

Purification & Characterization of a bacteriocin produced by a *Staphylococcus aureus* isolate

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Overview Antimicrobial peptides

Antimicrobial Peptides (AMPs)

- Ribosomally synthesized peptides produced by all living organisms
- Broad spectrum of antimicrobial activity
- Innate immune response

Bacteriocins

- Antimicrobial peptides produced by bacteria that kill other bacteria
- Immunity Protein

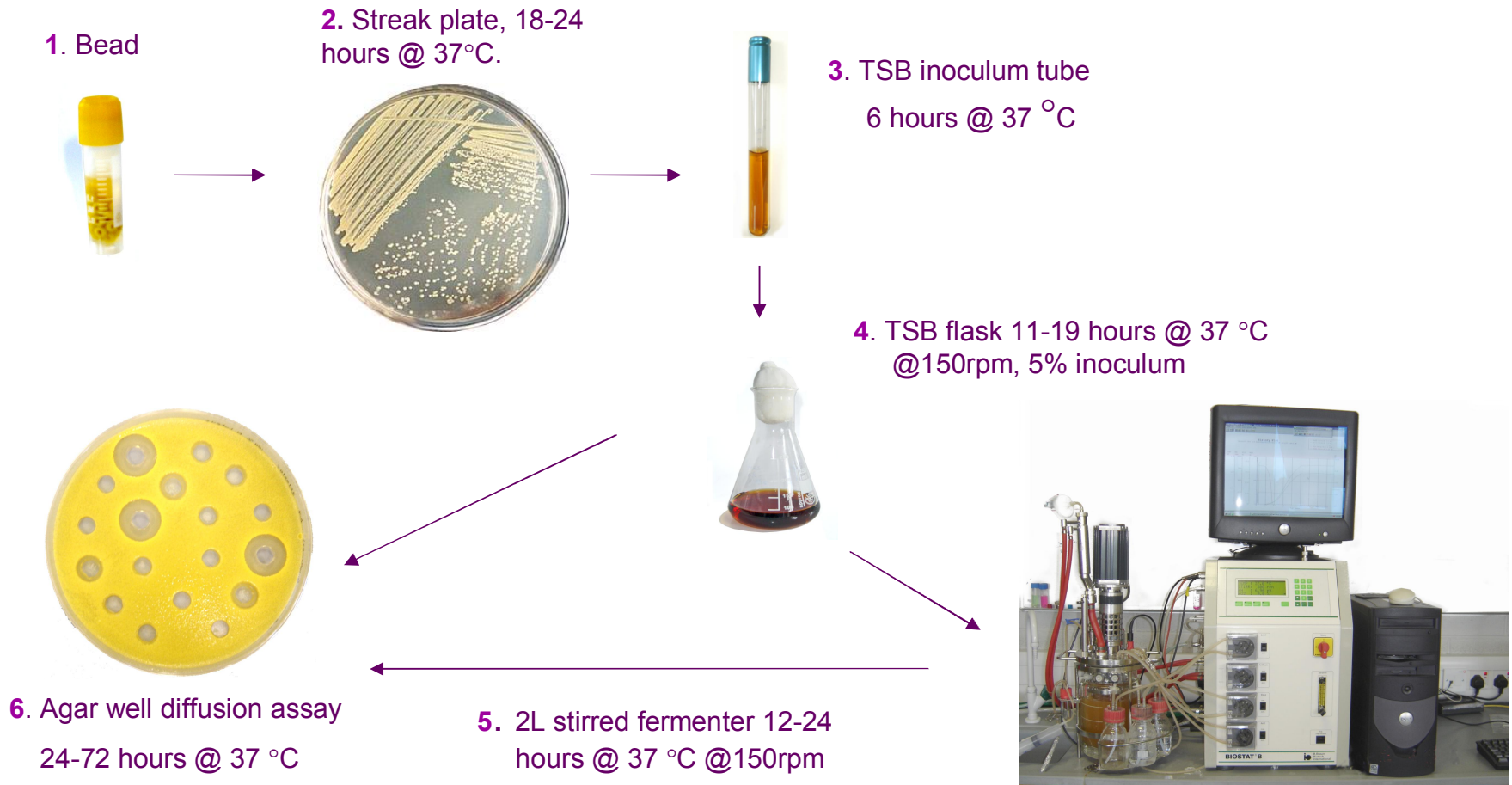
Applications of bacteriocins

- Food Preservation - Nisin
- Probiotics
- Clinical applications
 - Alternative to antibiotic
 - Treatment of antibiotic resistant bacteria
- Veterinary applications
 - Bovine mastitis

Objectives

- Production of antimicrobial activity
- Purification of the bacteriocin using a series of chromatographic techniques.
- Biochemical & Molecular characterization of the bacteriocin

Production of bacteriocin



Antimicrobial activity production by *S. aureus* isolate

Overview of Purification

Production by microbial fermentation



Filtration (Crossflow, 0.2µm)



Concentration (Tangential flow Filtration, (TFF) 10kDa)



Cation exchange chromatography (CIEC)

