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Broad-spectrum antimicrobial activity of secondary metabolites produced by *Serratia marcescens* strains

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ABSTRACT

The genus *Serratia* is a predominantly unexplored source of antimicrobial secondary metabolites. The aim of the current study was thus to isolate and evaluate the antimicrobial properties of biosurfactants produced by *Serratia* species. Forty-nine (n = 34 pigmented; n = 15 non-pigmented) biosurfactant producing *Serratia* strains were isolated from environmental sources and selected isolates (n = 11 pigmented; n = 11 non-pigmented) were identified as *Serratia marcescens* using molecular typing. The *swrW* gene (serrawettin W1 synthetase) was detected in all the screened pigmented strains and one non-pigmented strain and primers were designed for the detection of the *swrA* gene (non-ribosomal serrawettin W2 synthetase), which was detected in nine non-pigmented strains. Crude extracts obtained from *S. marcescens* P1, NP1 and NP2 were chemically characterised using ultra-performance liquid chromatography coupled to electrospray ionisation mass spectrometry (UPLC-ESI-MS), which revealed that P1 produced serrawettin W1 homologues and prodigiosin, while NP1 produced serrawettin W1 homologues and glucosamine derivative A. In contrast, serrawettin W2 analogues were predominantly identified in the crude extract obtained from *S. marcescens* NP2. Both P1 and NP1 crude extracts displayed broad-spectrum antimicrobial activity against clinical, food and environmental pathogens, such as multidrug-resistant *Pseudomonas aeruginosa,* methicillin-resistant *Staphylococcus aureus* and *Cryptococcus neoformans*. In contrast, the NP2 crude extract displayed antibacterial activity against a limited range of pathogenic and opportunistic pathogens. The serrawettin W1 homologues, in combination with prodigiosin and glucosamine derivatives, produced by pigmented and non-pigmented *S. marcescens* strains, could thus potentially be employed as broad-spectrum therapeutic agents against multidrug-resistant bacterial and fungal pathogens.

1. Introduction

The rapid spread and emergence of multidrug- (MDR) and extensive drug-resistant (XDR) bacteria and fungi is considered one of the major threats to global public health in the 21st century (Colombo et al., 2017; McCarthy et al., 2017). This has resulted in organisations, such as the World Health Organisation (WHO) and the Centres for Disease Control and Prevention (CDC), implementing vital strategies to combat the misappropriation of current antibiotic therapies and prioritise the research and development of alternative antimicrobial compounds (Centres for Disease Control and Prevention (CDC, 2013; World Health Organization (WHO, 2015). In this regard, several bacterial species have been reported to produce bioactive secondary metabolites, which are considered promising alternatives to antibiotics (Bérdy, 2005). Moreover, *Serratia* spp. represent a relatively unexploited source of valuable secondary metabolites with potential activity against MDR and XDR pathogens.

The *Serratia* genus consists of 18 species that have been isolated from various environmental sources, such as water and marine environments, contaminated soil, plants, animals or hospitalised patients (Grimont and Grimont, 2006; Su et al., 2016). The ubiquitous nature of this genus is due to the synthesis of numerous extracellular products, including exoenzymes, nucleases and secondary metabolites that aid in the adaption of *Serratia* to harsh environmental conditions (Harris et al., 2004). One such secondary metabolite includes a non-diffusible red pigment, identified as prodigiosin. Prodigiosin is produced by certain strains of *Serratia marcescens* (*S. marcescens*), *Serratia rubidaea* (*S. rubidaea*) and *Serratia surfactantfaciens* (*S. surfactantfaciens*), amongst others (Grimont and Grimont, 2006; Su et al., 2016) and displays antibacterial, antifungal, antiprotozoal, antitumor and immunosuppressant activities (Stankovic et al., 2014). *Serratia* spp. are capable of synthesising additional bioactive secondary metabolites, such as biosurfactants (serrawettins, stephensiolides, rubiwettins and rhamnolipids), althiomycin and bacteriocins (Clements et al., 2019a).

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Two species within the *Serratia* genus, namely *S. marcescens* and *S. surfactantfaciens*, have been reported to produce the lipopeptide biosurfactant class known as serrawettins (Matsuyama et al., 1985, 1990; Lindum et al., 1998; Su et al., 2016). Three molecular species of serrawettins have been identified thus far, including serrawettin W1 (also known as serratamolide A), serrawettin W2 and serrawettin W3 (Matsuyama et al., 1985). The general structure of serrawettin W1 includes a symmetric dilactone structure composed of two L-serine amino acids linked to two β-hydroxy fatty acids (comprised of 3-hydroxydecanoic acids) (Eckelmann et al., 2018). Numerous homologues of serrawettin W1 (serratamolide A) have also been detected, namely serratamolide B to G, which differ based on the variation in the length of the fatty acid chain (C_8 to C_{14}) and the presence of a double bond (Dwivedi et al., 2008; Zhu et al., 2018). The open reading frame (ORF) responsible for the biosynthesis of serrawettin W1 and homologues of this compound was identified as *swrW* and encodes for serrawettin W1 synthetase (Li et al., 2005; Thies et al., 2014).

The general structure of serrawettin W2 includes five amino acids (D-leucine/isoleucine-L-serine-L-threonine-D-phenylalanine-L-isoleucine/leucine) linked to a β-hydroxy fatty acid moiety (Matsuyama et al., 1992; Motley et al., 2016). Analogues of serrawettin W2 have been detected and differ based on the variation at the first, fourth or fifth amino acid positions or the length of the fatty acid chain (C_8 or C_{10}) (Motley et al., 2016; Su et al., 2016). The open reading frame (ORF) responsible for the biosynthesis of serrawettin W2, and analogues of this compound, was identified as *swrA*, encoding for non-ribosomal serrawettin W2 synthetase (Su et al., 2016). While the full chemical composition of serrawettin W3 has yet to be determined, the cyclodepsipeptide was found to be composed of a fatty acid (one dodecanoic acid) and five amino acids, including threonine, serine, valine, leucine and isoleucine (Matsuyama et al., 1986, 2011).

The most prominent properties displayed by serrawettin W1 and W2 include emulsification activity, surface activity, antitumor activity, antibacterial activity and antifungal activity (Clements et al., 2019a). Kadouri and Shanks (2013) investigated the antimicrobial activity of a serrawettin W1, produced by a *S. marcescens* strain, and found that this compound exhibited activity against primarily Gram-positive bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA). Similarly, serrawettin W2 has been shown to exhibit activity against Gram-positive bacteria, such as *S. aureus* and *Micrococcus* spp., whilst also exhibiting activity against a few Gram-negative bacteria, such as *Pseudomonas* and *Shigella* spp. (Su et al., 2016). Thus, while studies have indicated that serrawettins are effective against several Gram-positive bacteria, limited research has been conducted on the broad-spectrum antibacterial and antifungal activity of the serrawettins and their effectiveness against MDR and XDR Gram-negative bacteria. Accordingly, the primary aim of this study was to screen various environmental sources for *Serratia* isolates capable of biosurfactant production. A second aim was to chemically characterise the crude extracts produced by selected *Serratia* strains and assess the broad-spectrum antimicrobial activity of the extracts against pathogenic and opportunistic bacterial and fungal strains, including MDR and XDR clinical isolates. To the best of the author's knowledge, this is one of the first studies investigating the broad-spectrum antimicrobial potential of chemically characterised crude lipopeptide extracts obtained from *Serratia* spp. and exploring the activity of these compounds against MDR and XDR Gram-negative bacteria and fungi.

