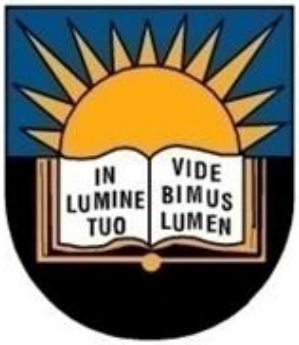
EVALUATION OF SOME *PSEUDOMONAS* SPECIES ISOLATED FROM HOGSBACK FOREST RESERVE FOR THE PRODUCTION OF ANTIBACTERIAL COMPOUNDS



University of Fort Hare
Together in Excellence

A Dissertation

Submitted in fulfilment of the requirements for the degree of

Master of Science (Biochemistry)

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By

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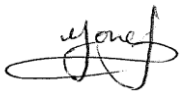
DECLARATION

I, Mkono Yonela Pelokazi, hereby declare that this dissertation submitted to the University of Fort Hare for the degree of Masters of Science in Biochemistry in the Faculty of Science and Agriculture and the work contained herein is my original work with exemption to the citations and that this work has not been submitted at any other University in partial or entirety for the award of any degree.

Signed: 

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DEDICATION

This work is dedicated to my mother Nopasika Mkono, my sister Odwa Mkono and niece Milani Mkono.

ACKNOWLEDGEMENT

I wish to express my deepest gratitude to God, the Lord my Saviour, who has kept me sane throughout all the struggles that came with the completion of my program, for providing strength that no person could have ever had the capacity to, and keeping me at peace until the end.

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LIST OF ABBREVIATIONS

Secondary metabolite(s)	SM
2,4–Diacetylphloroglucinol	DAPG
Methicillin-resistant *Staphylococcus aureus*	MRSA
Lateral Genetic Transfer	LGT
Quinolone-resistant protein	Qnr
Multi Drug Resistance	MDR
Genomic sampling library	GSL
Cluster identification library	CIL
Sodium dodecyl sulphate	SDS
Ethidium Bromide	EtBr

ABSTRACT

Pseudomonas species are Gram-negative bacteria most abundant in soil and water bodies, with the capacity to thrive in varied environments. They are largely associated with resistant pathogenic bacteria linked to human and plant diseases. Species such as *Pseudomonas aeruginosa* have been particularly targeted as case studies due to the extremity to which they pose a threat to human health. With more focus directed at using these species for biocontrol and bioremediation purposes, their role in bioactive compound production may be equally important. As the crisis on antimicrobial resistance still persists, the need for effective antimicrobial compounds is ever more urgent and solutions may possibly still be dormant in bacterial species whose potential has not been fully investigated. On a bid to source out potential antimicrobial compound producers, soil samples were collected from Hogback forest reserve in the province of the Eastern Cape, South Africa. For bacterial screening, M1 and R2A agar were used and the cultures grown at 37˚C for a period of seven days. After the presumed *Pseudomonas* species were identified, antimicrobial production was determined by submerged fermentation method using nutrient broth as media of choice. Active isolates were further studied to determine the optimum conditions which best facilitate for antimicrobial compound production, with parameters such as temperature (25˚C – 40˚C) and pH (4 – 9) considered. The role plasmids play in antimicrobial compound production was also investigated. Each isolate was grown in fermentation media containing Sodium dodecyl sulphate and Ethedium Bromide, at varying concentrations, to facilitate for plasmid curing. With each sample, distinct colonies were identified with varying pigmentations most dominant being a cream colour. The identity of the isolated strains was achieved through sequencing of 16S rDNA. Phylogenetic analysis showed that isolate A16 had 80% homology with *Pseudomonas plecoglossicida* strain P4 and share a close ancestor with isolates Y52 and Y81, also isolate Y89 showed a 90% homology with *Pseudomonas* sp. Co-11a.

With the exception of isolate A16, the isolates which were active against Gram-negative bacteria lost activity as the screening processes continued. When looking at temperature variations, isolates Y81 and A16 were highly active with maximum activity observed at 35˚C while Y89 performed best at 25˚C and Y52 showed constant activity across all studied temperatures. The plasmids in all isolates were found to be 48.5 kb in size with the exception of isolate Y89 which was 20 kb. The plasmids were cured at concentrations of (1 mg/ml; 5 mg/ml; 7 mg/ml; 10 mg/ml; 11 mg/ml) SDS and (125 µg/ml; 6.5 µg/ml; 5µg/ml) EtBr. The curing process also showed changes in both the antimicrobial activity of the isolates as well as their physical characteristics. The isolates are the first reported *Pseudomonas* species from Hogsback forest reserve with the ability to produce antimicrobial compounds which are active against Gram-positive and Gram-negative bacteria. These mesophilic bacteria also show that plasmids do not pay any role in the production of antimicrobial compounds and that the biosynthesis genes are highly likely to be chromosomal borne meaning that the production cannot be linked to horizontal transfer of genes. Therefore, these isolated *Pseudomonas* species provide a potential reservoir of antimicrobial compounds which may play an important role in the antimicrobial resistance phenomenon.

CHAPTER ONE

1. INTRODUCTION

The rise in drug resistance is increasing both morbidity and mortality at alarming rates, necessitating research which is aimed at solving problems associated with drug resistance. Antibiotic resistance has resulted in previously treatable and manageable diseases becoming unresponsive to first-line drugs. It is also, in addition, a pending crisis that antibiotic discovery has come to a halt (Katz *et al.*, 2006; Payne *et al.*, 2007). As it has been documented in history, epidemics such as the Spanish flu that resulted in an estimated death toll of 25 million have not surpassed the current antibiotic resistance health scare (Lederberg, 2000; Michael *et al.*, 2014). The present state, referred to as the post-antibiotic era, is reverting the human population back to old methods of treatment which are slower and less effective (Michael *et al.*, 2014; WHO, 2014).

In addressing the current situation, focus has been directed at sourcing the solutions from naturally abundant microorganisms. The genus *Pseudomonas*, first described by Migula (1894), is found in diverse environments particularly in soil and water bodies, and can be easily isolated. These aerobic gamma proteobacterial species are Gram-negative, non-sporulating and motile microbes of maximum 5 µm cell length (Palleroni, 2008; Couillerot *et al.*, 2009). They are characteristically rod-shaped and polar flagellated with a guanine-cytosine (GC) content of 58–69%. The flagella are associated with surface attachments, cell to cell interactions, mobility and phage absorption (Palleroni, 2008).