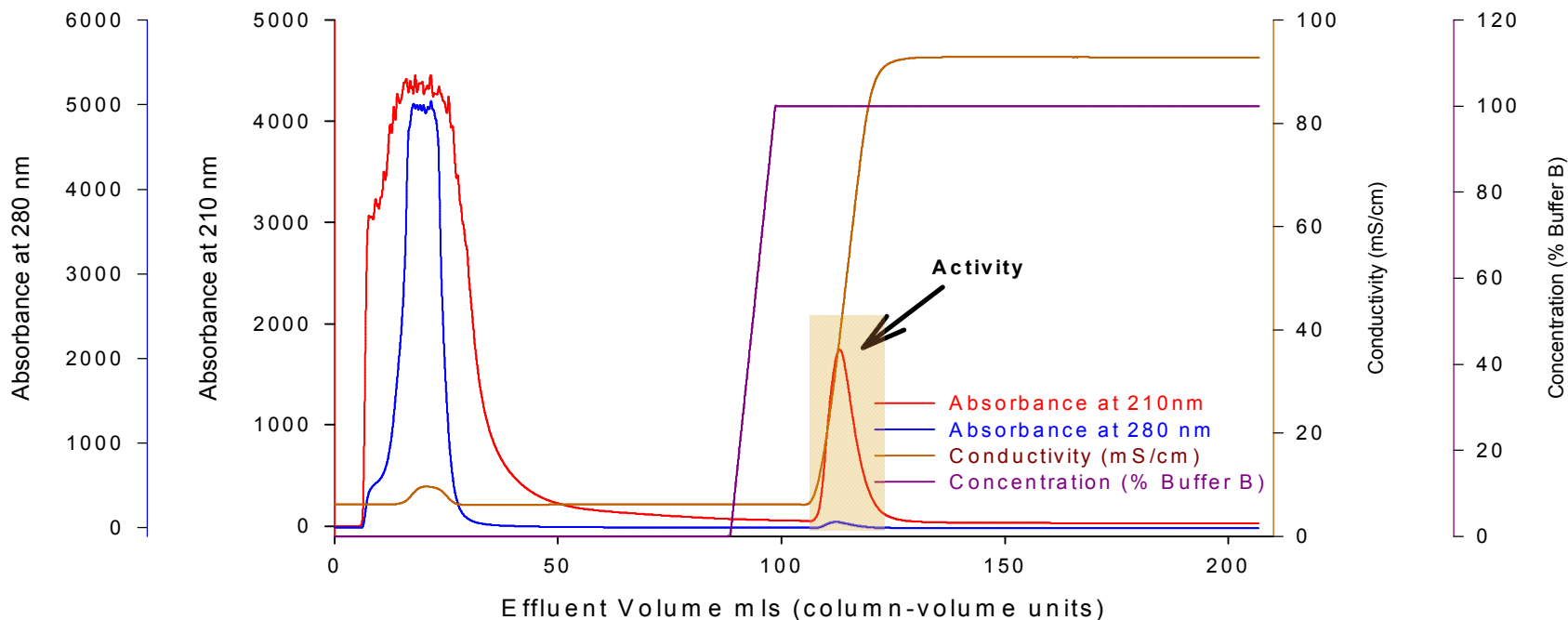


Reverse Phase Chromatography (RPC)



Size Exclusion Chromatography (SEC)