2. Materials and methods

2.1. Sampling sites

Various environmental sources were selected as the sampling sites for this study, including three municipal wastewater treatment plants (WWTPs) with samples collected from the influent point, aeration tanks, settling tanks and effluent points ($n = 12$). In addition, olive oil ($n = 4$) and wine ($n = 4$) effluent samples were collected at the points following the washing or crushing of olives and two wine grapes varietals, respectively. Samples ($n = 7$) were also collected from the inlet point, four different compartments of a bioreactor, sludge from one of the bioreactor compartments and an outlet point of an oil refinery treatment plant. Three river water samples (Plankenbrug River, Eerste River and Krom River) were collected within the Stellenbosch area and a sample of harvested rainwater was collected from a first flush diverter attached to the rooftop at Welgevallen experimental farm. Thus, 31 samples were collected in 1 L sterile schott bottles and samples were processed within 6 h of collection.

2.2. Isolation of biosurfactant producing Serratia species

In order to isolate *Serratia* spp. from each environmental sample, a serial dilution (10^{-1} to 10^{-4}) in 0.85% saline was prepared. Thereafter, 100 µL of the undiluted and each dilution was spread plated onto Caprylate-Thallous (CT) agar (Grimont and Grimont, 2006) in duplicate. The plates were incubated at 30 °C for 24–48 h. Following incubation, morphologically distinct colonies were selected and re-streaked onto nutrient agar (NA) (Merck, Biolab Diagnostic, South Africa) to obtain pure cultures. The isolates obtained were assigned a code identifier, which denoted the pigmentation of the isolate [P – pigmented (red); NP – non-pigmented] and each isolate was numbered.

2.3. Growth conditions and media composition for biosurfactant production

Purified pigmented and non-pigmented isolates were inoculated into 5 mL Peptone Glycerol (PG, pH 7.2 ± 0.2) broth composed of 5 g Bactopeptone (Merck) and 10 mL glycerol (Promega, Wisconsin, United States) in 1 L distilled water (Matsuyama et al., 1985). For the oil spreading method, the test tube broth cultures were incubated aerobically on a test tube rotator (MRCLAB, London, UK) at 30 °C for 48–96 h. For emulsification, surface tension and antimicrobial analysis, a seed culture of each biosurfactant producing strain was prepared by inoculation of a single colony into 5 mL of Luria Bertani (LB) broth (Merck) which was incubated at 30 °C for 18–24 h. Each seed culture was subsequently inoculated into a 500 mL baffled flask containing 100 mL PG broth, which was incubated on an orbital shaker (MRCLAB) at 30 °C for 120 h at 120 rpm. After incubation, the broth culture was centrifuged at 10 000 rpm for 20 min at 4 °C to obtain the cell free supernatant.

2.4. Screening for biosurfactant production: oil spreading method

The oil spreading method was used to screen the cell free supernatant obtained from the broth cultures of single bacterial colonies ($n = 596$) for the presence of biosurfactants as previously described by Youssef et al. (2004). The oil spreading analysis for all samples was conducted in duplicate.

2.5. Physico-chemical characterisation

2.5.1. Emulsification capacity assays

Based on the results obtained from the oil spreading method, the cell free supernatant from selected biosurfactant producing strains ($n = 22$) were subjected to emulsification capacity assays, as outlined by Ndlovu et al. (2016). The emulsification index (E_{24}) of the cell free supernatant obtained from each bacterial isolate was determined by adding 2 mL of the supernatant to an equal volume of diesel, kerosene, sunflower or mineral oil. Thereafter, the solution was vortexed for approximately 5 min. For all samples, the emulsification capacity assays were conducted in duplicate. The solution was left at room temperature for 24 h and the E_{24} for each substrate was measured and calculated using Eq. (1):

Table 1
The primer sequences and PCR cycling parameters used for the detection of genes encoding for the biosynthesis of serrawettin W1 and W2.

Biosurfactant type	Gene	Primer name	Primer (5' – 3')	Cycling parameter	Size (Bp)	Reference
Serrawettin W1	*swrW*	SW2-F3	GCGACAAAAGCAATGACAAA	94 °C for 5 min; 30 cycles: 94 °C for 45 s; 55 °C for 45 s; 72 °C for 3 min; 72 °C for 10 min.	915 to 975 bp	Apao et al. (2012)
		SW2-R3	GTCGGCGTATTGTTCCAACT			
Serrawettin W2	*swrA*	SRA-F	ACTTCAGCAGCCAGGAATAC		398 bp	This study
		SRA-R	GGACGAATAAGGGACGAGTTT			

$$\text{Emulsification index } (E_{24})\% = \frac{\text{Height of the emulsion layer}}{\text{Total height of the solution}} \text{x } 100 \quad (1)$$

2.5.2. Surface tension measurements

The surface tension of the cell free supernatant was measured using a Du Nouy ring tensiometer, as previously outlined by Youssef et al. (2004). The surface tension of the biosurfactant compounds present in the cell free supernatant of each bacterial isolate was measured at room temperature. Prior to surface tension measurements of each cell free supernatant sample and sterile PG broth, calibration was performed using distilled water to ensure validity of the measurements. All samples were measured in triplicate and an average value was recorded as the surface tension of the sample.

2.6. Molecular identification of biosurfactant producing Serratia spp

The extraction of genomic deoxyribonucleic acid (DNA) was performed for the identification of the biosurfactant producing bacterial strains ($n = 22$) using the High Pure Polymerase Chain Reaction (PCR) Template Preparation Kit (Roche Diagnostics, Risch-Rotkreuz, Switzerland) as per the manufacturer's instructions. Deoxyribonucleic acid samples were stored at -20 °C until utilised for PCR analysis.

The biosurfactant producing isolates ($n = 22$) were subjected to PCR amplification of the *pfs* gene using *Serratia* specific primers Fpfs1 (5′ CCGGCATCGGCAAAGTCT 3′) and Rpfs2 (5′ ATCTGGCCCGGCTCG TAGCC 3′) (Zhu et al., 2008). The *pfs* gene encodes for an S-adenosylhomocysteine nucleosidase enzyme and is involved in quorum-sensing within *Serratia* spp. (Zhu et al., 2008). The reaction mixture consisted of 1X Green GoTaq® Flexi buffer (Promega), 2.0 mM $MgCl_2$ (Promega), 0.1 mM of each dNTP (Thermo Fisher Scientific, Waltham, Massachusetts, United States), 0.3 µM of each primer, 1.5 U of GoTaq® G2 DNA polymerase (Promega) and 2 µL of template DNA, which was made up to a final volume of 25 µL using sterile nuclease-free water. Amplification was performed using the T100™ thermal cycler (Bio-Rad Laboratories, Netherlands) and the PCR cycling parameters consisted of initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 45 s, 55 °C for 30 s, and 72 °C for 15 s, and then a single final extension step of 72 °C for 5 min. Sterile nuclease free water was used as a negative control, while genomic DNA extracted from *S. marcescens* American Type Culture Collection (ATCC) 13880 was used as a positive control.

The 193 bp PCR products were electrophoresed on a 1.0% agarose gel stained with ethidium bromide (0.5 µg/mL) in 1X tris/acetate/ethylenediaminetetraacetic acid (EDTA) (TAE) buffer and were visualised through UV illumination, with the images captured using the MiniBIS Pro (Bio-Imaging Systems, California, USA). All the PCR products ($n = 22$) were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) as per manufacturer's instructions and were sequenced in accordance with the BigDye Terminator Version 3.1 Sequencing Kit (Applied Biosystems, USA) at the Central Analytical Facility (CAF), Stellenbosch University (Stellenbosch, South Africa). Chromatograms for each sequence were examined using the Finch TV Version 1.4.0 software and were identified using the National Centre for Biotechnological Information (NCBI) Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov).