In addition to the ability of *Pseudomonas* species to adapt to varied nutritional and physicochemical settings, they have the capacity to alleviate endogenous and exogenous stresses through the development of protective systems and also contribute to the microbial production of bioactive compounds (Kim and Park, 2014; Nikel *et al.*, 2014). Apart from the prior mentioned, *Pseudomonas* species are notably used as biocontrol agents which target soil-borne pathogens as well as bioremediation agents which are used as detoxificants (Gao *et al.*, 2012; Wasi *et al.*, 2013).

Majority of the documented species are primarily pathogenic, however it is of interest to look into prospective strains which may have the potential to produce bioactive compounds with antibacterial properties. Studies on the molecular framework of some antimicrobials derived from Pseudomomas have a number of gaps which restrict a forward plan in tackling the present antibiotic resistance problem (Gurney and Thomas, 2011). As it is with any antibiotic producing species, the location of biosynthesis clusters is of crucial importance since production may be tampered if necessary precautions are not taken into consideration. The importance of genetic studies, in terms of whole-genome sequencing, of potential species facilitates a better understanding of key pathways as well as gene locations in secondary metabolite (SM) production. Such knowledge is essential in up-scaling whereby producer strains do not possess the production capacity required on an industrial scale as well as in gene manipulation to improve SM yields as well as the efficiency of a particular SM (Baltz, 2006).

1.1 STUDY RATIONALE

The documented exceptional numbers of antimicrobial drugs produced on the market are no longer considered valuable due to antibiotic resistance. Only a small fraction of the documented 75% and 60% of these drugs used in medicine and agriculture, respectively, is effective against bacterial infections (Chaudhary *et al.*, 2013). Although different environments have been explored for potential producer strains, a limited number has been discovered and isolated, with the same species being frequently screened resulting in an inevitable rediscovery of known bioactive compounds (Busti *et al.*, 2006; Singh *et al.*, 2014).

The exploration of new environments has brought promise of the identification of new and rare species which hold promise of producing novel bioactive compounds. With the abundance of Psuedomonads in the environment, there is a high probability that Hogsback woodlands in the Raymond Mhlaba municipality in the Eastern Cape provide a potential source of pharmaceutically important species.

1.2 RESEARCH HYPOTHESIS

The present study hypothesises that swamp-soil from Hogsback woodland is not a reservoir of antimicrobial producing *Pseudomonas* species.

1.3 RESEARCH AIM AND OBJECTIVES

1.3.1. Aim

This study aims at the evaluation of antibacterial compounds produced by some gamma gamma proteobacteria isolated from Hogsback forest reserve.

To achieve the above stated research aim, the following sets of objectives were considered;

1.3.2. Objectives

- To collect swamp-soil samples from Hogsback woodland and isolate *Pseudomonas* species.
- To screen isolated *Pseudomonas* species for the production of antibacterial compounds and identify positive isolates.
- To evaluate fermentation conditions optimum for the production of antimicrobial production.
- To evaluate the role of plasmids in the antibiotic production potentials of selected *Pseudomonas* isolates.

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CHAPTER TWO

2. LITERATURE REVIEW

The genus *Pseudomonas* falls under the subclass ɤ-Proteobacteria in the order Pseudomonadales and the family Pseudomonadaceae (Couillerot *et al.*, 2009). It is abundant in natural settings and is frequently isolated from soil and aquatic ecosystems. These microorganisms do not have complex nutritional requirements and can utilise broad range of compounds as their energy and carbon source. In addition to their complex enzymatic systems, the prior mentioned is attributed to their ability to adapt and survive in different environmental niches (Palleroni, 2008; Peix *et al.*, 2009; Uğur *et al.*, 2012). Over the years there have been continual taxonomic revisions which caused uncertainty in the correct number of species within this particular genus, however, to date there are 211 described species. Some species which have been reclassified are located under the following genera; *Delftia*, *Ralstonia*, *Comamonas*, *Acidovorax*, *Burkholderia* and *Hyrodenophaga* (Anzai *et al.*, 2000; Scales *et al.*, 2014).

2.1 THE GENUS – *PSEUDOMONAS*

The identification of species within this genus came with a few challenges as it was, for a number of years, aligned with unrelated species (Peix *et al*., 2009). However, this is not a past concern as presently there are a number of discrepancies at the species level which still need to be resolved (Peix *et al*., 2009). The first record of the genus *Pseudomonas* was in the 1800s by a German botanist by the name of Friedrich August Walter Migula (Peix *et al*., 2009). The first known species to be described was *Pseudomonas aeruginosa* which was initially known as *Pseudomonas pyocyanae*, it is an abundant, motile and rod-shaped bacteria (Sokatch, 1986; Yamamoto *et al.*, 2000; Rehm, 2008; Peix *et al.*, 2009). *P. aeruginosa* has since become one of the most documented species as it falls under classified pathogens within the genus. Identified as an opportunistic pathogen, this bacterium targets immune compromised individuals who suffer from diseases such as AIDs, burnt wounds, cystic fibrosis and cancer (Pukatzki *et al.*, 2002; Rehm, 2008).

In the earlier stages, the taxonomical description of the genus did not allow for a clear distinction from other Gram-negative bacteria as classifications solely relied on phenotypical characterisation. To establish precise classifications, nutritional characterizations followed by DNA renaturation were carried out. However, it was not until RNA-DNA relatedness had been considered that there were two groups set apart to remain under the genus Pseudomonas (Peix *et al*., 2009).

Based on rRNA-DNA hybridization, the genus *Pseudomonas* was reclassified into five groups, which differ based on inability and/or ability to fluoresce under UV light (Palleroni, 2008). Fluorescent species fell under rRNA homology group I and produce a water soluble, yellow-green pigment known as pyoverdine which fluoresces under UV. Apart from differentiating the group from the rest, the pigment plays an essential role in iron transport and its uptake for the functioning of these aerobic microbes. This is, however, not a constant marker as the production of pyoverdine depends on iron deficiency (Meyer, 2000; Gurney and Thomas, 2011). The non-fluorescent species are relatively a small group, representing 7–10% of *Pseudomonas* species, which comprises of species such as *Pseudomonas corrugata* and *Pseudomonas mediterranea* which has been associated with tomato pith necrosis (Bossis *et al.*, 2000; Kaszubiak, 2000; Moura *et al.*, 2006). As compared to fluorescent species, the antagonism of non-fluorescent species against actinobacteria and some *Pseudomonas* species is not frequent but when studying their interrelationships with actinobacteria the activity, though lower than that of their counterparts, is at times more recurring (Kaszubiak, 2000) **Table 2.1** below list the known *Pseudomonas* species which have remained under the phylum.