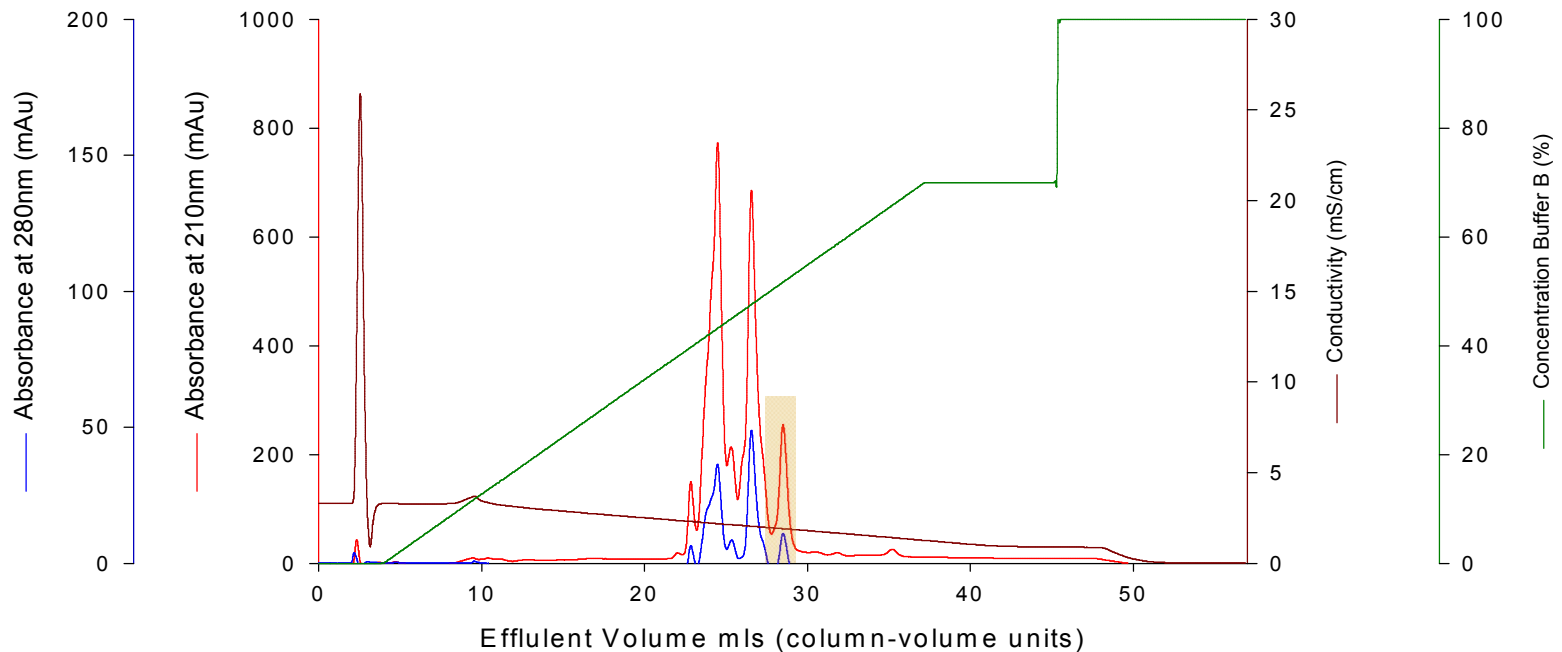
Cation Exchange



Cation Exchange Chromatography

5ml of the buffer exchange sample was applied to the Hi Prep 16/10 SP FF cation exchange column. A sharp linear gradient from 0 (Buffer A: 50mM Sodium Phosphate pH7) to 100% buffer B (50mM Sodium Phosphate pH 7 + 1M NaCl) over one C V. 1.5ml fractions were collected and absorbance was monitored at 210 and 280nm. Antimicrobial activity was analysed by the agar plate diffusion assay. Chromatography was performed on the Äkta Explorer™

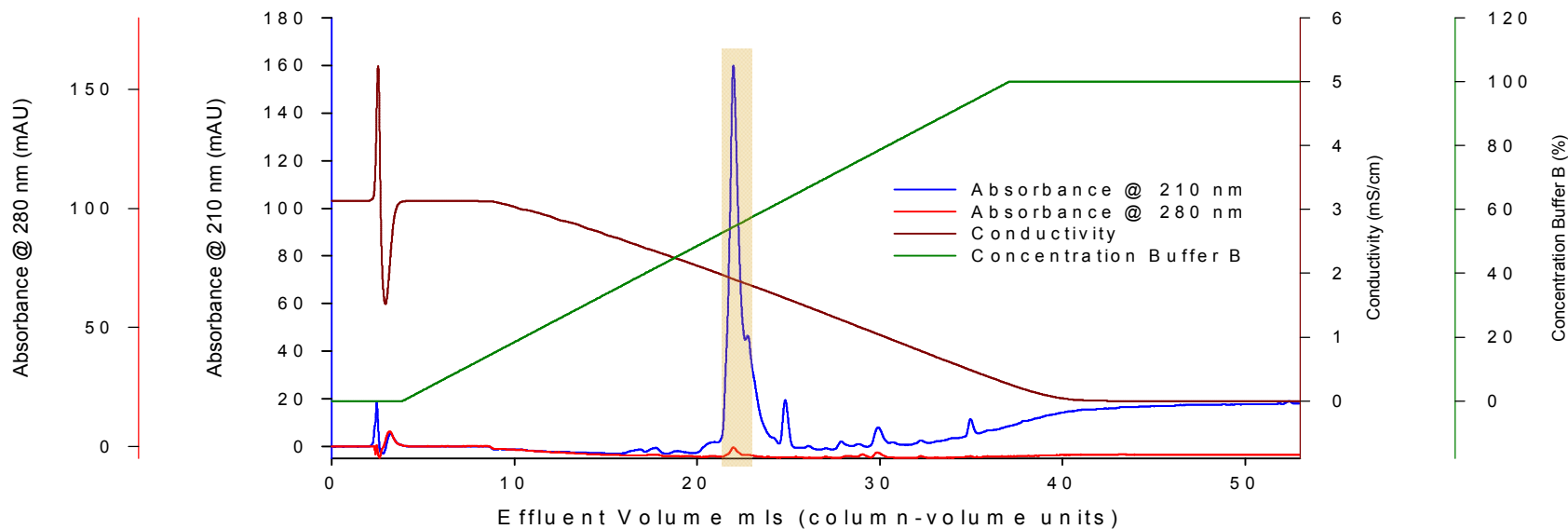
Reverse Phase Chromatography



Reverse Phase Chromatography

500µl of concentrated cation exchange fractions was applied to a Source 15RPC ST 4.6/100 reverse phase column. A linear gradient from 0.065 % TFA in H₂O to 70% Buffer B (0.05% TFA in Acetonitrile) over 20 column volumes followed by an 8ml delay before adjusting to 100% buffer B. 1.5ml fractions were collected and absorbance was monitored at 210 and 280 nm. Chromatography was performed on Äkta Explorer™ FPLC system.

