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The characterisation of the antimycobacterial activity of a Pseudomonas isolate

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Appendix A:

Media:

Luria Broth (LB) (Sambrook *et al*, 1989) (Biolab):

Tryptone Extract	1 g
Yeast	0.5 g
NaCl	0.5 g
dH_20	100 ml

Tryptone soy broth (TSB) (Biolab):

Trytone Soy Broth media	3g
dH_20	100 ml

Difco Middlebrook 7H9 (Broth):

7H9 powder	0.5222 g
Glycerol	0.555 ml
dH_20	100 ml

Supplements (For 20ml M. smegmatis mc^{2}155 cultures):

25% Tween 80	*40 µl*
25% Glucose Stock	*1.6 ml*
25% NaCl	*680 µl*

Difco Middlebrook 7H10 (Agar):

7H10 powder	0.5222 g
Glycerol	0.555 ml
dH_20	100 ml

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SOB media (Sambrook *et al*. 1989)

Bacto tryptone	20g	Shake till dissolved
Bacto yeast	5g	
NaCl	0.5g	
250mM KCl	10ml	

250mM KCl [0.93g to 50ml dH20, adjust pH to 7 with 5N NaOH(0.2µl) and adjust volume to 1 litre and autoclave for 20 minutes.]

Before use add 5ml of 2MMgCl2 [3.8g MgCl2 + 18ml dH2O, adjust to 20ml dH20 and autoclave for 20 minutes]

Buffers:

PIPES Buffer (100mmol)

CaCl2.2H2O	20.2g
Glycerol	150 ml
dH20	(made up to 1000ml)

Filter sterilise the buffer and store at 4ºC.

TAE Buffer (50×) (Sambrook *et al*, 1989)

Tris Base	242 g
Glacial acetic acid	57.1 ml
*0.5M Na_2 EDTA (pH8.0)	100 ml
dH_20	(made up to 1000 ml)

*0.5M Na_2 EDTA (pH.8.0) :

Na_2 EDTA	186.1 g
dH_20	800 ml
NaOH	(Use to adjust pH to 8.0)

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Solution I:

50mM glucose
25mM Tris. Cl (pH =8)
10mM EDTA (pH=8)
Autoclave for 15 minutes and store at 4 ºC

Solution II (freshly prepared):

0.2N NaOH
1% SDS

Solution III (3M potassium and 5M acetate):

*5M potassium Acetate (Autoclaved)	60 ml
Glacial Acetic acid	11.5 ml
sdH_2O	28.5 ml

Store at 4ºC.

*3M Potassium Acetate	
Sodium Acetate	49.075g
pH with glacial acetic acid	7.0
dH20	(make up to 100ml)

Autoclave to sterilise

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Appendix B:

16s rDNA sequence of the soil isolate and the Pseudomonadaceae sequence :

GACGCTGGCGGCAGGCCTTAACACATGCAAGTCTAGCGGATGACGGGAGCTTGC
TCCTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGG
GGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGC
AGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTG
GTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATC
AGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG
AATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAG
GTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGCTAATACCT
TGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCC
GCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGC
GTAGGTGGTTCGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCA
TCCAAAACTGGCGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGC
GGTGAAATGCGTAGATATAAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGG
ACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGAATCCTTGAGATTTT
AGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAAGTA
AAACTCAATGAATTGACTGGGTCCGCACAACGGTGAGCATGGGTTAATTCGACCA
CCCGAAAATCG

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Table 10: Scores obtained from the BLASTn search from the NCBI database using the 16s rDNA sequence of the *Pseudomonas* isolate.

Bacteria from Genbank database	**Bit score**	**E value**
*Pseudomonas putida GB-1**	1616	0.0
*Pseudomonas putida F1**	1616	0.0
*Pseudomonas entomophila L48****	1616	0.0
*Pseudomonas putida KT2440**	1611	0.0
*Pseudomonas fluorescens PfO-1***	1539	0.0
*Pseudomonas fluorescens Pf-5*****	1413	0.0
*Pseudomonas aeruginosa PAO1****	1411	0.0

Bit scores and E values of the comparison between the 16s rDNA sequence of the soil isolate and the Pseudomonadaceae sequence database on NCBI. The primer set used for the amplification of 16S rDNA were as follows U1392R (5'-ACG GGCGGT GTG TRC-3') (Lane *et al*,1991; Ferris *et al,0* 1996; McGarvey *et al*, 2004) and Bac27F(5'-AGA GTT TGA TCM TGG CTC AG-3')(Inagaki *et al*, 2003; McGarvey *et al*, 2004). The stars indicate strains that are associated to bioremediation (*), biodegradation (**), pathogenic traits (***) and biosurfactants (****).