Table 2.1: *Pseudomonas* species known to date (Euzéby, 2008):

CURRENT CLASSIFIED *PSEUDOMONAS* SPECIES					
P. abietaniphila	*P. aminovorans*	*P. beijerinckii*	*P. cissicola*	*P. endophytica*	*P. gladioli*
P. acidovorans	*P. amygdali*	*P. benzenivorans*	*P. citronellolis*	*P. entomophila*	*P. glareae*
P. aeruginosa	*P. andropogonis*	*P. beteli*	*P. cocovenenans*	*P. extremaustralis*	*P. glathei*
P. aestusnigri	*P. anguilliseptica*	*P. borbori*	*P. coleopterorum*	*P. extremorientalis*	*P. glumae*
P. agarici	*P. antarctica*	*P. boreopolis*	*P. composti*	*P. facilis*	*P. graminis*
P. alcaligenes	*P. antimicrobica*	*P. brassicacearum*	*P. congelans*	*P. ficuserectae*	*P. granadensis*
P. alcaliphila	*P. argentinensis*	*P. brenneri*	*P. corrugate*	*P. flava*	*P. grimontii*
P. alkylphenolica	*P. arsenicoxydans*	*P. caeni*	*P. costantinii*	*P. flavescens*	*P. guangdongensis*
P. aminovorans	*P. asplenii*	*P. cannabina*	*P. cremoricolorata*	*P. flectens*	*P. guariconensis*
P. amygdali	*P. asturiensis*	*P. carboxydohydrogena*	*P. cuatrocienegasensis*	*P. fluorescens*	*P. guineae*
P. andropogonis	*P. asuensis*	*P. caricapapayae*	*P. deceptionensis*	*P. formosensis*	*P. halophila*
P. abietaniphila	*P. aurantiaca*	*P. caryophylli*	*P. delafieldii*	*P. fragi*	*P. helmanticensis*
P. acidovorans	*P. aureofaciens*	*P. cattleyae*	*P. delhiensis*	*P. frederiksbergensis*	*P. hibiscicola*
P. aeruginosa	*P. avellanae*	*P. cedrina*	*P. diminuta*	*P. fulva*	*P. hussainii*
P. aestusnigri	*P. avenae*	*P. cepaciap.*	*P. donghuensis*	*P. fuscovaginae*	*P. huttiensis*
P. agarici	*P. azotifigens*	*P. chloritidismutans*	*P. doudoroffii*	*P. gelidicola*	*P. indica*
P. alcaligenes	*P. baetica*	*P. chlororaphis*	*P. duriflava*	*P. geniculata*	*P. indigofera*
P. alcaliphila	*P. balearica*	*P. cichorii*	*P. echinoides*	*P. gessardii*	*P. iners*
P. alkylphenolica	*P. bauzanensis*				

CURRENT CLASSIFIED *PSEUDOMONAS* SPCEIS					
P. japonica	*P. mixta*	*P. parafulva*	*P. pseudomallei*	*P. segetis*	*P. thivervalensis*
P. jessenii	*P. mohnii*	*P. paucimobilis*	*P. psychrophila*	*P. seleniipraecipitans*	*P. tolaasii*
P. jinjuensis	*P. monteilii*	*P. pelagia*	*P. psychrotolerans*	*corrig.*	*P. toyotomiensis*
P. kilonensis	*P. moorei*	*P. peli*	*P. punonens*	*P. simiae*	*P. tremae*
P. knackmussii	*P. moraviensis*	*P. perfectomarina*	*P. putida*	*P. solanacearum*	*P. trivialis*
P. koreensis	*P. mosselii*	*P. pertucinogena*	*P. pradiora*	*P. soli*	*P. tuomuerensis*
P. kunmingensis	*P. multiresinivorans*	*P. phenazinium*	*P. reinekei*	*P. spinosa*	*P. umsongensis*
P. kuykendallii	*P. nautica*	*P. pickettii*	*P. resinovorans*	*P. stanieri*	*P. vancouverensis*
P. lanceolata	*P. nitritireducens*	*P. pictorum*	*P. rhizosphaerae*	*P. straminea corrig.*	*P. veronii*
P. lemoignei	*P. nitroreducens*	*P. plantarii*	*P. rhodesiae*	*P. stutzeri*	*P. vesicularis*
P. matsuisoli	*P. oleovorans*	*P. plecoglossicida*	*P. rubrilineans*	*P. synxantha*	*P. viridiflava*
P. mediterranea	*P. oryzihabitans*	*P. poae*	*P. rubrisubalbicans*	*P. syringae*	*P. vranovensis*
P. meliae	*P. otitidis*	*P. pohangensis*	*P. sabulinigri*	*P. syzygii*	*P. woodsii*
P. mendocina	*P. pachastrellae*	*P. prosekii*	*P. saccharophila*	*P. taeniospiralis*	*P. xanthomarina*
P. mephitica	*P. palleroniana*	*P. protegens*	*P. salegens*	*P. taetrolens*	*P. xiamenensis*
P. meridiana	*P. palleronii*	*P. proteolytica*	*P. salina*	*P. taiwanensis*	*P. xinjiangensis*
P. mesophilica	*P. panacis*	*P. pseudoalcaligenes*	*P. salomonii*	*P. testosteroni*	*P. yamanorum*
P. migulae	*P. panipatensis*	*P. pseudoflava*	*P. saponiphila*	*P. thermotolerans*	*P. zeshuii*
			P. savastanoi		*P. zhaodongensis*

2.2 THE DIVERSE ROLE OF *PSEUDOMONAS*

Cognisance of the importance of *Pseudomonas* in the environment and the key roles which establish their existence in nature is imperative. The ability to adapt to varied habitats suggests that the genus holds a significant amount of genomic multiplicity as well as genetic versatility. These microbes are most notable for the important roles they play in nitrogen and carbon recycling (Khan *et al.*, 2010).