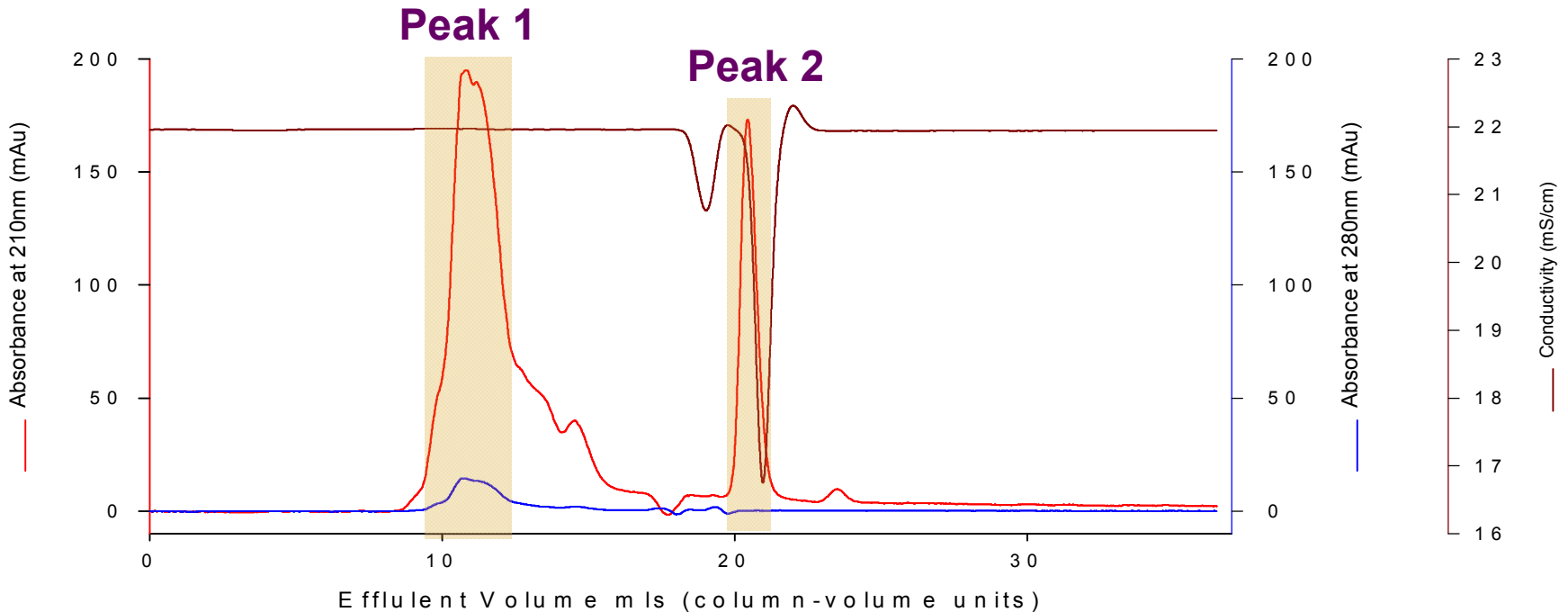
Micro Reverse Phase



Micro Reverse Phase Chromatography

200 μ l of concentrate reverse phase samples were applied to the micro reverse phase source C18 4.6/100ST. A linear gradient from 0% Buffer A (0.065% TFA in H₂O) to 100% Buffer B (0.05% TFA in ACN) over 20 column volumes. 1.5ml fractions were collected and absorbance was monitored at 210nm. Antimicrobial activity was analysed using the agar plate diffusion assay. Chromatography was preformed on Äkta Explorer™ FPLC system.