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Table 11: Solvents used for the extraction of different antibiotics (Tingyue. 2000)

Antibiotic	Medium	Solvent	pH
Actinomycin	Cake	1MeOH+2 methylene chloride	2.5
Adrianimycin	Cake	Acetone	Acidic
Bacitracin	Broth	n-Butanol	7
Chloraphenicol	Broth	Ethyl acetate	N-alkaline
Clavulanic acid	Broth	n-Butanol	2
Cycloheximid	Broth	Methylene chloride	3.5-5.5
Erthromycin	Broth	Amylacetate	Alkaline
Fusidic acid	Broth	Methylisobutylketone	6.8
Griseofulvin	Cake	Butylacetae, mhylene chloride	Neutral
Macrolides	Broth	Methylisobutylketone, ethyl acetate	Alkaline
Nisin	Broth	CHCl3+ sec-Oct.alc	4.5
Nisin	Mash	CHCl3	2
Oxytetracycline	Broth	1 Butanol	
Penicillin G	Broth	Butylacetate, amylacetate	2
Salinomycin	Cake	Butyl acetate	9.0
Tetracycline	Broth	1-Butanol	
Tyllosin	Broth	Butylacetate, amylacetate	
Viginiamycin	Broth	Methylisobutylketone	Acidic

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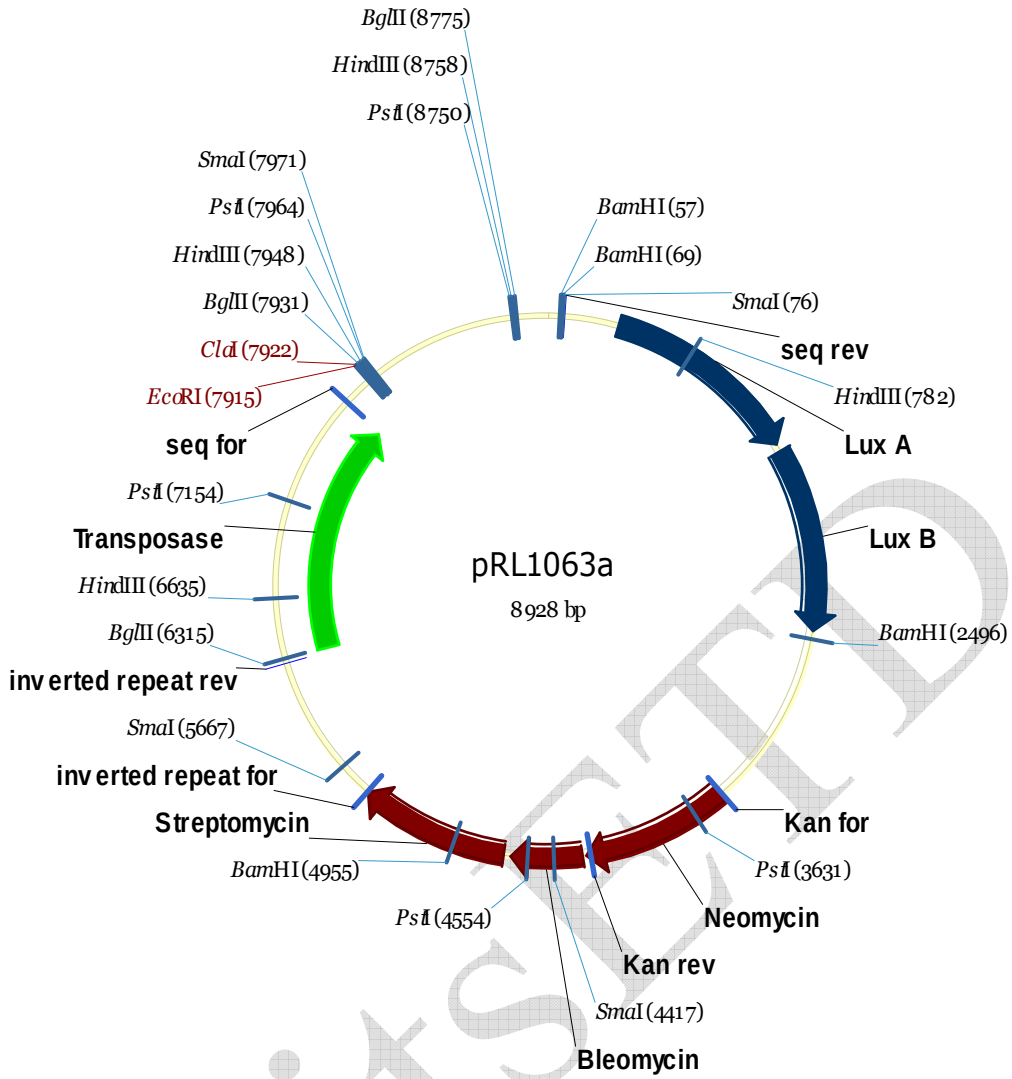