2.7. Detection of genes encoding for the biosynthesis of serrawettins

A primer set was designed for the detection of the *swrA* gene that encodes for non-ribosomal serrawettin W2 synthetase. The two available gene sequences for *swrA* were obtained from the Genbank (http://www3.ncbi.nlm.nih.gov) database (Accession numbers: JX667980.1; AF039572.1) and were aligned using CLC Main Workbench 7.6.2 software (CLC Bio, Aarhus, Denmark) to obtain the consensus sequence. Consensus regions were used to design specific primers using the IDT PrimerQuest Tool software (https://eu.idtdna.com/PrimerQuest/Home/Index). The primers were further analysed in IDT OligoAnalyzer 3.1 (https://eu.idtdna.com/calc/analyzer) and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure the primer sequences are able to detect the available genes.

Hereafter, the DNA extracted from all biosurfactant producing isolates ($n = 22$) was subjected to PCR amplification using primers to detect the *swrW* and *swrA* genes (Table 1), known to be involved in the biosynthesis of serrawettin W1 and serrawettin W2, respectively. The primer sequences and cycling parameters used to amplify each target gene are indicated in Table 1. For both the *swrW* and *swrA* genes, the reaction mixture consisted of 1X Green GoTaq® Flexi buffer (Promega), 2 mM $MgCl_2$ (Promega), 0.1 mM dNTP mix (Thermo Scientific), 0.1 µM of each primer, 1.5 U of GoTaq® G2 DNA Polymerase (Promega) and 2.5 µL of template DNA. All reaction mixtures were made up to a final volume of 25 µL with sterile nuclease-free water. The PCRs were performed using a T100™ thermal cycler (Bio-Rad Laboratories), with the cycling parameters outlined in Table 1.

The DNA extracted from *S. marcescens* ATCC 13880 (*swrW* gene) and a sequence verified gene of *swrA* were used as positive controls in the PCR assays, while sterile nuclease free water was used as a negative control. After PCR amplification, representative PCR products for *swrW* and *swrA* genes were purified, concentrated and sent for sequencing as described in the "Molecular identification of biosurfactant producing *Serratia* spp." section.

2.8. Extraction and partial purification of biosurfactant compounds

Based on the physico-chemical properties and molecular analysis, two pigmented (P1 and P4) and five non-pigmented strains (NP1 – NP5) were selected for preliminary antimicrobial testing (using the methodology outlined in the "Antimicrobial and haemolytic activity of the crude extracts" section; results not shown). Thereafter, the P1, NP1 and NP2 strains were selected for further chemical characterisation and broad-spectrum antimicrobial testing, as the crude extracts obtained from P1 and NP1 strains (containing the gene encoding for serrawettin W1) displayed the highest antimicrobial activity for the pigmented and non-pigmented strains analysed, respectively, while the NP2 strain (containing the serrawettin W2 gene) displayed the second highest antimicrobial activity for the non-pigmented strains. Therefore, the *S. marcescens* P1, NP1 and NP2 strains were selected for biosurfactant production, purification and partial chemical characterisation as previously outlined by Ndlovu et al. (2017). Briefly, the cell free supernatants of P1, NP1 and NP2 obtained in the "Growth conditions and media composition for biosurfactant production" section were lyophilised and dissolved in 70% (*v/v*) acetonitrile (Romil, Darmstadt, Germany). The acetonitrile soluble fraction was transferred into a sterile

McCartney bottle and lyophilised. This step was repeated three times to further purify the biosurfactant compounds. The weighed extracts were utilised for chemical characterisation and were stored at -20 °C until used for antimicrobial analysis.

2.9. Ultra-performance liquid chromatography coupled to mass spectrometry

The compounds present in the crude extracts produced by *S. marcescens* P1, NP1 and NP2 were analysed by the Liquid chromatography-mass spectrometry (LC–MS) unit at the CAF (Stellenbosch University). A Waters Quadrupole Time-of-Flight Synapt G2 (Waters Corporation, Miliford, USA) mass spectrometer was utilised for the electrospray ionisation mass spectrometry (ESI-MS) for direct mass analysis and was coupled to an Acquity ultra-performance liquid chromatography (UPLC) for the UPLC-ESI-MS analysis.

Briefly, 3 µL of the acetonitrile soluble extract obtained from P1, NP1 and NP2 strains were injected and separated on an UPLC C18 reverse-phase analytical column (Acquity UPLC® HSS T3, 1.8 µm particle size, 2.1 × 150 mm, Waters corporation, Dublin, Ireland) at a flow rate of 0.300 mL/min using a 0.1% formic acid (A) to acetonitrile (B) gradient (60% A from 0 to 0.5 min for loading, gradient was from 40 to 95% B from 0.5 to 11 min and then 95 to 40% B from 15 to 18 min) (Ndlovu et al., 2017). The analytes were subjected to a capillary voltage of 3 kV, cone voltage of 15 V and a source temperature of 120 °C. Data acquisition in the positive mode was performed by MS scanning a second analyser through the *m/z* range of 200–3000 daltons. The UPLC-ESI-MS profiles of the crude extracts were then compared to literature for the partial characterisation of the compounds produced by the selected strains. The data obtained was analysed using the Masslynx software version 4.1 (Waters Corporation).

2.10. Antimicrobial and haemolytic activity of the crude extracts

The crude extracts produced by the *S. marcescens* P1, NP1 and NP2 strains were subjected to broad-spectrum antimicrobial testing. The lyophilised P1, NP1 and NP2 crude extracts obtained in the "Extraction and partial purification of biosurfactant compounds" section were dissolved in 15% (*v/v*) methanol (Sigma Aldrich, United States) to obtain a 1.00 mg/mL (extract) concentration for antimicrobial disc susceptibility testing.

The antimicrobial activity of the crude extracts produced by the three strains were tested against various actively growing microorganisms available in the Water Resource Laboratory Culture Collection, including ATCC, environmental, and clinical bacterial and fungal strains. In addition, fungal strains isolated from surface water (Benadé et al., 2016) and clinical samples were obtained from the Environmental Biotechnology laboratory in the Department of Microbiology. All test microorganisms were inoculated into Mueller Hinton Broth (MHB, Merck) and were incubated at 37 °C for 18–24 h, with the exception of *Legionella* spp., *Enterococcus* spp. and *Listeria* spp. Following incubation, 100 µL of the bacterial suspension was spread plated onto Mueller Hinton agar (MHA, Merck) to create a microbial lawn. The *Legionella* spp. isolates were grown in Lennox broth [10 g/L Tryptone (Biolab, Merck), 5 g/L Yeast Extract (Biolab, Merck), 5 g/L sodium chloride (NaCl; Saarchem, Durban, South Africa)] supplemented with *Legionella* BCYE growth supplement [*N*-(2-acetamido)-2-aminoethanesulfonic acid buffer/potassium hydroxide (10.0 g/L), ferric pyrophosphate (0.25 g/L), alpha-ketoglutarate (1.0 g/L) and *L*cysteine HCl (0.4 g/L) (Oxoid, Basingstoke, United Kingdom)] and were incubated for 48 h at 30 °C. Following incubation, a 100 µL of each *Legionella* suspension was spread plated onto Buffered Charcoal Yeast Extract (BCYE) agar (Oxoid) supplemented with *Legionella* BCYE growth supplement (Oxoid) to create a bacterial lawn. The *Enterococcus* and *Listeria* isolates were grown in Tryptone Soy Broth (Merck) supplemented with 6 g/L yeast extract (Merck) ($TSBYE_{0.6\%}$) and were incubated at 37 °C for 18–24 h. Following incubation, 100 µL of each *Enterococcus* and *Listeria* suspension was spread plated onto Tryptone Soy agar (Merck) supplemented with 6 g/L yeast extract (Merck) ($TSAYE_{0.6\%}$) to create a bacterial lawn.

