treated filters did, however, have an effect on the morphology of the seedlings. The 50 µg/mL filter caused a slight loss of biomass indicative of growth inhibition. The 5 µg/mL treated filters showed the best results promoting root length which is preferred when transferring seedlings from germination to plant tissue culture.

The Trc CL treated filters are therefore not adequate for the use of sterilization and can rather be considered to be used within a combination prevention strategy. The 5 µg/mL filter does however promote root growth which correlates with what was previously been observed that 15 µg/mL Trcs solution enhanced the roots, foliage and overall growth of grapevine tissue culture plants [21].

5.3 Future Studies

The solid surfaces within this study were treated with a standard tyrocidine extract as part of a pilot study to determine the antimicrobial activity of these surfaces. Further studies could include the treatment of filters with a peptide extract obtained from amino acid supplemented growth cultures, also the treatment of filters with tyrocidines in combination with other antimicrobials, especially focussing on combatting Gram-negative organisms, fungi, food pathogens and biofilm forming organisms. For this combination of peptides to be used, antagonism/synergism studies need to be completed to determine the effect the antimicrobials

have on each other's activity. Isotopically labelled peptides can also be produced, using previous studies as reference [22], and applied to various solid surfaces to study the interaction between the peptides and solid surfaces with solid phase nuclear magnetic resonance (NMR). The range of solid surfaces tested can be extended to the likes of polycarbonate, polystyrene and different electro-spun polymers such as surfaces electro-spun EVOH (polyethylene-co-vinyl-alcohol on polyethylene terephthalate film) in combination with chitin.

The quantification of the amount of peptide adsorbed can be extended to the elemental analysis of the treated cellulose filters and selected treated materials specifically looking at nitrogen levels that could directly indicate the amount of peptide present. Also to be developed and optimised is a set of physical quality tests set on determining the surface integrity such as tensile strength and porosity.

Antimicrobial activity within this study was test against *M. luteus* with both the CFU agar assay and the cell viability assay. A preliminary study showed that the Trc and gramicidin treated filters have limited activity against Gram-negative *Escherichia coli* and *Pseudomonas fluorescence*. Future work for the antimicrobial activity of peptide treated surfaces can be extended to biofilm forming Gram-positive *Listeria monocytogenes* and Gram-negative *Pseudomonas aeruginosa*, as well as determining the anti-biofilm activity of the treated solid surfaces against *L. monocytogenes* strains, *Bacillus subtilis*, *Candida albicans* and *Pseudomonas* species. Attention can also be given to mixed cultures of target organisms since biofilms rarely contains just one organism but is rather a mixture of different organisms [23].

The stability of the adsorbed tyrocidines were determined by subjecting treated CL filters to either 12 wash steps, changes in pH and temperature. Future studies in determining the stability of the treated solid surfaces can be extended to a range of solvents modified with

salts, weak organic bases and acids, strong acids and bases and organic solvents. The loss of antimicrobial activity can also be observed over a period of time to determining the half-life of the product should it be implemented in an industrial or agricultural application. Interaction between the peptides and solid surfaces can also be investigated with solid phase NMR and high sensitivity FTIR.

5.4 Last word

The rising resistance of pathogens to known treatments and persistent infections that cause continued loss in various industries have resulted in a need for new antimicrobials but also antimicrobial surfaces to prevent adhesion to certain surfaces. Tyrocidines holds great promise in this application since it has a broad spectrum of activity and due to its membranolytic mode of action resistance is less likely to develop. It has been shown that Trcs maintain its antimicrobial activity when adsorbed to different surfaces and remain relatively stable. Pilot studies into applications have shown that tyrocidines hold great promise in combating the rising resistance and persistent infections associated with surface colonization.

5.5 References

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