Figure 33: Restriction enzyme sites on plasmid pRL1063a. The restriction site map was created using Vector NTI version 11 (Invitrogen). The respective primer sites are shown in green and the selectable marker region for kanamycin is shown in red. One cutter restriction enzyme sites are depicted in blue i.e EcoRI

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The following tables (Table 12-14) contain information obtained using New England Biolabs incorporated, virtual digest program, Nebcutter.

Table 12: Predictive sizes of double restriction digest of Pst I-BglII using Nebcutter:

Fragment	Ends	Coordinates	Length (bp)
1	BglII-PstI	8775-3630	3784
2	PstI-BglII	4554-6314	1761
3	PstI-PstI	3631-4553	923
4	BglII-PstI	6315-7153	839
5	PstI-PstI	7964-8749	786
6	PstI-BglII	7154-7930	777
7	BglII-PstI	7931-7963	33
8	PstI-BglII	8750-8774	25

Table 13: Predictive sizes of fragments form a restriction digest of Pst I using Nebcutter:

Fragment	Ends	Coordinates	Length (bp)
1	PstI-PstI	8750-3630	3809
2	PstI-PstI	4554-7153	2600
3	PstI-PstI	3631-4553	923
4	PstI-PstI	7154-7963	810
5	PstI-PstI	7964-8749	786

Table 14: Predictive sizes of fragments from a restriction digest with BglII using Nebcutter

Fragment	Ends	Coordinates	Length (bp)
1	BglII-BglII	8775-6314	6468
2	BglII-BglII	6315-7930	1616
3	BglII-BglII	7931-8774	844

WITHDRAWN

Appendix C:

Table 15: Colony forming units of bacterial cultures incubated for a specific period

Strain	*Bacillus DLS*	*Bacillus aureus*	*Staphylococcus aureus*	*Salmonella*	*Pseudomonas putida 317*	*Pseudomonas putida dss2*
Average cfu/ml	2.53E+09	1.08E+08	2.80E+12	4.20E+09	2.03E+09	1.72E+10
STD error of mean	1101514109	101823377	1.13E+12	1907878403	351188458	28616079396
Required dilution	1.00E+06	1.00E+06	1.00E+06	1.00E+06	1.00E+06	1.00E+06
Times to be diluted	3	2	6	3	3	4
Hours propagated	20	20	1.95E+01	19.5	23	23

Strain	*Our isolate*	*Nocardia*	*M.fortuitum*	*M. smegmatis*	*Ecoli 29*	*Ecoli 32*
Average cfu/ml	5.47E+09	3.70E+09	9.77E+10	9.13E+08	3.30E+09	2.33E+09
STD Error of mean	2203028219	2066397832	1.22E+16	272472629	700000000	4196682420
Required dilution	1.00E+06	1.00E+06	1.00E+06	1.00E+06	1.00E+06	1.00E+06
Times to be diluted	3	3	4.00E+00	2	3	3
Hours propagated	22	22	20	20	20.5	20.5

Poisson Equation used for the calculation of reversion frequencies:

Frequency = h/N , h= ln[no. of revertants ÷ sample size], N= cfu/ml ÷ 0.1

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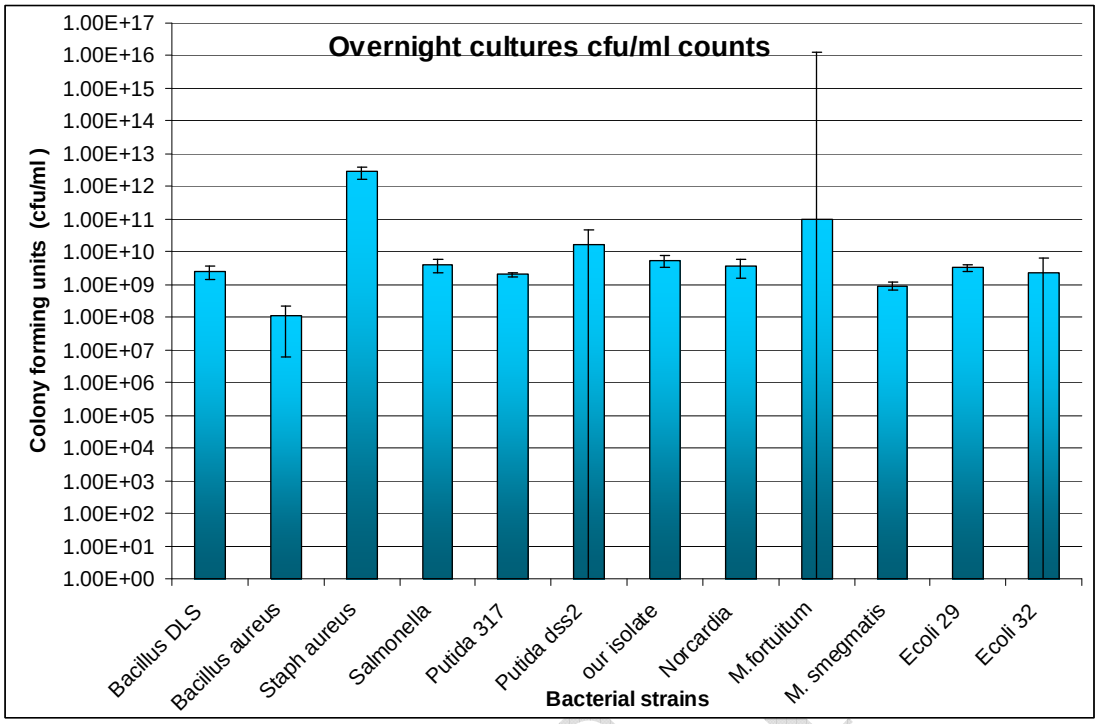


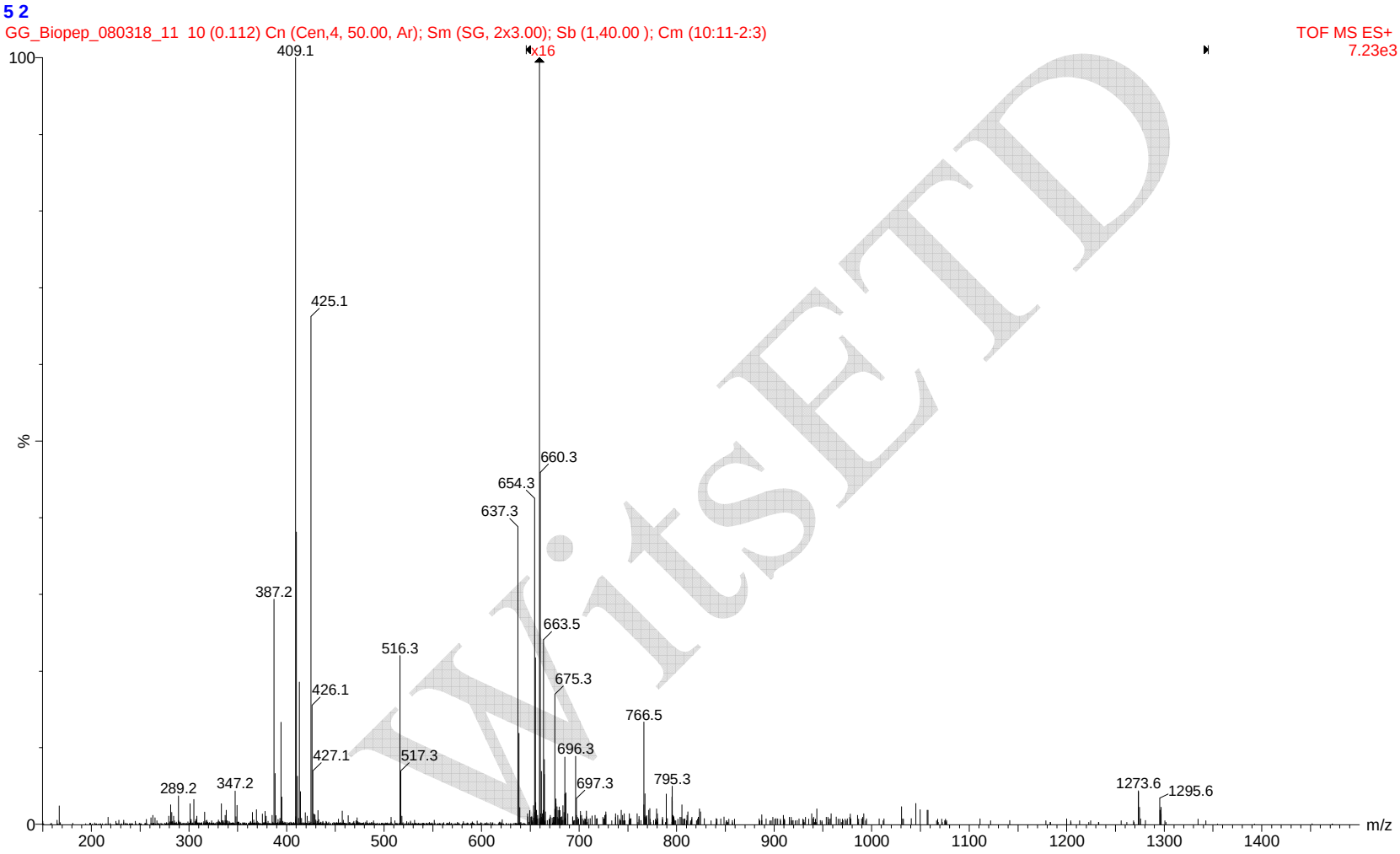
Figure 34: Graph depicting the average cfu/ml with standard error of mean values of overnight cultures of the respective environmental strains.