For all the isolates, 6 mm filter paper discs (Oxoid) were placed onto the respective lawns using a sterile needle and 50 µL of each crude extract (1.00 mg/mL) was pipetted directly onto the filter paper in order to create an antimicrobial disc. A negative control of 50 µL of 15% (*v/v*) methanol was included for each test strain. All tests were performed in triplicate. The MHA and $TSAYE_{0.6\%}$ plates were incubated for 24–48 h at 37 °C, while the BCYE plates were incubated for 48 h at 30 °C. Thereafter, the diameter of the zone of inhibition around the inoculated paper disc was measured (Ndlovu et al., 2017). The average of the triplicates and standard deviation of each crude extract was determined against the selected test microorganism.

The haemolytic activity of each crude extract was also assessed as described by Das et al. (2008). The P1, NP1 and NP2 crude extracts (20 µL of 1.00 mg/mL crude extract concentration) were spot plated onto sheep blood agar (Selecta-Media, Johannesburg, South Africa) plates in duplicate. In addition, a surfactin standard (20 µL of 1.00 mg/mL; Sigma Aldrich) was included as a positive control and 15% methanol was included as a negative control. The plates were incubated for 24 h at 30 °C and were analysed for the zone of haemolysis following overnight incubation.

3. Results

3.1. Screening of Serratia spp. for biosurfactant production

All the presumptive *Serratia* strains ($n = 569$) isolated from the various environmental samples were screened using the oil spreading method (preliminary screening for biosurfactant production), with oil displacement observed for 49 isolates ($n = 34$ pigmented; $n = 15$ non-pigmented; 8.6%). Of the 49 presumptive *Serratia* isolates capable of biosurfactant production, 85.7% were isolated from the WWTP samples, 12.2% were isolated from the river (Eerste River and Krom River) water samples and 2.1% were isolated from the oil refinery samples. However, no biosurfactant producing strains were isolated from the Plankenbrug River water sample, wine or olive oil effluent samples and the first flush diverter rainwater sample.

3.2. Physico-chemical characterisation

Based on the zone diameter of the dispersed oil (oil spreading method), 11 pigmented and 11 non-pigmented presumptive *Serratia* isolates were selected and analysed for their ability to emulsify diesel, kerosene, sunflower oil and mineral oil. The emulsification indices obtained for the cell free supernatant of the 22 isolates are indicated in Table 2. The overall E_{24} for the pigmented *Serratia* isolates (P1 to P11) ranged from 0 to 63.7%, 0 to 58.9%, 0 to 16.3% and 0 to 59.3% with mineral oil, kerosene, diesel and sunflower oil as substrates, respectively. Similarly, the overall E_{24} for the non-pigmented *Serratia* isolates (NP1 to NP11) ranged from 0 to 56.7%, 0 to 46.5%, 0 to 59.3% and 0 to 61.5% with mineral oil, kerosene, diesel and sunflower oil as substrates, respectively. Twenty (91%) of the 22 presumptive *Serratia* isolates screened for biosurfactant production using the emulsification capacity assay were thus able to emulsify at least one hydrocarbon analysed in this study (Table 2).

The 22 presumptive *Serratia* isolates ($n = 11$ pigmented; $n = 11$ non-pigmented) were then tested for their ability to reduce the surface tension of sterile PG broth (Table 2). The highest reduction in surface tension for pigmented isolates was from 61.7 ± 0.5 mN/m to 32.0 ± 0.0 mN/m (P2, P4, P7, P8 and P9), while the highest reduction in surface tension for the non-pigmented isolates was from 61.7 ± 0.5 mN/m to 29.7 ± 0.0 mN/m (NP4 and NP6) (Table 2). Based on the results obtained, the 22 (100%) presumptive *Serratia*

Table 2
Genus specific sequence identification and emulsification indices of representative *Serratia* isolates producing surface-active compounds.

Isolate number	Organism (Genebank accession no.)	% ID	Source	Surface tension (mN/m)	Emulsification indices (%)			
					MO	K	D	SO
P1	*S. marcescens* (CP005927.1)	100	OS	32.7 ± 0.3	34.1	24.6	14.8	3.7
P2	*S. marcescens* (CP016032.1)	100	WWTP	32.0 ± 0.0	20.3	14.7	0.0	0.0
P3	*S. marcescens* (CP018927.1)	100	WWTP	35.7 ± 0.3	63.7	21.0	0.0	23.1
P4	*S. marcescens* (CP016032.1)	99	WWTP	32.0 ± 0.0	7.3	58.9	3.8	59.3
P5	*S. marcescens* (CP013046.2)	100	WWTP	33.0 ± 0.3	23.6	30.8	1.8	14.3
P6	*S. marcescens* (CP018927.1)	100	WWTP	32.7 ± 0.3	0.0	0.0	0.0	33.3
P7	*S. marcescens* (CP018927.1)	100	WWTP	32.0 ± 0.0	0.0	0.0	0.0	0.0
P8	*S. marcescens* (CP021984.1)	100	WWTP	32.0 ± 0.0	24.1	27.6	16.3	0.0
P9	*S. marcescens* (CP005927.1)	100	WWTP	32.0 ± 0.0	32.3	21.4	1.9	0.0
P10	*S. marcescens* (CP021984.1)	98	WWTP	33.3 ± 0.0	30.9	16.7	5.6	35.7
P11	*S. marcescens* (CP013046.2)	100	WWTP	33.8 ± 0.0	16.2	3.9	1.9	29.6
NP1	*S. marcescens* (CP021984.1)	100	KR	32.9 ± 0.0	56.7	24.1	48.2	53.9
NP2	*S. marcescens* (CP018917.1)	100	WWTP	30.2 ± 0.0	3.7	16.7	52.7	37.7
NP3	*S. marcescens* (CP018923.1)	100	KR	30.2 ± 0.0	1.9	25.9	37.0	40.7
NP4	*S. marcescens* (CP018928.1)	100	KR	29.7 ± 0.0	14.8	44.4	59.3	61.5
NP5	*S. marcescens* (CP018929.1)	100	WWTP	30.2 ± 0.0	16.1	46.5	42.1	55.6
NP6	*S. marcescens* (CP018930.1)	100	WWTP	29.7 ± 0.0	0.0	14.8	21.6	7.7
NP7	*S. marcescens* (CP018929.1)	100	WWTP	30.9 ± 0.3	14.0	0.0	0.0	0.0
NP8	*S. marcescens* (CP018930.1)	100	WWTP	30.6 ± 0.0	3.9	5.6	5.7	53.6
NP9	*S. marcescens* (LT575490.1)	100	WWTP	30.3 ± 0.3	25.9	0.0	0.0	46.3
NP10	*S. marcescens* (CP012639.1)	100	ER	32.9 ± 0.0	7.7	21.4	1.9	0.0
NP11	*S. marcescens* (HG738868.1)	100	ER	30.9 ± 0.5	0.0	0.0	0.0	0.0

ID – Identity, OS – Oil sludge from an oil refinery bioreactor, WWTP - municipal wastewater treatment plant, KR – Krom River, ER – Eerste River, MO – Mineral oil, K – Kerosene, D – Diesel and SO – Sunflower oil.

Table 3
Summary of the compounds identified in the crude extracts obtained from *S. marcescens* P1 and NP1 strains, detected using high-resolution mass spectrometry (UPLC-ESI-MS analysis) (< 10 ppm).