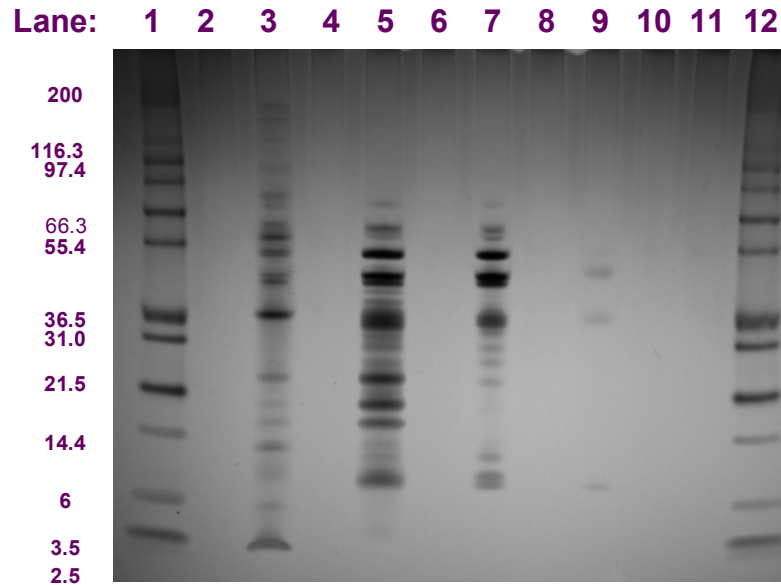
Size Exclusion



Gel Filtration Chromatography

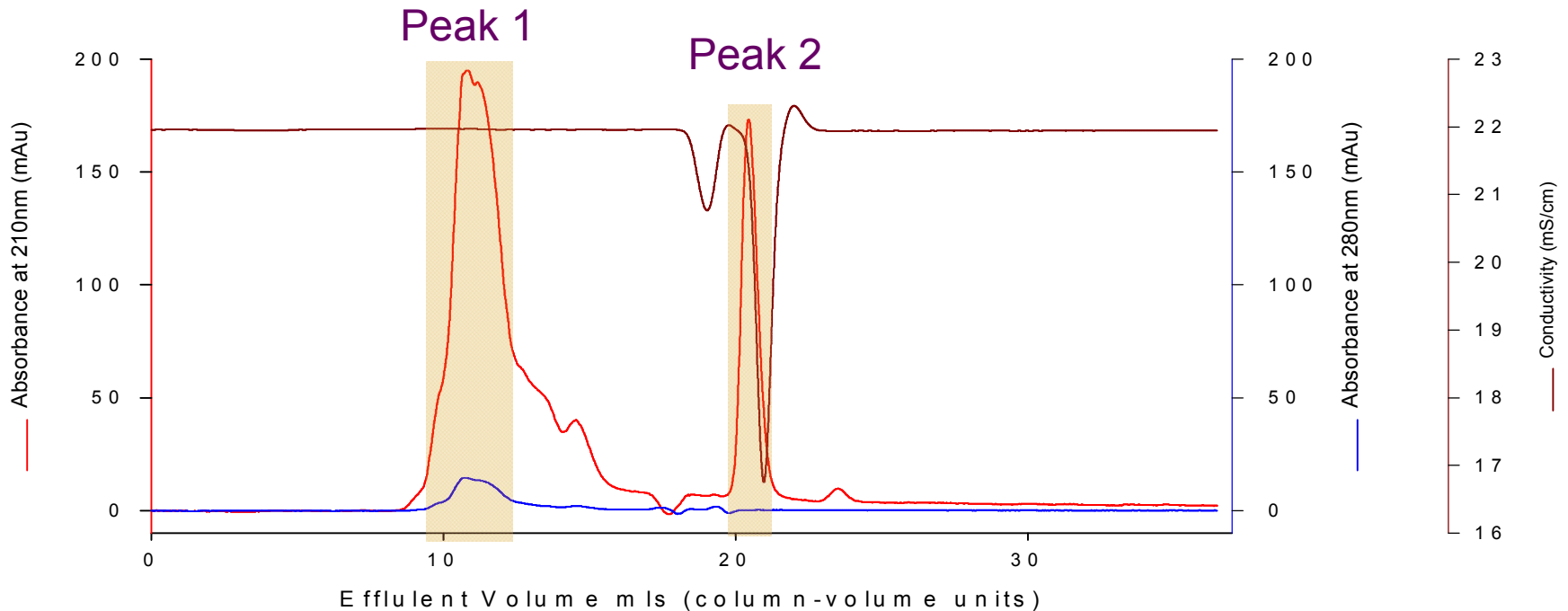
500ul of concentrate reverse phase samples were applied to the Superdex 75 10/300 GL column. An isocratic gradient for 1.5 column volumes, Buffer 50mM Sodium Phosphate pH7. 1.5ml fractions were collected and absorbance was monitored at 210 and 280 nm. Antimicrobial activity was analysed using the agar plate diffusion assay. Chromatography was performed on Äkta Explorer™ FPLC system.

SDS PAGE analysis of purification



Lane 1 & 12: Invitrogen Mark 12 molecular weight marker,
Lane 3: CFS, Lane 5: cation exchange,
Lane 7: reverse phase,
Lane 9: Size Exclusion Peak 1
Lane 10: Size Exclusion Peak 2

Mass Spectrometry Analysis Gel Filtration



Gel Filtration Chromatography

500ul of concentrate reverse phase samples were applied to the Superdex 75 10/300 GL column. An isocratic gradient for 1.5 column volumes, Buffer 50mM Sodium Phosphate pH7. 1.5ml fractions were collected and absorbance was monitored at 210 and 280 nm. Antimicrobial activity was analysed using the agar plate diffusion assay. Chromatography was performed on Äkta Explorer™ FPLC system.

Mass Spectrometry Analysis

Size Exclusion **Peak 1**- 5 proteins were identified.

- gi|81859498|sp|Q5HCM7|LIP1_STAAC
Lipase 1 precursor
- gi|81649764|sp|Q6GBB1|LTAS_STAAS
Glycerol phosphate lipoteichoic acid synthase
- gi|126333|sp|P10335|LIP2_STAAU
Lipase 2 precursor
- gi|81774927|sp|Q931U5|ATL_STAAM
Bifunctional autolysin precursor
- gi|8181932|sp|Q5HKP6|LIP_STAEQ
Lipase Precursor