The standard deviation figures for *M. fortuitum* and *E. coli* were not of acceptable values, however, they were only used for a crude estimation of colony forming units of the cultures therefore there was no need for them to be repeated.



Figure 35: Sequence alignment of sequences attained from *E. coli* DH5α M1 clone. Sequences attained from Inqaba biotech after sequencing of clone M1 via the Sanger method. Sequence alignment was done using Vectro NTI version 10 (trial version). Sequences corresponded to each other from position 7783 to 8021.

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Figure 36: ESI-MS spectra of fractionated crude extract sample diluted Fraction 5 for sample A.

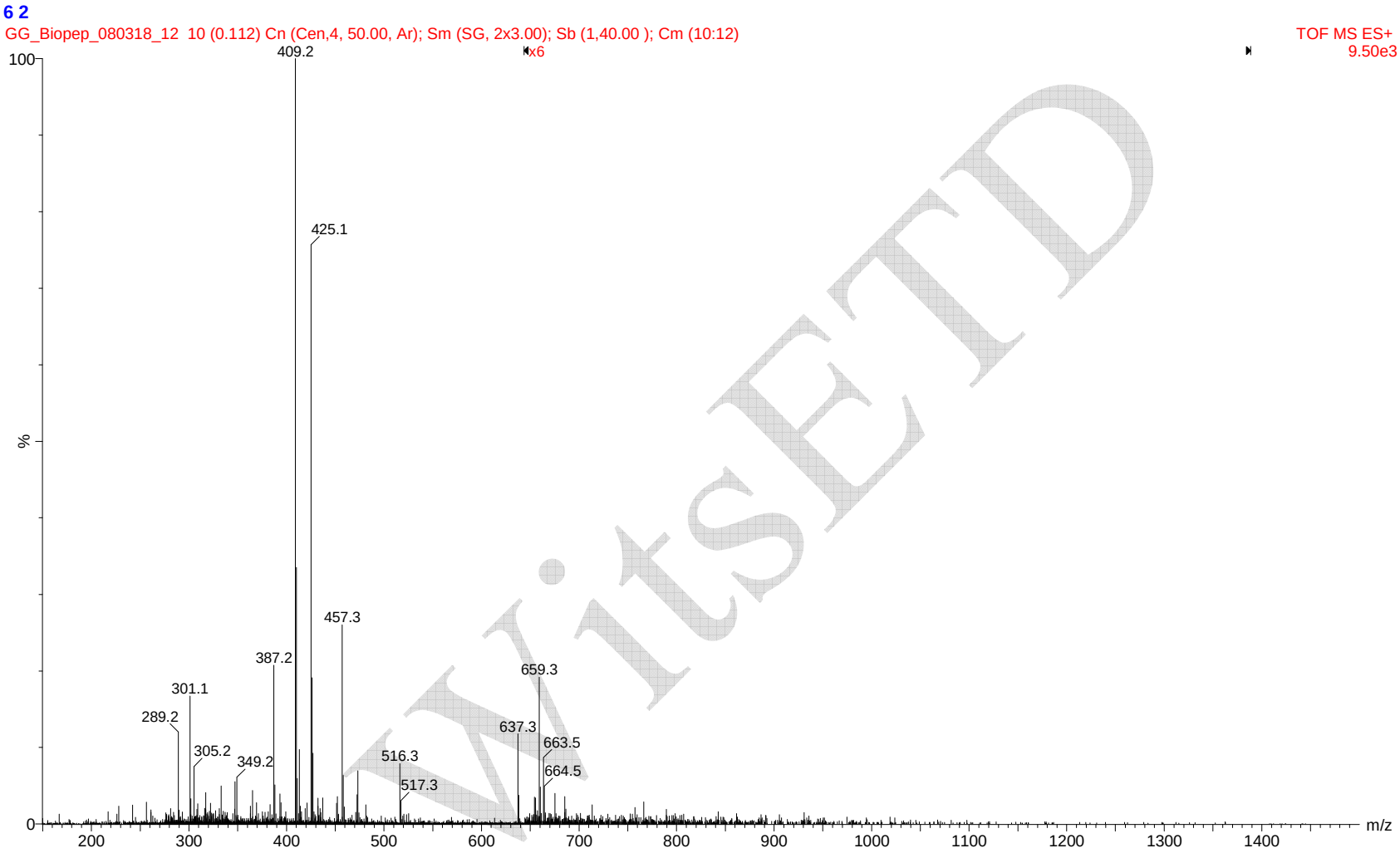
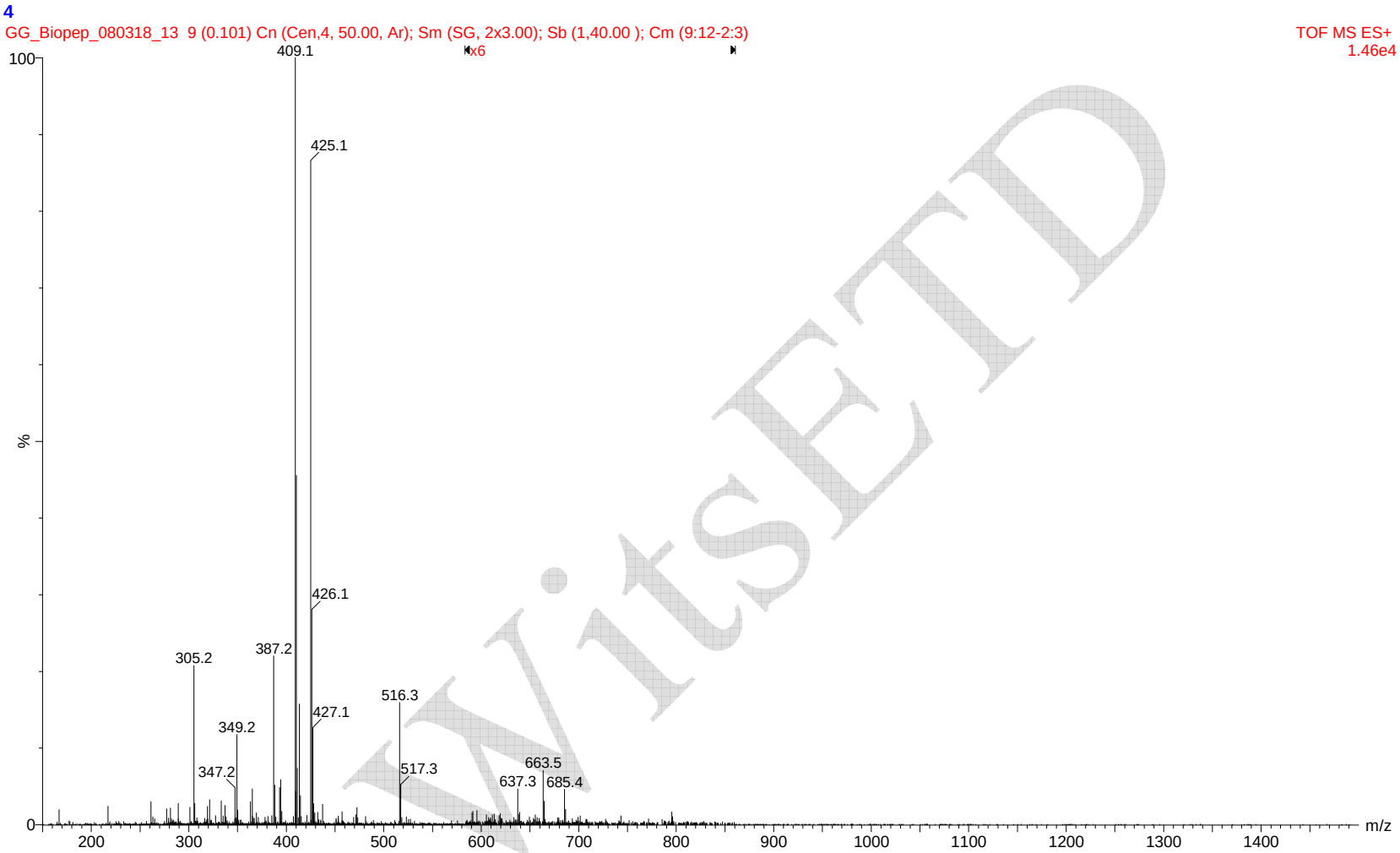