Compound	UPLC Rt (min)	UPLC Rt (min)	Proposed fatty acid chain length	Monoisotopic mass (M_r)	Theoretical/ Detected Protonated specie (m/z)		Theoretical/ Detected Sodiated specie (m/z)	
	P1 crude extract	NP1 crude extract			P1 crude extract	NP1 crude extract	P1 crude extract	NP1 crude extract
Prodigiosin	5.11	N/D	–	323.1998	324.2077 324.2065	N/D	N/D	N/D
Serratamolide A (Serrawettin W1)	7.14 7.36	7.14 7.36	$C_{10}+C_{10}$	514.3254	515.3333 515.3325	515.3333 515.3325	537.3152 537.3103	537.3152 537.3204
Serratamolide B	8.21 8.49	8.21 8.49	$C_{10}+C_{12:1}$	540.3411	541.3490 541.3463	541.3490 541.3463	563.3309 563.3271	563.3309 563.3271
Serratamolide C	8.74 9.00	8.74 9.00	$C_{10}+C_{12}$	542.3567	543.3646 543.3700	543.3646 543.3598	565.3465 565.3500	565.3465 565.3500
Serratamolide E	5.74	5.74	C_8+C_{10}	486.2941	487.3020 487.3048	487.3020 487.3048	509.2839 509.2740	509.2839 509.2740
Glucosamine derivative A	N/D	9.54	–	584.4073	N/D	585.4116 585.4073	N/D	607.3935 607.3953

N/D – not detected.

isolates screened for biosurfactant production using the Du Nouy ring tensiometer were able to reduce the surface tension of the growth medium by ≥ 26.0 ± 0.0 mN/m.

3.3. Molecular characterisation of the biosurfactant producing bacteria

Sequencing of the *pfs* PCR product (193 bp) and BLAST analysis identified the 22 bacterial isolates (screened for emulsification and surface tension properties) as *S. marcescens* strains (Table 2). All the identified *S. marcescens* strains (n = 22) were further screened for the presence of the *swrW* and *swrA* genes encoding for the biosynthesis of serrawettin W1 and serrawettin W2, respectively. Results indicated that the pigmented strains isolated in this study primarily contained the *swrW* gene encoding for serrawettin W1 synthetase (n = 11), while 82% (n = 9) of the non-pigmented strains contained the *swrA* gene encoding for non-ribosomal serrawettin W2 synthetase (Supplementary Table S1). One non-pigmented isolate (*S. marcescens* NP1) contained the *swrW* gene encoding for serrawettin W1. Furthermore, *S. marcescens* NP10 did not contain the *swrW* or *swrA* genes, while none of the *S. marcescens* strains (n = 22) contained both the *swrW* and *swrA* genes.

3.4. Ultra-performance liquid chromatography coupled to mass spectrometry

Based on the physico-chemical properties, molecular analysis, and preliminary antimicrobial testing (results not shown), three *S. marcescens* strains (P1, NP1 and NP2) were selected for the production and extraction of biosurfactant crude extracts. Following solvent extraction, the crude extracts were subjected to chemical characterisation utilising UPLC coupled to ESI-MS analysis for identification of potential antimicrobial compounds present in the crude extracts.

3.4.1. P1 crude extract

The positive mode ESI-MS analysis of the crude extract obtained

from the *S. marcescens* P1 strain revealed a cluster of *m/z* peaks with a difference of approximately 2, 26 or 28 atomic mass units (amu) in their molecular ion species, revealing four groups of homologues. The spectra in positive mode revealed the main groups of molecular ions at *m/z* 487.30, 515.33, 541.35 and 543.37 $[M+H]^+$ (Table 3; Supplementary Fig. S1a 1–5), which corresponded to the protonated singly charged species. Their corresponding sodium adducts were also detected at *m/z* 509.27, 537.31, 563.33 and 565.35 $[M + Na]^+$ (Table 3). Additionally, the detected molecular ions at *m/z* 487.30, 515.33, 541.35 and 543.37 $[M+H]^+$ corresponded to serratamolide E, A, B and C, respectively (Table 3), as previously reported by Dwivedi et al. (2008). The protonated singly charged specie was observed at *m/z* 324.21 $[M+H]^+$ (Supplementary Fig. S1a 5) and corresponded to the *m/z* of prodigiosin as previously reported by Eckelmann et al. (2018).

The UPLC-MS analysis revealed five significant peaks between 5 and 10 min (Supplementary Fig. S2). The five peak clusters observed corresponded to prodigiosin [retention time (Rt) 5.11 min], serratamolide E (Rt 5.74 min), serratamolide A (Rt 7.14 and 7.36 min), serratamolide B (Rt 8.21 and 8.49 min) and serratamolide C (Rt 8.74 and 9.00 min) (Dwivedi et al., 2008; Thies et al., 2014) (Table 3). The main peak observed at 7.36 min corresponded to serratamolide A, based on the M_r 514.33 corresponding to the detected protonated molecule (*m/z* 515.33) and sodiated molecule (*m/z* 537.31) (Dwivedi et al., 2008), as indicated in Table 3.

3.4.2. NP1 crude extract

Similar to the crude extract obtained from *S. marcescens* P1, the positive mode ESI-MS analysis of the crude extract obtained from *S. marcescens* NP1 revealed a cluster of *m/z* peaks with a difference of 2, 26, or 28 amu in their molecular ion species, revealing four groups of homologues. The spectra in the positive mode revealed the main groups of molecular ions at *m/z* 487.30, 515.33, 541.35 and 543.36 $[M+H]^+$ (Table 3; Supplementary Fig. S1b 1–5) which corresponded to the protonated singly charged species. Their corresponding sodium adducts were also detected at *m/z* 509.27, 537.32, 563.33 and 565.35 $[M + Na]^+$ (Table 3). The detected molecular ions at *m/z* 487.30, 515.33, 541.35 and 543.36 corresponded to serratamolide E, A, B and C, respectively (Table 3), as previously reported by Dwivedi et al. (2008). However, *m/z* 585.41 $[M+H]^+$ and *m/z* 607.40 $[M + Na]^+$ were also detected and corresponded to a protonated and sodiated singly charged glucosamine derivative A (Dwivedi et al., 2008).

The UPLC-MS analysis revealed five significant peaks between 5 and 10 min (Supplementary Fig. S3). The peaks eluted corresponded to serratamolide E (Rt 5.74 min), serratamolide A (Rt 7.14 and 7.36 min), serratamolide B (Rt 8.21 and 8.49 min) and serratamolide C (Rt 8.74 and 9.00 min), as reported in literature (Dwivedi et al., 2008; Thies et al., 2014) (Table 3). However, a peak was observed at a retention time of 9.54 min that corresponded to a glucosamine derivative A (Dwivedi et al., 2008), while prodigiosin was not detected. The main peak observed at 7.36 min corresponded to serratamolide A, based on the M_r 514.33 corresponding to the detected protonated molecule (*m/z* 515.33) and sodiated molecule (*m/z* 537.32), as indicated in Table 3.

3.4.3. NP2 crude extract

The positive mode ESI-MS analysis of the crude extract obtained from the *S. marcescens* NP2 strain revealed a cluster of *m/z* peaks with a difference of approximately 14 and 16 amu in their molecular ion species, revealing five groups of homologues. The spectra in positive mode revealed the main groups of molecular ions at *m/z* 690.41, 704.42, 718.44, 732.45 and 748.46 $[M+H]^+$ (Table 4; Supplementary Fig. S4) which corresponded to the protonated singly charged species. Their corresponding sodium adducts were also detected at *m/z* 712.39, 726.41, 740.43, 754.44 and 770.43 $[M + Na]^+$ (Table 4). The detected molecular ions were denoted serrawettin W2 C (*m/z* 704.42), serrawettin W2 B (*m/z* 718.44), serrawettin W2 A (*m/z* 732.45) and serrawettin W2 D (*m/z* 748.46) (Table 4). Therefore, serrawettin W2 A (*m/z* 732.45), serrawettin W2 C (*m/z* 704.42) and serrawettin W2 D (*m/z* 748.46) corresponded to serrawettin W2, serrawettin W5 and serrawettin W6, respectively, as previously reported by Motley et al. (2016), while serrawettin W2 B (*m/z* 718.44) corresponded to an analogue, referred to as sw-2 or serrawettin W4 (Motley et al., 2016; Su et al., 2016). Lastly, a molecular ion species was detected at *m/z* 690.41 $[M+H]^+$; however, it did not correspond to the *m/z* of any previously reported analogues of serrawettin W2.