2.2.1 *PSEUDOMONAS* – BIOREMEDIATION PROCESS

Bioremediation is a process where potentially harmful pollutants are eliminated or neutralised through microbial activities (Wasi *et al.*, 2013). In this regards, both organic and inorganic pollutants are convert into clean or less toxic substances (Wasi *et al.*, 2013). These pollutants result from human activities such as excessive extraction of natural resources as well as industrialization. In other instances these pollutants are as a result of natural activities such as forest fires, volcanic eruptions and excretions from rocks into water bodies (Rita and Ravisankar, 2014). This method is natural and cost effective and a number of species have been identified as being effective tools in the bioconversion of toxic pollutants to innocuous substances (Jariyal *et al.*, 2015). **Table 2.2** shows some of the species that have been chosen for such processes.

Table 2.2: *Pseudomonas* species with bioremediation abilities.

SPECIES	POLLUTANT	EFFICIENCY	REFERENCE
Pseudomonas sp. strain Imbl 5.1	Phorate	Complete degradation	Jariyal *et al*. (2015)
Pseudomonas aeruginosa	metallic ions (Fe, Cr, Mn, Cd)	Cr (70–75%), Mn (85–90%), Fe (50–55%), Cd (0–90%)	Singh *et al.* (2013)
Pseudomonas sp. NRRL B-12227	cyanuric acid	> 60%	Shiomi *et al.* (2006)
Pseudomonas taiwanensis strain SJ9	caprolactam	--	Hong *et al.* (2016)
Pseudomonas sp. BTEX-30	BTEX (benzene, toluene, ethylbenzene and xylenes)	Complete degradation	Khodaei *et al.* (2017)
Pseudomonas pseudoalcaligenes CECT5344	Cyanide and cyano-derivatives	--	Wibberg *et al.* (2016)
Pseudomonas putida	Phenanthrene	--	Jing *et al.* (2017)
Pseudomonas mendocina NSYSU	octachlorodibenzo-*p*-dioxin (OCDD)	75%	Tu *et al.* (2014)
Pseudomonas sp. BZ-3	phenanthrene	75%	Lin *et al.* (2014)
Pseudomonas sp. WJ6	*n*-alkanes and polycyclic aromatic hydrocarbons	--	Xia *et al.* (2014)

2.2.2 *PSEUDOMONAS* – BIOLOGICAL CONTROL AGENTS

Research on the negative impacts of agrochemicals on both human and other life forms has been widely reported (Walsh *et al.*, 2001; Winchester *et al*., 2009; Ferreira *et al*., 2010). These chemicals are associated with fish endocrine disorders as they inhibit steroidogenic enzymes (Ferreira *et al.*, 2010). Also, the link between elevated concentrations of pesticides in surface water and birth defects in humans was established in a study by Winchester *et al.* (2009).

Apart from the detrimental effects of agrochemicals on health, their use for selective control is a failing task. A number of herbicides that are in use, such as those used to target the growth of *Poa annua* L., ABG (**Table 2.3**), which are specifically intended to suppress the growth of unwanted weeds also affect the growth of other plants (Kennedy, 2016). *Pseudomonas* species. are, on the other hand, a very suitable choice as biocontrol agents, as can be seen in **Table 2.3**. They colonize plant rhizosphere and do not temper with germination or development of the plant (Agaras *et al.*, 2015). There are a number of species which act as biocontrol agents and the mechanism of the microbial products and/or the whole organism is action discussed in subsequent sections.

2.2.2.1 PRODUCTION OF BIOACTIVE COMPOUNDS

The bacterial strains inhabit intercellular spaces of plants which result in the production of inhibitors that will in turn suppress growth of pathogenic bacteria and/or fungi. The compounds which are often produced are the secondary metabolites including hydrogen cyanide and 2,4–Diacetylphloroglucinol (DAPG) and the production of the fluorescent siderophore pyoverdin (Rachidi and Ahmed, 2005; Mavrodi *et al.*, 2007; Michelsen and Stougaard, 2012; Kennedy, 2016).

2.2.2.2 MICROBE-PLANT INTERACTION

Pseudomonas species partner with plants to induce systemic resistance in response to invasion by plant pathogens (De Vleesschauwer *et al.*, 2008). They co-partner with plants to trigger or enhance the action of plant-borne transduction pathways in which hormones such as ethylene, salicylic acid and jasmonic acid play a crucial role in response to biotic and abiotic stress response (Turner *et al.*, 2002; Bouchez *et al.*, 2007; De Vleesschauwer *et al.*, 2008; War *et al.*, 2011)

2.2.2.3 PROMOTION OF PLANT GROWTH

The production and secretion of essential regulatory chemicals by bacterial strains assist in plant growth by regulating plant hormones which is directly and indirectly achieved. The bacteria may directly produce a hormone such as auxin which stimulates cell elongation or secrete cell wall degrading enzymes, resulting in the release of peptides and carbohydrates such as oligosaccharides which are involved in plant growth (Preston, 2004). They also hinder the effects and impacts of pathogens as well as assisting the plants to acquire phosphorous, minerals and nitrogen (Ahemad and Kibret, 2014). The overall use of *Pseudomonas* strains, in place of hazardous chemicals, has contributed to plant health and productivity (Ahemad and Kibret, 2014)

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Table 2.3: Psuedomonas species used as biocontrol agents

PSEUDOMONAS **SPECIES**	***PSEUDOMONAS*** **STRAIN**	**TARGET**	**HOST**	**PRODUCT**	**REFERENCES**
Pseudomonas chlororaphis	*P. chlororaphis* (aureofaciens) strain 30-84	*Gaeumannomyces graminis* var. tritici.	Wheat	Phenazines	Maddula *et al.* (2008)
	P. chlororaphis GP72	Broad spectrum activity against bacteria	Green pepper	Phenazine-1-carboxylic acid 2-hydroxyphenazine	Liu *et al.* (2007)
Pseudomonas fluorescens	*P. fluorescens* XJ3	Annual Bluegrass		--	Kennedy *et al*. (2016)
	P. fluorescens CHA0	Root disease and soilborne fungi	--	Hydrogen cyanide	Laville *et al.* (1998)
	P. fluorescens strain CL145A	*Dreissena polymorpha* and *Dreissena rostriformis bugensis*	Aquatic environment	Heat-labile secondary metabolite	Molloy *et al.* (2013)
Pseudomonas protegens	*P. protegens*Pf-5	Soil-borne oomycete, fungi and bacteria	–	--	Xie *et al.* (2016)
Pseudomonas aurantiaca	*P. aurantiaca* strain JD3	*Fusarium*	Algarrobo Blanco	--	Felker *et al.* (2005)
Pseudomonas brassicacearum	*P. brassicacearum* MA250	*Microdochium nivale*	Wheat	--	Holmberg *et al.* (2009)