Figure 37: ESI-MS spectra of fractionated crude extract sample diluted Fraction 6 for sample A.

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Figure 38: ESI-MS spectra of fractionated crude extract sample Fraction 4 for sample A.

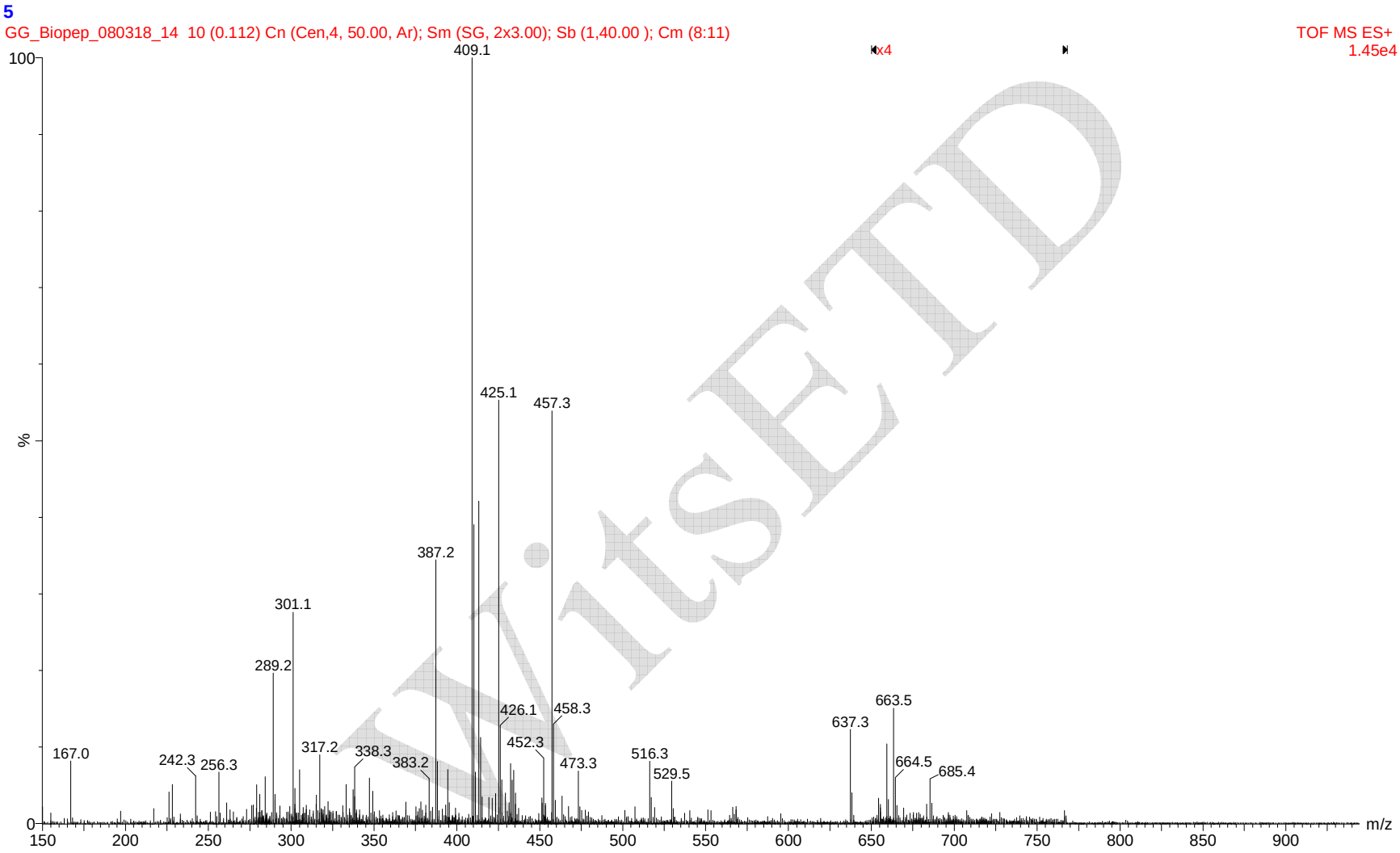