The UPLC-MS analysis revealed five significant peaks between 5 and 10 min (Supplementary Fig. S5). The peaks eluted corresponded to serrawettin W2 D (Rt 6.88 min), serrawettin W2 C (Rt 6.80 and 7.02 min), serrawettin W2 B (Rt 7.81 min) and serrawettin W2 A (Rt 8.22 and 8.46 min) as reported in literature (Motley et al., 2016; Su et al., 2016). In contrast, the peak detected at a retention time of 6.34 min did not correspond to a serrawettin W2 analogue previously reported in literature. The main peak observed at 8.46 min corresponded to serrawettin W2, based on the M_r 731.45 corresponding to the detected protonated molecule (*m/z* 732.45) and sodiated molecule (*m/z* 754.44) (Su et al., 2016), as indicated in Table 4.

3.5. Antimicrobial and haemolytic activity of the crude extracts

The chemically characterised crude extracts produced by *S. marcescens* P1, NP1 and NP2 were subjected to antimicrobial assays against a broad range of bacterial and fungal strains (Table 5). The crude extract produced by the pigmented *S. marcescens* P1 strain (oil refinery isolate) displayed activity against 63.6% ($n = 7$) of the Gram-negative bacteria tested in the current study (Table 5). The Gram-negative bacterial strains that were the most susceptible were *P. aeruginosa* S1 68 (environmental strain) and PA3 (clinical strain) with inhibition zones of 18.0 ± 1.0 mm recorded, respectively, while the clinical *A. baumannii* AB3 strain was the least susceptible with an inhibition zone of 9.7 ± 0.6 mm. The P1 crude extract also displayed activity against 90% ($n = 9$) of the Gram-positive bacteria tested in the current study (Table 5). The *L. monocytogenes* G1 food isolate was the most susceptible with an inhibition zone of 24.7 ± 0.6 mm recorded, while the least susceptible strain was *B. cereus* ATCC 10876 with an inhibition zone of 12.3 ± 0.6 mm. Lastly, the P1 crude extract displayed activity against 100% ($n = 6$) of the *C. albicans* and *C. neoformans* strains tested. The most susceptible fungal strain was *C. albicans* ATCC 66027 with an inhibition zone of 25.0 ± 0.0 mm recorded, while the least susceptible was the environmental *C. neoformans* CAB831 strain with an inhibition zone of 12.0 ± 0.0 mm.

The crude extract produced by the non-pigmented *S. marcescens* NP1 strain (Krom River isolate) displayed activity against 45.5% ($n = 5$) of the Gram-negative bacteria tested in the current study (Table 5). The most susceptible Gram-negative bacterial strain was *P. aeruginosa* S1 68 with an inhibition zone of 15.0 ± 1.7 mm, while the least susceptible strain was *P. aeruginosa* PA3 with an inhibition zone of 11.0 ± 1.0 mm. The NP1 crude extract also displayed activity against 70% ($n = 7$) of the Gram-positive bacteria tested in the current study (Table 5). The most susceptible Gram-positive bacterial strain was *L. monocytogenes* ATCC 13932 with an inhibition zone of 21.0 ± 1.0 mm recorded, while the least susceptible strain was *B. cereus* ATCC 10876 with an inhibition zone of 10.3 ± 0.6 mm. Lastly, the NP1 crude extract displayed activity against 100% ($n = 6$) of the *C. albicans* and *C. neoformans* strains tested. The most susceptible fungal strains were *C. albicans* ATCC 66027 and *C. neoformans* CAB844 (environmental strain) with inhibition zones of 15.0 ± 0.0 mm and 15.0 ± 1.7 mm recorded, respectively. The clinical *C. albicans* CAB8911 and *C. neoformans* CAB1055 strains were the least susceptible with inhibition zones of 11.3 ± 1.5 mm and 11.3 ± 0.5 mm recorded, respectively.

The crude extract produced by the non-pigmented *S. marcescens* NP2 strain (WWTP isolate) displayed activity against 36.4% ($n = 4$) of the Gram-negative bacteria tested in the current study (Table 5). *Acinetobacter baumannii* AB3 was the most susceptible Gram-negative

Table 4
Summary of the analogues identified in the crude extract obtained from the *S. marcescens* NP2 strain, detected using high-resolution mass spectrometry (UPLC-ESI-MS analysis) (< 10 ppm).

Compound name	Literature name of compound	UPLC Rt (min)	Proposed analogue structure (Literature)	Monoisotopic mass (M_r)	Theoretical/ Detected Protonated specie (m/z)	Theoretical/ Detected Sodiated specie (m/z)
Serrawettin W2 E	Unidentified	6.34	–	N/A	N/A	N/A
					690.4106	712.3941
Serrawettin W2 D	Serrawettin W6	6.88	C_{10}OH-Ile/Leu-Ser-Thr-Tyr-Ile/Leu	747.4418	748.4497	770.4316
					748.4564	770.4290
Serrawettin W2 C	Serrawettin W5; sw-1	6.80	C_8OH-Ile/Leu-Ser-Thr-Phe-Ile/Leu	703.4156	704.4235	726.4054
		7.02	C_{10}OH-Abu/Aib-Ser-Thr-Phe-Ile/Leu		704.4213	726.4014
Serrawettin W2 B	sw-2;	7.81	C_{10}OH-Val-Ser-Thr-Phe-Ile/Leu	717.4313	718.4392	740.4211
	Serrawettin W4		C_{10}OH-Ile/Leu-Ser-Thr-Phe-Val		718.4441	740.4263
Serrawettin W2 A	Serrawettin W2; sw-3; sw-4;	8.22	C_{10}OH-Leu/Ile-Ser-Thr-Phe-Ile/Leu	731.4469	732.4548	754.4367
	sw-5	8.46			732.4514	754.4413

N/A – Not applicable.

bacterial strain with an inhibition zone of 14.0 ± 1.0 mm recorded, while the least susceptible strain was *A. baumannii* ATCC 19606 with an inhibition zone of 10.0 ± 1.0 mm. Similarly, the NP2 crude extract displayed activity against 30% ($n = 3$) of the Gram-positive bacteria tested (Table 5). The most susceptible strain was *L. monocytogenes* G1 with an inhibition zone of 17.7 ± 1.5 mm, while the least susceptible was the environmental *Bacillus* sp. S8 38 strain with an inhibition zone of 8.7 ± 0.6 mm. Lastly, the NP2 crude extract only displayed activity against one (16.7%) of the fungal strains (*C. neoformans* CAB1055), with an inhibition zone of 7.7 ± 0.6 mm recorded.

In addition to antimicrobial activity, the P1, NP1 and NP2 crude extracts were tested for haemolytic activity on sheep blood agar. The crude extracts were found to be γ-haemolytic on sheep blood agar, indicating that the extracts did not lyse the red blood cells in comparison to the positive control, surfactin (Sigma-Aldrich), which displayed prominent β-haemolytic (clear lysis zone) activity.