Mass Spectrometry Analysis

1 MAKKFNYKLP SMVALTLVGS AVTAHQVQAA ETTQDQTTNK NVLDSNKVKA
 51 TTEQAKAEVK NPTQNISGTQ VYQDPAIVQP KTANNKTGNA QVSQKVDTAQ
 101 VNGDTRANQS ATTNNTQPVA KSTSTTAPKT NTNVTNAGYS LVDDDDNSE
 151 NQINPELIKS AAKPAALETQ YKAAAPKAAAT TSAPKAKTEA TPKVTTFSAS
 201 AQP RSVAATP KTS LPKYK PQ VNSSINDYIR KNMLKAPKIE EDYTSYFPKY
 251 AYRNGVGRPE GIVVHDTAND RSTINGEISY MKNNYQNAFV HAFVDGDRII
 301 ETAPT DYLSW GVGAVGNPRF INVEIVHTHD YASFARSMNN YADYAATQLQ
 351 YYGLKPDSAE YDNGTGVWTH YAVSKYLG GT DHADPHGYLR SHNYSYDQLY
 401 DLIN EKYL I K MGKVAPWGTQ STTPTTTPSK PTTSPKPS TG KLTVAANNV
 451 AQIKPTNSGL YTTVYDKTGK ATNEVQKTF A VSKTATLGNQ KFYLVQDYN S
 501 GNKFGWVKEG DVVYNTAKSP VNVNQSYSIK PGTKLYTVPW GTSKQVAGSV
 551 SSGNQTFKA SKQQIDKSI YLYGSVNGKS GWSKAYLVD TAKPTPTPTP
 601 KPSTPTTNK LTVSSLNGVA QINAKNGLF TTVYDKTGKP TKEVQKTF AV
 651 TKEASLGGNK FYLVKDYNSP TLIGWVKQGD VIYNNAKSPV NVMQTYTVKP
 701 GTKLYSVPWG TYKQEAGAVS GTGNQTFKAT KQQQIDKSIY LFGTVNGKSG
 751 WSKAYLAVP AAPKKAVAQP KTAVKAYTVT KPQTQT VSK IAQVKPNNTG
 801 IRASVYEKTA KNGAKYADRT FYVTKERAHG NETYVLLNNT SHNIPLGWFN
 851 VKDLNVQNLG KEVKTTQKYT VNKSNNGLSM VPWGTKNQVI LTGN NIAQGT
 901 FNATKQVSVG KDVYLYGTIN NRTGWVNAKD LTAPTAVKPT TSAAKDNYT
 951 YVIKNGNGYY YVTPNSDTAK YSLKAFNEQP FAVVKEQVIN GQTWYYGKLS
 1001 NGKLAWIKST DLAKELIKYN QTGMTLNQVA QIQAGLOQYP QVQRVPGKWT
 1051 DANFNDVKHA MDTKRLAQDP ALKYQFLRLD QPQNISIDKI NQFLKGGKVL
 1101 ENQGA AFNKA AQMYGINEVY LISHALLETG NGTSQLAKGA DVVNNKVVTN
 1151 SNTKYHNVFG IAAVDNDPLR EGIKYAKQAG WDTVSKAIVG GAKFIGNSYV
 1201 KAGQNTLYKM RWNPAHPGTH QYATDIDWAN INAKI IKGY DKIGEVGKYE
 1251 DIPQYE

Size Exclusion Peak 2 – single protein identified
gi|21204103 - autolysin

Antimicrobial Spectrum

Test Organism	CFS Antimicrobial Activity (AU/ml)	RPC Antimicrobial Activity (AU/ml)
<i>K. rhizophilia</i> (ATCC 9341)	640	320
<i>S. xylosus</i> (ATCC 29971)	5120	640
<i>E. coli</i> (ATCC 4157)	160	20
<i>E. coli</i> (ATCC 10536)	160	20
<i>E. aerogenes</i> (ATCC 13408)	20	20
<i>B. cerus</i> (ATCC 10876)	20	20
Cystic Fibrosis Pathogens		
<i>B. cenocepacia</i> (J2315)	5120	640
<i>P. apista</i> (LMG 16407)	320	160
<i>P. pnomenusa</i> (LMG 18087)	80	40
<i>B. cenocepacia</i> (LMG 18830)	80	40
<i>P. pulmonicola</i> (LMG 18106)	40	20

Clinical Antimicrobial Spectrum

Test Organism	CFS Antimicrobial Activity (AU/ml)	RPC Antimicrobial Activity (AU/ml)
<i>Pseudomonas spp</i>	100	50
<i>Achromobacter spp</i>	400	400
<i>Stenotrophomonas spp</i>	200	100
<i>A. haemolyticus</i>	100	50
<i>E. Coli</i>		
<i>E. Coli</i>	200	50
Resistant <i>E. coli</i>	200	100
Resistant <i>E. coli</i>	200	50
ESBL <i>E. coli</i>	200	100

Bacteriocin Stability

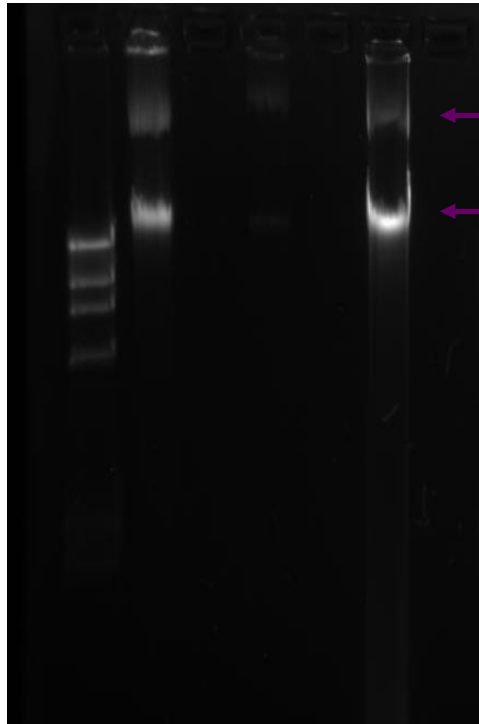
- Stability was examined over a range of both temperature and pH.
- Crude CFS was stable up to 55°C for 24hr
- 70% pure RPC was stable up to 37°C for 24hr

- Crude CFS was stable at pH 6-10 for 24hr
- 70% pure RPC was stable at pH 2 for 24hr.