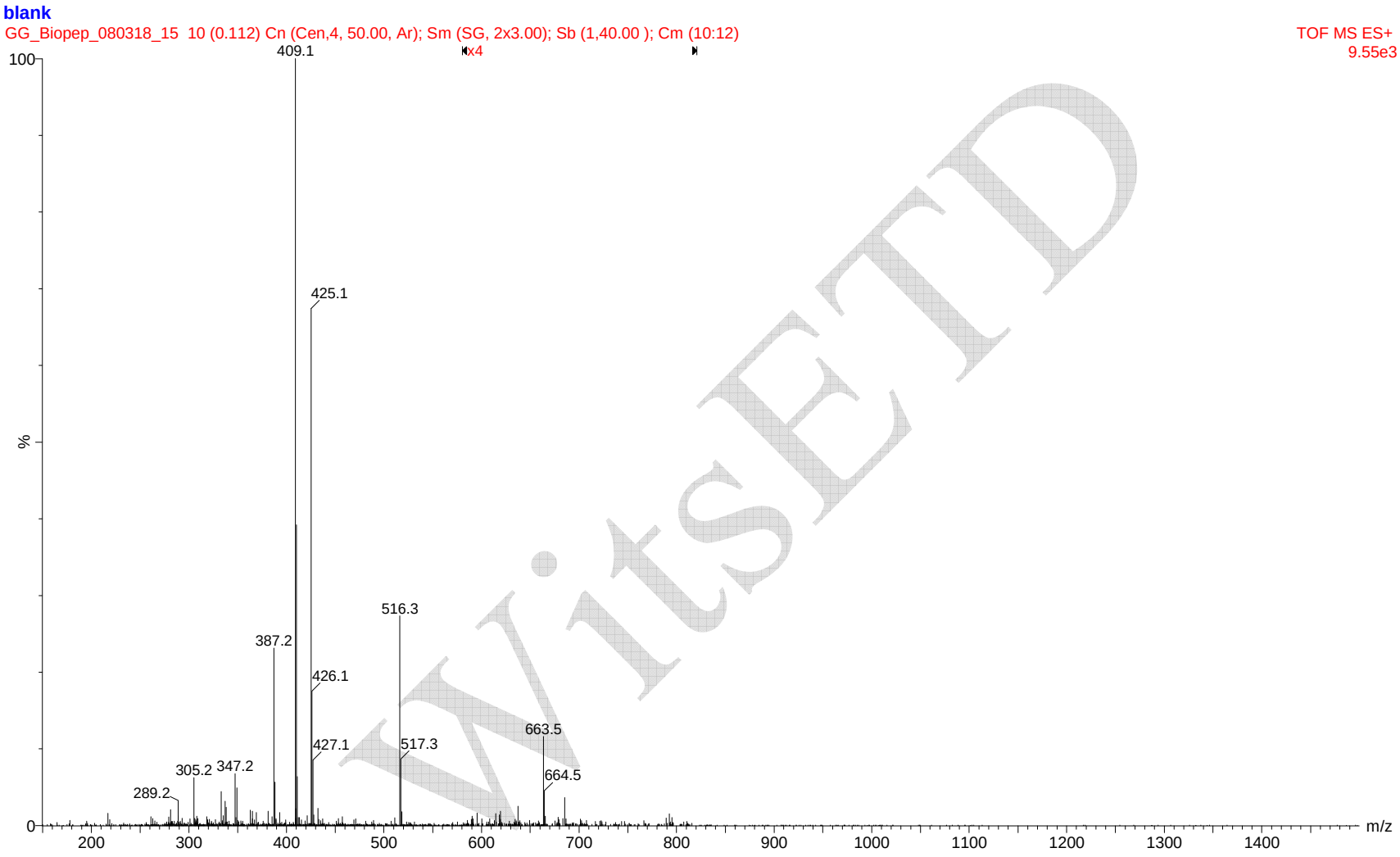


Figure 39: ESI-MS spectra of fractionated crude extract sample Fraction 5 for sample A.

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Figure 40: ESI-MS spectra of sample blank for fractionated crude extract samples.