4. Discussion

Based on the physico-chemical and molecular analysis, one pigmented (P1; isolated from oil refinery effluent) and two non-pigmented [NP1 (isolated from the Krom River) and NP2 (isolated from a WWTP sample)] *S. marcescens* strains were selected for UPLC-ESI-MS analysis to identify potential antimicrobial compounds present in the crude extracts. The major peak detected in both *S. marcescens* P1 and NP1 crude extracts corresponded to serrawettin W1 (serratamolide A; m/z

Table 5
Activity of the biosurfactant extracts (1.00 mg/mL) against a panel of Gram-negative and Gram-positive bacterial and fungal isolates as determined by agar disc diffusion method.

S. marcescens strain number		P1	NP1	NP2
Test microorganism	Source	Antimicrobial zone of inhibition (mm; mean ± standard deviation)		
Gram-negative bacteria				
Escherichia coli (ATCC 13706)	ATCC	17.0 ± 1.0	0.0	0.0
Enterohaemorrhagic E. coli (O157:H7)	ATCC	0.0	0.0	0.0
Legionella longbeachae (ATCC 33462)	ATCC	0.0	0.0	12.0 ± 0.0
Legionella pneumophila (ATCC 33152)	ATCC	12.7 ± 0.6	13.7 ± 0.6	0.0
Pseudomonas aeruginosa (ATCC 27853)	ATCC	15.0 ± 1.0	14.7 ± 1.2	0.0
P. aeruginosa (S1 68)	Environmental	18.0 ± 1.0	15.0 ± 1.7	0.0
**P. aeruginosa* (PA3)	Clinical	18.0 ± 1.0	11.0 ± 1.0	11.0 ± 1.0
Klebsiella pneumoniae (ATCC 10031)	ATCC	0.0	0.0	0.0
**K. pneumoniae* (KP3)	Clinical	0.0	0.0	0.0
Acinetobacter baumannii (ATCC 19606)	ATCC	12.3 ± 0.6	12.3 ± 0.6	10.0 ± 1.0
***A. baumannii* (AB3)	Clinical	9.7 ± 0.6	0.0	14.0 ± 1.0
Gram-positive bacteria				
Bacillus cereus (ATCC 10876)	ATCC	12.3 ± 0.6	10.3 ± 0.6	0.0
B. cereus (S1 77)	Environmental	18.3 ± 0.6	15.7 ± 0.6	12.7 ± 0.6
Bacillus sp. (S8 38)	Environmental	0.0	0.0	8.7 ± 0.6
Enterococcus faecalis (ATCC 7080)	ATCC	15.0 ± 0.0	12.7 ± 0.6	0.0
E. faecalis (S1)	Clinical	18.0 ± 0.0	16.7 ± 0.6	0.0
Listeria monocytogenes (ATCC 13932)	ATCC	20.7 ± 0.6	21.0 ± 1.0	0.0
L. monocytogenes (G1)	Food	24.7 ± 0.6	0.0	17.7 ± 1.5
Staphylococcus aureus (ATCC 25923)	ATCC	14.0 ± 0.0	19.0 ± 0.6	0.0
Staphylococcus equorum (SP2)	Environmental	22.3 ± 0.6	0.0	0.0
Methicillin resistant *S. aureus* (MRSA) (Xen 30)	Clinical	19.3 ± 0.6	14.0 ± 0.0	0.0
Fungal strains				
Candida albicans (ATCC 66027)	ATCC	25.0 ± 0.0	15.0 ± 0.0	0.0
C. albicans (CAB8911)	Clinical	12.3 ± 0.6	11.3 ± 1.5	0.0
C. albicans (CAB1085)	Environmental	14.3 ± 0.6	12.6 ± 1.5	0.0
Cryptococcus neoformans (CAB831)	Environmental	12.0 ± 0.0	12.0 ± 0.6	0.0
C. neoformans (CAB1055)	Clinical	12.6 ± 1.2	11.3 ± 0.5	7.7 ± 0.6
C. neoformans (CAB844)	Environmental	13.0 ± 1.7	15.0 ± 1.7	0.0

* MDR strain.
** XDR strain.

515.33 $[M+H]^+$), while homologues of serratamolide A (namely, serratamolide B, C and E) were also detected. The chemical characterisation of the compounds produced by the *S. marcescens* P1 and NP1 strains thus correspond to the molecular analysis, where the *swrW* gene that encodes for serrawettin W1 synthetase (serratamolides) was detected in both strains. The detected serratamolides are comprised of a cyclic peptide moiety of two serine amino acids and are linked to two β-hydroxy fatty acids that vary in chain length, thus resulting in various homologues (Eckelmann et al., 2018). Thies et al. (2014) identified various serrawettin W1 homologues, including serratamolide C, D, E and F as well as two novel homologues (*m/z* of 557 and 571), in a crude extract obtained from a pigmented *S. marcescens* DSM12481 strain, while Dwivedi et al. (2008) found that a pigmented *Serratia* sp. strain SHHRE645 produced serratamolide A, B, C, D and F homologues. In the current study, *S. marcescens* P1 was also found to produce prodigiosin and previous studies have similarly isolated *S. marcescens* strains that are capable of co-producing prodigiosin and serratamolide homologues (serrawettin W1) (Eckelmann et al., 2018; Hage-Hülsmann et al., 2018). As expected, the non-pigmented strain did not produce prodigiosin; however, the NP1 strain produced another secondary metabolite referred to as glucosamine derivative A. This corresponds to literature, where Dwivedi et al. (2008) reported on the co-production of serratamolides with glucosamine derivatives by a pigmented *Serratia* sp. strain SHHRE645 previously isolated from the rhizosphere of wheat.

In contrast, the major peak detected in the *S. marcescens* NP2 crude extract corresponded to serrawettin W2 (*m/z* 732.45 $[M+H]^+$), while analogues of this compound were also detected. The detected serrawettin W2 is comprised of a cyclic peptide moiety of five amino acids (D-leucine/isoleucine-L-serine-L-threonine-D-phenylalanine-L-isoleucine/leucine) linked to a β-hydroxy fatty acid moiety of varying chain length (Matsuyama et al., 1992). However, further chemical characterisation is required to determine the exact amino acid composition of each analogue and confirm the identity of the potential novel analogue of serrawettin W2 with a molecular ion detected at *m/z* 690.41 $[M+H]^+$. The chemical characterisation of the compounds within the NP2 crude extract also corresponded to the molecular analysis, where the *swrA* gene that encodes for non-ribosomal serrawettin W2 synthetase was detected. Moreover, as the chemical characterisation of the crude extract indicated that serrawettin W2 and analogues of this lipopeptide were produced, the primers designed in this study successfully provided an indication of the biosurfactant produced by the *Serratia* strain. *Serratia* spp. capable of producing serrawettin W2 and analogues of this lipopeptide have been isolated by various research groups (Motley et al., 2016; Su et al., 2016; Heise et al., 2019). A study by Heise et al. (2019) isolated and identified a *S. marcescens* strain from the gut of a burying beetle, *Nicrophorus vespilloides*, and discovered that the *S. marcescens* strain was able to produce serrawettin W2 (*m/z* 732.45 $[M+H]^+$). Similarly, Su et al. (2016) isolated a pigmented *S. surfactantfaciens* YD25^T strain from rhizosphere soil and found that the strain produced various serrawettin W2 analogues, including *m/z* 704.3, 718.3, 732.2 and 746.4 $[M+H]^+$, amongst others.