Plasmid curing resulted in loss antimicrobial activity

Lane: 1 2 3 4 5 6

23,130
9,416
6,557
4,361



Open Circular form
of plasmid DNA

Supercoiled form
of plasmid DNA



Curing of Plasmid results in
loss of Antimicrobial Activity

Lane 1: Lamda DNA Hind III digest

Lane 2: ITT 37, Lane 4: Cured ITT 37

Lane 6: Plasmid PSK 1, Lane 3 & 5: Blank

Summary

- Wide spectrum on antimicrobial activity against both Gram positive and Gram negative bacteria including *Burkholderia cenocepacia* J2315 and ESBL *E. coli*
- Purification scheme - cation exchange, reverse phase and size exclusion chromatography
- Micro reverse phase analysis - 70% pure after the reverse phase chromatography step
- Mass spectrometry analysis of the size exclusion peak 2 identified as a porin protein as gi|21204103 autolysin
- Retains its activity over a wide range of temperatures & pHs
- Antimicrobial activity of the *S. aureus* isolate is linked to a large plasmid (>23 kb)

Future Studies

- Southern blot analysis
- Chemical synthesis of the peptide

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- Biology Technicians

Thank you for Listening!

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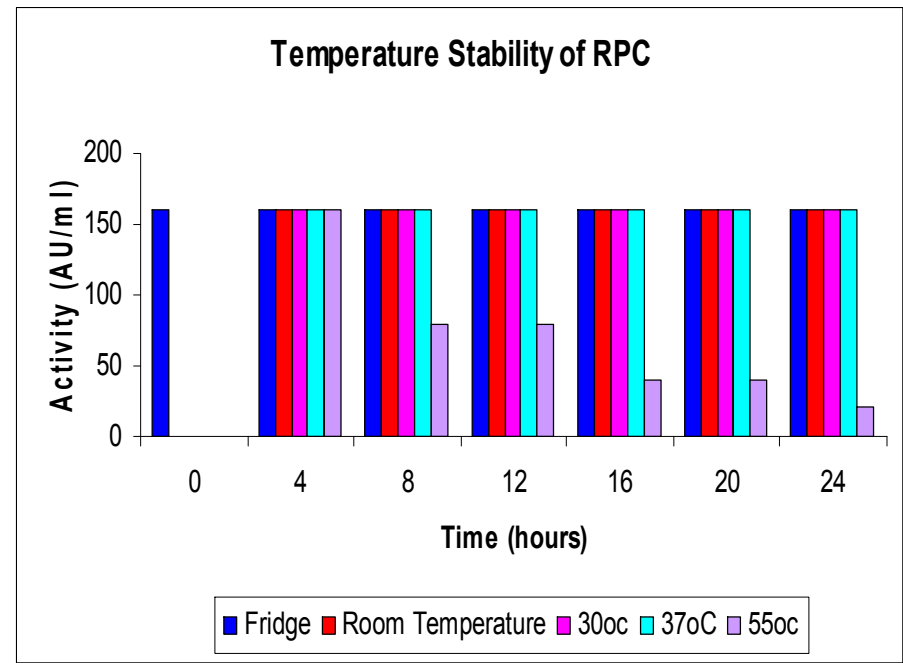
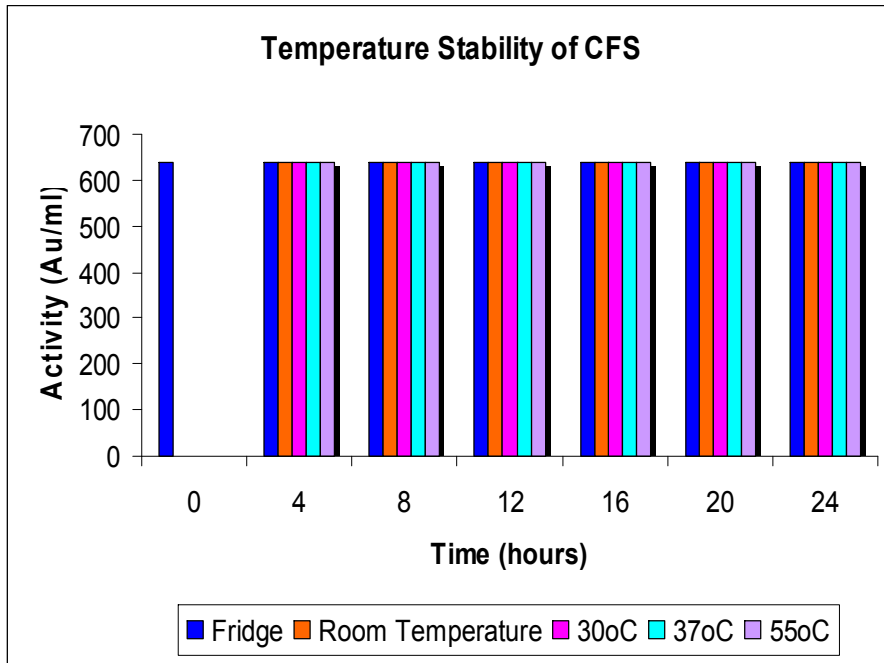