2.3 *PSEUDOMONAS* PROUCTION OF ANTIMICROBIALS

The antimicrobial substances produced by *Pseudomonas* species play a role in plant health, growth and development and it is for this reason they are intensely studied (Weller, 2007). Although there is limited information of antimicrobial production, there are a few documented species which have shown activity against Gram-positive and Gram-negative bacteria (Weller, 2007; Matthijs *et al.*, 2014). The antibiotic mupirocin, produced by *P. fluorescens*, inhibits isoleucyl-tRNA synthetase (IleRS) in target bacteria (Hurdle *et al*., 2005; Matthijs *et al*., 2014). This particular antimicrobial compound is widely used as a topical antibiotic and targets one of the most resistant and life threatening microbe, the Methicillin-resistant *Staphylococcus aureus* (MRSA) (Boyce, 2001).

Surprisingly, *P. aeruginosa* which is counted amongst the most virulent microorganisms threatening human health has also been recorded to produce antimicrobial agents such as phenazines, which act against MRSA, and other biological compounds which target the plant bacterial pathogen *Xanthomonas* spp (Darabpour *et al.*, 2010; Cardozo *et al.*, 2013; Spago *et al.*, 2014). Another "insecticide" which is pathogenic to insects in both the juvenile and adult stages is the *P*. *entomophila* L48 which produces proteases and hydrogen cyanide. This particular strain is amongst the few recorded Gram-negative bacteria which acts against insect invasion at these growth stages (Gross and Loper, 2009).

2.4 *PSEUDOMONAS* ANTIMICROBIAL COMPOUND PRODUCTION MECHANISM: A CASE OF MUPIROCIN

Mupirocin hydrolyses when in contact with plasma resulting in the production of monic acid, which has no antimicrobial effect on bacteria and therefore is restricted to external uses. The structure is a made up of monic acid and 9-hydroxynonanoic acid (9-HN), linked together by ester bonds, which may be synthesised separately or resulting from the elongation of a starter unit to produce 9-HN (El-Sayed *et al.*, 2003; Hurdle *et al.*, 2004, 2005; Gurney and Thomas, 2011). Interestingly, the biosynthesis cluster, which codes for protein with acyltransferase and for polyketides, exhibits an order in the gene layout that does not correspond to the order of biosynthesis steps (Gurney and Thomas, 2011).

The initial biosynthetic steps for the production of mupirocin involve the synthesis of a monic acid precursor, C_{17}-heptaketide, which is the end result of six condensation reactions catalysed by two key players namely; MmpD and MmpA (Gurney and Thomas, 2011). The reaction begins with an acetate derived starter unit (acetyl-coenzyme A), and furthers on to modification and condensation steps on the MmpD module. MmpA is associated with an important role as the antimicrobial activity may be lost if ACP domains and atypical KS, which make up module 1 of MmpA, are structurally compromised (Gurney and Thomas, 2011; Gao *et al.*, 2014). On the other hand, synthesis of 9-HN is not clearly understood as studies have not inconclusively identified involved all enzymes, proteins and genes (Gurney and Thomas, 2011). The origin of 9-HN structure is, however, associated with 3-hydroxypropionate starter unit which is extended by MmpB condensations (Gurney and Thomas, 2011; Gao *et al.*, 2014).

2.5 *PSEUDOMONAS* - EXTRACHROMOSOMAL MATERIAL FUNCTIONALITIES

The looming antimicrobial resistance epidemic is one that was predicted back in 1945 by Alexander Fleming. In an interview he specified that the misuse of penicillin, one of two available classes of drugs at the time, would lead to mutant strains which would cause serious disease (Alanis, 2005). Since then, bacterial strains have been adapting at exceeding rates and counteracting the effects of available antimicrobial compounds. With the existing 17 classes of antimicrobial compounds, reports suggest that there is at least a single resistant mechanism per class which makes treatment an almost impossible task (Alanis, 2005). Seemingly, these wonder drugs which had the reputation of preventing infections in immune compromised patients as well as elevating life expectancy through treatment are no longer potent (Ventola, 2015).

Within the genus *Pseudomonas*, *P. aeruginosa* is most noted for its resistance to most available antimicrobial compounds. This particular species is commonly isolated in hospital settings and is associated with nosocomial infections as immune compromised individuals have a high risk factor of acquiring infection (Mesaros *et al.*, 2007; Pinheiro *et al.*, 2008; Jafari *et al.*, 2013). The mechanism of resistance observed in *P. auruginosa* is either through acquired mutational changes or through intrinsic mechanisms (Pinheiro *et al.*, 2008; Strateva and Yordanov, 2009). What makes this particular bacteria a difficult pathogen is the ability to exhibit almost all known mutational and enzymatic mechanisms resulting in multi-resistance (Strateva and Yordanov, 2009). Infections caused by this species lead to an increase in morbidity and mortality rates (Lister *et al.*, 2009).

Extrachromosomal materials, such as plasmids, carry resistance genes which are transported through processes such as Lateral Genetic Transfer (LGT). This process occurs through conjugation, where plasmids act as the mobile elements (Stokes and Gillings, 2011; Strauss *et al.*, 2015). These plasmids are not species specific thus transfer can be found in mixed microbial populations (Svara and Rankin, 2011).Amongst many others, *Pseudomonas* associated plasmids carry multiple resistance determinants such as the quinolone-resistant protein (Qnr). As has also been observed in other Gram-negative bacteria, the plasmids containing Qnr have been isolated from species such as *P. putida* and *P. aeruginosa*, with the ability to produce quinolone-resistant mutants at a higher rate (Robicsek *et al.*, 2006; Cayci *et al.*, 2014).