The antimicrobial activity of the crude extracts obtained from the three *S. marcescens* strains was subsequently assessed against pathogenic and opportunistic bacterial and fungal strains (including MDR and XDR clinical isolates), using disc diffusion assays. The P1 and NP1 crude extracts were both effective against a broader range of Gram-positive bacteria (90% and 70%, respectively) in comparison to Gram-negative bacteria (64% and 45%, respectively), while both crude extracts displayed activity against all fungal strains tested. These results corroborates previous research where it was indicated that serrawettin W1 (serratamolide) exhibits antimicrobial activity towards Gram-positive bacteria, such as MRSA strains (Kadouri and Shanks, 2013) and fungal strains, such as *C. albicans* (Zhu et al., 2018). However limited research on the activity of serrawettin W1 (and its homologues) against Gram-negative bacteria has been reported. In contrast, in the current study, the NP2 crude extract was effective against 36% of the Gram-negative bacteria and 30% of the Gram-positive bacteria analysed, and a low antifungal activity was recorded (17%). Thus, in comparison to the P1 and NP1 crude extracts, a narrow-spectrum of antimicrobial activity was observed for the secondary metabolites produced by the *S. marcescens* NP2 strain. Literature has however, indicated that serrawettin W2 displays antimicrobial activity against Gram-positive and Gram-negative bacteria, such as *S. aureus, P. aeruginosa, Micrococcus luteus* (*M. luteus*) and *Shigella dysenteriae* (*S. dysenteriae*), amongst others (Su et al., 2016). A study by Motley et al. (2016) also reported that serrawettin W2 analogues displayed antifungal activity against *C. albicans*.

Moreover, despite the fact that serrawettin W1 and serrawettin W2 were discovered more than 30 years ago (Wasserman et al., 1962; Matsuyama et al., 1986), limited information is currently available on the antimicrobial mode of action of these compounds. It is however, well-known that the primary target of lipopeptides is the cell membrane (Schlusselhuber et al., 2018). The proposed general mode of action is driven by the interaction of the peptide moiety of the lipopeptide with the polar head groups of the phospholipids. Thereafter, the hydrophobic moiety of the lipopeptide (fatty acids) is incorporated into the hydrophilic moiety (lipopolysaccharides) of the cell membrane (Jenssen et al., 2006; Schlusselhuber et al., 2018). A few studies have also revealed that the length of the fatty acid chain affects the antimicrobial potency of the lipopeptide due to the changes in the hydrophobic interaction of the lipopeptide with the cell membrane (Malina and Shai, 2005; Chu-Kung et al., 2010; Schlusselhuber et al., 2018). As the P1 and NP1 crude extracts were comprised of four serratamolide homologues with two fatty acid chains of varying lengths (C_8 to C_{12}) per homologue, the combination of the lipopeptides with varying lengths could provide an explanation for the broad-spectrum activity observed for these crude extracts against both bacteria and fungi. In contrast, the NP2 crude extract was comprised of serrawettin W2 analogues with one C_{10} fatty acid chain (with the exception of one analogue that has a C_8 fatty acid chain) attached to a peptide moiety, which could elucidate the reduced broad-spectrum activity.

As indicated, an additional secondary metabolite, prodigiosin, was detected in the P1 crude extract and has been reported to display antimicrobial activity against *S. aureus, B. subtilis* and *Streptococcus pyogenes* (*S. pyogenes*), amongst others (Darshan and Manonmani, 2015). Danevčič et al. (2016) investigated the mode of action of prodigiosin and found that this compound interacts with the cytoplasmic membrane and increases the membrane permeability in *B. subtilis*, as well as uncouples proton transport and disrupts energy generation, ultimately leading to cell lysis and death. Hage-Hülsmann et al. (2018) further found that prodigiosin and serrawettin W1, produced by a *S. marcescens* DSM12481 strain, displayed greater antimicrobial activity in combination against a soil bacterium, *Corynebacterium glutamicum* (*C. glutamicum*), compared to the individual activity of these two compounds. Moreover, the NP1 crude extract was also found to contain a secondary metabolite known as glucosamine derivative A, which could have contributed to the observed antibacterial and antifungal activity. Although limited information is available on the antifungal activity of glucosamine derivatives produced by *Serratia* spp., Dwivedi et al. (2008) has indicated that all compounds, including serratamolides A to F, open ring-serratamolides B and D and glucosamine derivatives A to C, produced by a pigmented *Serratia* sp. SHHRE645 strain, exhibited antimycobacterial activities. Based on the results obtained in the current study, many of the water- and foodborne pathogens and opportunistic pathogens selected as test organisms, e.g. *L. monocytogenes*, *S. aureus*, *Bacillus* spp. virulent *E. coli*, *Enterococcus* spp., *Legionella* spp. and *Pseudomonas* spp. (Sharma et al., 2003; Mayrhofer et al., 2004; Moritz et al., 2010; Clements et al., 2019b), were susceptible to the metabolites present in the P1 and NP1 crude extracts. Moreover, MDR and XDR bacteria frequently associated with hospital-acquired infections, such as *A. baumannii*, *P. aeruginosa*, *Enterococcus* spp. and MRSA (Khan et al., 2015, 2017), as well as fungal opportunistic pathogens,

such as *C. albicans* and *C.neoformans* were also inhibited by the P1 and NP1 crude extracts. It is thus hypothesised that the broad-spectrum antimicrobial activity observed for the P1 and NP1 crude extracts was due to the synergistic effect of serratamolide homologues and prodigiosin (P1) and the glucosamine derivative A (NP1), which effectively inhibited the proliferation of these water- and foodborne opportunistic pathogens and clinical strains. In comparison, a narrow or limited activity against the test strains was observed for the NP2 crude extract, where serrawettin W2 analogues only were identified.

Previous studies have however, indicated that serratamolides exhibit cytotoxic and haemolytic activity (Shanks et al., 2012; Petersen et al., 2017). A study by Shanks et al. (2012) investigated the haemolytic and cytotoxic activity of purified serratamolide A and found that the lipopeptide haemolysed sheep and murine red blood cells and was cytotoxic to human airway and corneal limbal epithelial cells *in vitro*. Furthermore, Su et al. (2016) demonstrated that serrawettin W2 exhibits cytotoxicity to cancer cell lines (such as Hela cells), while reduced activity towards non-malignant cell lines (such as Vero cells) was recorded. Results obtained in the current study however, indicated that at a concentration of 1.00 mg/mL, the P1, NP1 and NP2 crude extracts did not display haemolytic activity on sheep blood agar. While further analysis of the purified compounds and crude extracts' cytotoxicity and haemolytic activity against human red blood cells is required, the secondary metabolites produced by *S. marcescens* P1, NP1 and NP2 strains may still have the potential to be applied as broad- or narrow-spectrum antimicrobial agents.

5. Conclusions

The crude extracts obtained from *S. marcescens* P1 and NP1 exhibited broad-spectrum antibacterial and antifungal activity against MDR *P. aeruginosa,* MRSA and clinical *C. neoformans*, amongst others. Although the mode of action of serrawettins has not yet been fully elucidated, secondary metabolites produced by specifically *S. marcescens* strains P1 and NP1 have thus emerged as promising candidates for the treatment of infections caused by Gram-negative and Gram-positive pathogens and opportunistic pathogens as well as fungal pathogens of clinical and industrial significance. In comparison, whilst displaying activity against MDR *P. aeruginosa* and XDR *A. baumannii*, the crude extract obtained from *S. marcescens* NP2 exhibited a narrow-spectrum antimicrobial activity.

It is also important to note that limited information on the acquired resistance of pathogens and opportunistic pathogens to these secondary metabolites has been recorded. This implies that these bioactive compounds could serve as promising antibacterial and antifungal agents for therapeutic application. It is however, recommended that the crude extracts be further purified to elucidate the detailed structure of each homologue of serrawettin W1 and analogue of serrawettin W2. It is further recommended that the biofilm disruption and antiadhesive potential of the extracts against bacterial and fungal strains be investigated for application as antifouling agents in the food, water and medical industries.

Ethical approval

This article does not contain any studies with human participants or animals

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.126329.

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