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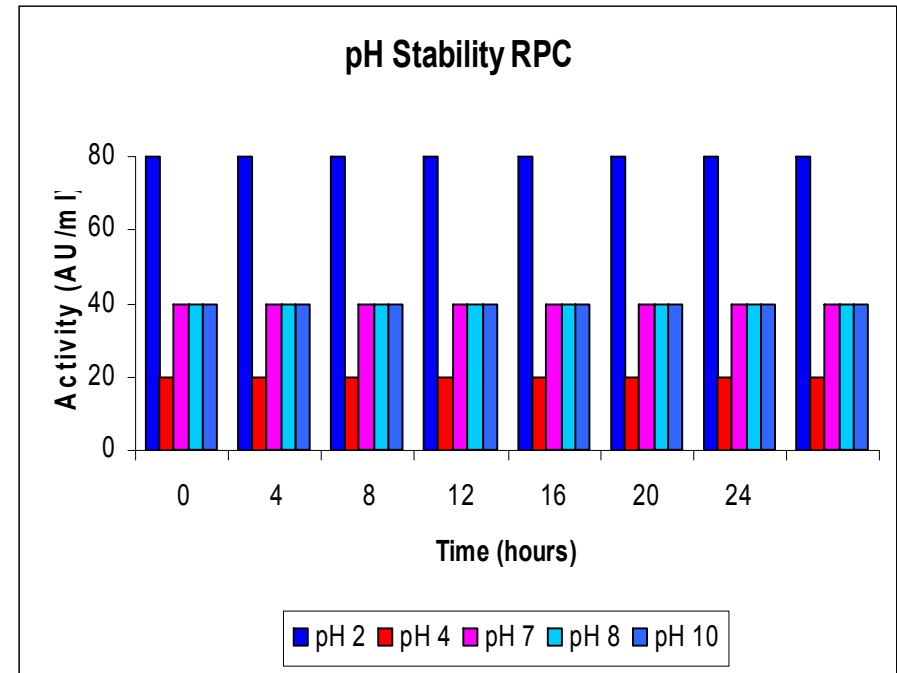
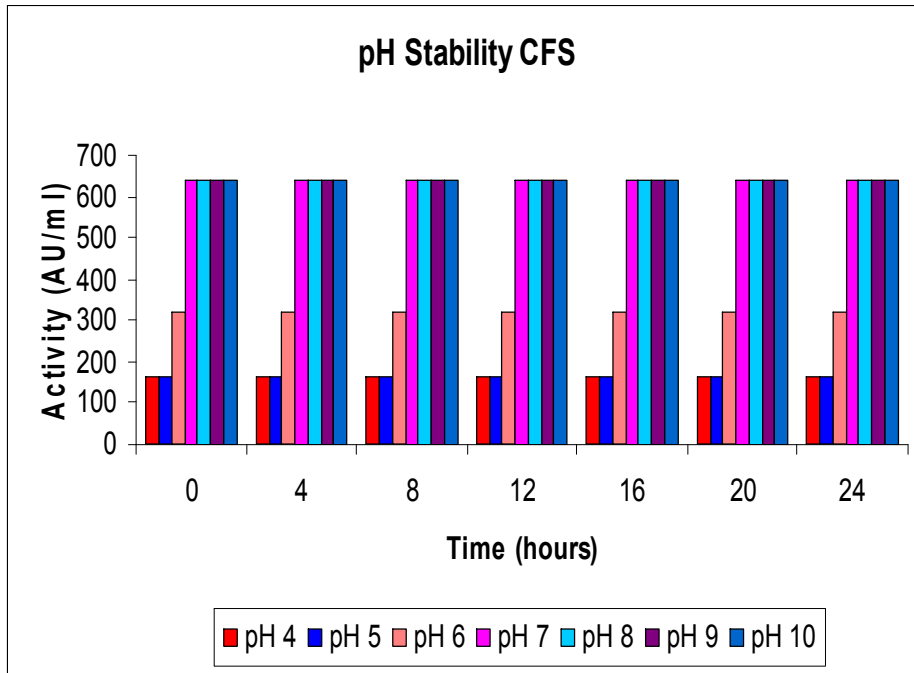
Temperature Stability



Temperature Stability of crude CFS and semi-pure Reverse phase (RPC)

A sample of CFS and pooled reverse phase sample were incubated at 4°C, room temperature, 30°C, 37°C and 50°C. An sample was taken every 2 hours for 24 hours and examined for antimicrobial activity using the plate diffusion assay.

pH Stability



pH Stability study of crude CFS and semi-pure RPC

The pH of CFS altered to 4, 5, 6, 7, 8, 9 and 10. The pH of the pooled RPC sample was altered to pH 2, 4, 7, 8, 10.. The samples were then filtered (and stored at 2 - 8°C. Samples were assayed every two hours for 24 hours for activity using the plate diffusion assay.