Pseudomonas plasmids are associated with antibiotic resistance genes, however, they are also linked to roles such as the degradation of pollutants and the production of plant hormones (Gross and Loper, 2009; Kumar *et al.*, 2010). There has been, however, an assumed link in the antagonistic action of *P. aeruginosa* FP6 towards *Colletotrichum gloeosporioides* and *Rhizoctonia solani* (Bakthavatchalu *et al.*, 2013). With reference to actinobacteria, there has been evidence of the involvement of plasmids in antimicrobial production (Novakova *et al.*, 2013). The presence of these genes was also said to facilitate for horizontal gene transfer amongst the bacterial population (Novakova *et al.*, 2013). This may also be considered for the genus *Pseudomonas* as the biosynthetic pathways for its metabolites share a commonality with the well-studied phylum (Gross and Loper, 2009). Also, as observed in bacterial strains which produce antimicrobial compounds, plasmids play a significant role in genetic instability during production and therefore the role they play in biosynthesis should be fully understood.

2.6 THE FUTURE FOR ANTIMICROBIAL COMPOUNDS DISCOVERY

The importance of bioactive compounds derived from microorganisms cannot be stressed enough. These compounds serve as derivatives for new pharmaceutical products used against diseases (Gontang *et al.*, 2010). The use of traditional methods in the search for antimicrobial compounds seemingly does not hold much promise for sustainable discovery and supply of novel compounds (Zazopoulos *et al.*, 2003). Due to the slow discovery rate in comparison with the high resistance rate, the study into new and/or innovative ways to improve discovery and production of SM (importantly antibiotics) is a topical research theme as the moment. The use of methods such as metabolic engineering, bioinformatics-based approaches, Genome and DNA sequencing are some prospects that have gained much attention. These methods will allow for the unearthing of new products, reduction in the re-discovery of known products and improvement in production (Udwary *et al.*, 2007; Gontang *et al.*, 2010; Weber and Kim, 2016).

2.7 GENOME, DNA SEQUENCE AN GENOME SCANNING

Genome sequencing certainly reveals tremendous information about microbial species including the adaptations to environmental conditions which may result in complex life cycles. These processes provide wealth of information about how bioactive compounds are synthesized and the specific genes responsible for their synthesis which then allows for their manipulation to obtain favourable results (Udwary *et al.*, 2007; Gomez-Escribano *et al.*, 2016).

The success of applying genomic scanning is determined by the accurate identification and separation of primary and secondary metabolite coding genes. This process involves the use of short randomly occurring genome tags referred to as GSTs which originate from genes involved in bioactive product synthesis. Due to their size, GSTs enable for a genomic scan which is likely to cover an organism's whole genome. The protocol involves the use of a high molecular weight DNA molecule to generate a genomic sampling library (GSL) and cluster identification library (CIL). These are then used to obtain GSTs which are translated to amino acid sequences and compared to biosynthetic genes from an appropriate data base (Zazopoulos *et al*., 2003).

2.8 METABOLIC ENGINEERING

The chemical synthesis and large extraction of bioactive compounds is achievable through genetic manipulation as microorganisms synthesize what will be sufficient for their individual needs (Adrio and Demain, 2005). Metabolic engineering is important in drug discovery and product improvement as it focuses on the use of resources to manipulate biosynthetic pathways rather than rely exclusively on the central metabolic pathways (Khosla and Keasling, 2003). The resultant enhanced target strain as well as fermentation processes are therefore directly attributed to greater product output (Weber *et al.*, 2015).

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CHAPTER THREE

3. MATERIALS AND METHODS

3.1.1 STUDY AREA AND SAMPLE COLLECTION

Samples were collected from Hogsback Forest reserve (43.4960° N, 75.2275° W) located in the Raymond Mhlaba municipality in the province of the Eastern Cape, South Africa. The samples were aseptically collected using sterile 50 ml, appropriately labelled, stored at 4˚C, plastic capped containers and transported to the laboratory within 6 hours where they were processed for further analysis.

3.1.2 ISOLATION MEDIA FOR PRESTIVE *PSEUDOMONAS* SPECIES

About 5 ml of sterile normal saline was added to 0.5 g soil sample and serially diluted. In triplicates, aliquots of each dilution were spread respectively on M1 and R2A agar plates, which were seeded with antibiotics to prevent the growth of fungi and none targeted bacterial species. The plates were incubated at 37°C for 7 days to allow for colony formation during which periodical examinations were made. Presumed *Pseudomonas* colonies were selected, isolated and purified by repeated streaking on the same media and incubated at the same temperature for 7 days. The pure cultures were maintained at 4˚C in nutrient agar plates, for short term preservation and in 10% glycerol stored at –80°C for long term preservation (modified Jensen *et al.*, 2005)

3.1.3 ANTIMICROBIAL SCREENING

3.1.3.1 SUBMERGED FERMENTATION AND AGAR-WELL DIFFUSION METHOD

An overnight culture was used for the fermentation process. In a 100 mL Erlenmeyer flask, 10 mL of nutrient broth was prepared, sterilized and inoculated with 10 mL of culture and kept for 7 days at 37˚C. After sufficient growth, a volume of 1 to 2 mL of the fermented culture was harvested by centrifugation to separate the cell mass from the supernatant. To determine the ability of antimicrobial production, antagonistic activity was determined against selected Gram-positive and Gram-negative bacteria (modified Jensen *et al*., 2005).

3.1.4 MOLECULAR CHARACTERIZATION OF *PSEUDOMONAS* ISOLATES

Using the ZR Fungal/Bacterial DNA Kit™ (Zymo Research), DNA was extracted from the chosen isolates. To amplify the target 16S rDNA region, DreamTaq™ DNA polymerase (Thermo Scientific™) as well as the specified primers presented in Table 1 were used. The extraction of the PCR products was achieved by using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research) and sequenced using the ABI PRISM™ 3500xl Genetic Analyser. Purification of sequence products was achieved by using the ZR-96 DNA Sequencing Clean-up Kit™ (Zymo Research) and analysed using CLC Main Workbench 7 and BLAST analysis (Altschul *et al*., 1997).

Table 3.1: Primers used in amplification of the targeted sequences (Lane *et al*., 1991; Turner *et al*., 1999).

NAME OF PRIMER	TARGET	SEQUENCE (5' TO 3')
16S-27F	16S rDNA sequence	AGAGTTTGATCMTGGCTCAG
16S-1492R	16S rDNA sequence	CGGTTACCTTGTTACGACTT

3.1.5 TEMPERATURE AND pH

All parameters were studied following the modified protocol of Singh *et al*. (2013).The culture (1 ml) was inoculated into 10 ml of fermentation media (pH 7.0) in a 100 ml Erlenmeyer flask and incubated for 7 days at varying temperatures from 25˚C to 40˚C. Following the above steps, the effects of pH on productivity was evaluated at different pH ranges (4–9). To adjust the fermentation media, 0.1 M hydrochloric acid and 0.1 M sodium hydroxide were used

3.1.6 PLASMID CURING

To activate the cells, isolates were grown for a 24 hour period on fermentation media (nutrient broth). A volume of 1 ml was transferred to 10 ml of fresh media containing different concentrations Ethidium bromide and Sodium dodecyl sulphate used as curing agents After a five day period, the cured cultures were plated on nutrient agar, to determine the effects of the chemical agents on the phenotypical characteristics. The antimicrobial activity of all cured strains was also determined (Das and Dash, 2014).

Table 3.2: The different concentrations used in curing plasmids in *Pseudomonas* strains.

CHEMICAL	LOW CONCENTRATIONS	HIGH CONCENTRATIONS
Sodium dodecyl sulphate	1 mg/ml	10 mg/ml
	5 mg/ml	11 mg/ml
	7 mg/ml	
Ethidium Bromide	2.5 µg/ml	100 µg/ml
	4.5 µg/ml	115 µg/ml
	6.5 µg/ml	125 µg/ml

3.1.7 PLASMID EXTRACTION

Plasmid extraction was done following the method of Kado and Liu (1981). Cells grown in nutrient broth were pelleted by centrifugation at 5 700 × rpm for 4 minutes (Labnet Z233 M-2 High capacity microcentrifuge).The cell pellet was suspended in 1 ml of buffer E. In the same solution, 2 ml of lysing buffer was added to disrupt the cell for release of intracellular contents. This was then mixed gently by agitation and heated at 65˚C for 20 minutes.

Two volumes of phenol-chloroform solution was added, mixed by briefly shaking and centrifuged at 6 000 × rpm for 15 minutes (Labnet Z233 M-2 High capacity microcentrifuge).The samples were used directly for gel electrophoresis on a 0.7% agarose gel.

REFERENCES

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CHAPTER FOUR

4. RESULTS

4.1 SAMPLE DESCRIPTION

The total number of samples obtained from Hogsback forest reserve totalled 22, and were processed according to a modified method by Jensen *et al*. (2005). After serially diluting the samples down to 10^{-4}, samples were spread on agar plates to facilitate for colony formation. The chosen samples were largely moist soil with a few samples collected from semi-dry areas and some had decaying matter (**Table 4.1**).

4.2 COLONY ISOLATION

Colony distinctions were recorded based on their phenotypic characterisation such as cell shape, colour and texture. Individual colonies were isolated and purified by re-streaking unto M1 and R2A media. With each sample, there were different distinct colonies that could be identified with varying pigmentations, most dominant being cream, and textures. However, all formed colonies were circular in shape (**Table 4.2**).

4.3 16S rDNA IDENTIFICATION

The identity of the isolated strains was achieved through sequencing of 16S rDNA. The sequences obtained were subjected to BLAST analysis and the relationship between known *Pseudomonas* species determined using MUSCLE software. The construction of the phylogenetic tree, which illustrates relativeness, was achieved by using MEGA6 software. The phylogenetic analysis illustrates that isolate A16 showed 80% homology with *Pseudomonas plecoglossicida* strain P4 and share a close ancestor with isolates Y52 and Y81. Isolate Y89 showed a 90% homology with *Pseudomonas* sp. Co-11a, and all isolates were closely related as they share a common ancestor (**Figure 4.1**)

Table 4.1: Detailed description of collected samples from different areas in Hogsback woodland.

SAMPLE CODES	DESCRIPTION
HB7	Moist soil and wood mixture
HBS8	Moist soil
HBS9	Moist soil
HBS10	Moist soil
HB11	Moist soil and decaying leaves
HBS14	Moist soil
HBS15	Semi-dry soil
HBS16	Moist soil
HBS17	Moist soil (from beneath decaying wood)
HBS20	Moist soil
HBS22	Muddy soil
HB23	Moist soil with decaying leaves
HBS25	Moist soil
HBS26	Moist soil
HBS27	Moist soil
HBS32	Moist soil
HBS33	Moist muddy soil
HBS35	Muddy soil
HB36	Moist soil and decaying wood
HB40	Soil particles and decaying wood
HBS42	Muddy soil
HBS43	Moist soil

Table 4.2: Observed phenotypic characterizations of different colonies formed on M1 and R2A Agar.

SAMPLE CODES	COLOUR	SHAPE	TEXTURE
HBS27	Cream, Yellow Brownish, Pink	Round	Smooth-edged Slimy
HBW29	Cream-white, Orange Pink, Yellow	Round	Elevated with smooth edges
HBS26	Orange, Yellow Cream, Pink Brownish	Round	Slimy
HBS10	Yellow, Cream-white	Round	Smooth
HBS25	Cream-white, Orange-brown, Yellow	Round	Smooth
HB23	Cream-white, Brown, Light-yellow, Orange-brown	Round	
HBS20	Brown, Cream, and creamy, Pink	Round	Slimy
HBS33	Yellow, Orange, Cream-white	Round	
HBS36	White, Light-yellow, Orange-yellow, Cream-white	Round	Uneven edges
HB11	Cream-white, Cream-brown, Greyish-brown	Round	Smooth edges
HBS35	White, Light yellow, Cream-white, Orange, Brownish-orange, Cream	Round	Slimy
HBS9	Cream-white	Round	Smooth
HBS43	Orange, Cream, Brown-orange, White	Round	Irregular edges Elevated, Smooth edges
HBS8	Light yellow,	Round	Smooth edges, Elevated in the centre
HBS42	Cream-white, Light yellow	Round	Irregular edges
HBS28	Cream-white, Yellow		Elevated edges

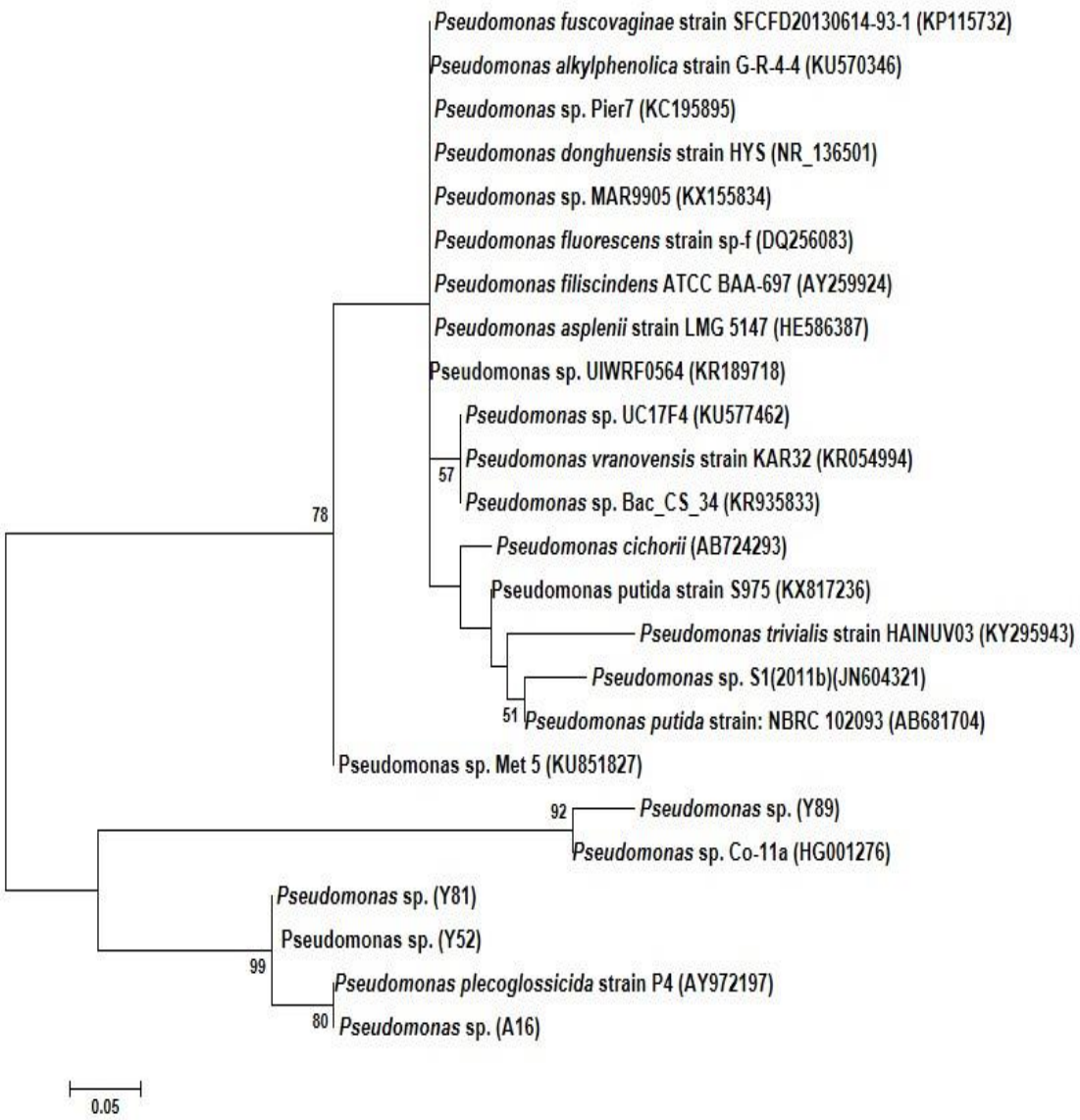

Figure 4. 1: Phylogenetic analysis by Maximum Likelihood method which shows the relationship between identified species based on partial 16S rRNA gene sequences.

4.4 ANTIMICROBIAL SCREENING

A total of 500 presumed *Pseudomonas* species were isolated and screened for their production of antibacterial compounds. The agar-well diffusion method was used to determine the varying activity and zones of inhibition measured against both Gram-positive and Gram-negative bacteria. Isolates that showed antimicrobial activity were recorded. A few isolates, such as A105, A107 and A16, showed excellent antimicrobial activity against both Gram-positive and Gram-negative bacteria with a maximum measurement of 21 mm. With the exception of isolate A16, the isolates lost activity as the screening processes continued (**Table 4.3**). Although the highlighted isolates were not the most active isolates, they showed reproducibility and insignificant change under optimisation conditions (**Table 4.4**).

Table 4. 3: Isolates which showed antimicrobial activity against selected bacteria.

	ZONE OF INHIBITION (mm)								
Isolates	**Gram-negative Bacteria**						**Gram-positive bacteria**		
	***E.coli* (1)**	***V. fluvialis***	***S.enteritidis***	***S.typhi***	***E.coli* (2)**	***P.aeruginosa***	***L. ivanovii***	***L. mono***	***E. faecalis***
A 105	---	20	12	---	14	18	18	16	---
A 107	---	21	---	---	15	20	20	---	---
A 92	---	10	14	---	13	15	11	---	15
A 86	---	---	---	---	---	---	10	---	---
A 78	---	---	---	---	---	---	---	---	12
A 61	---	---	---	---	---	19	17	---	---
A 33	---	---	---	---	---	---	10	---	---
A 22	---	20	---	27	---	---	15	---	---
A 66	---	---	---	---	---	---	---	24	---
A 16	---	21	14	---	---	---	---	---	---
A 70	---	11	---	15	11	---	---	---	---

A 17	---	20	---	---	---	---	---	---	---
Y 52	---	---	---	---	---	---	---	---	14
Y 69	---	---	---	---	---	---	---	---	13
Y 106	---	---	---	---	---	---	11	---	---
Y 78	---	---	---	---	---	---	---	---	14
Y 81	---	---	---	---	---	---	---	---	16
Y 79	---	---	---	---	---	---	---	---	15
Y 83	---	---	---	---	---	---	---	---	15
Y 96	---	18	---	---	---	---	17	24	---
Y 118	---	15	13	15	0	15	10	---	20
Y 155	---	---	---	---	---	---	---	---	12
Y 158	---	---	---	---	---	---	---	---	9
Y 86	---	---	---	---	---	---	---	---	19
Y 89	---	---	---	---	---	---	---	---	14
Y 90	---	---	---	---	---	---	---	---	15
Y 125	---	10	15	15	16	14	14	---	---

Table 4.4: Four identified Pseudomonas strains showed consistent antimicrobial activity and were chosen for further studies. These isolated wereoptimised for maximum production.

	ZONES OF INHIBITION (mm)								
Isolates	**Gram-negative Bacteria**						**Gram-positive bacteria**		
	***E.coli*(1)**	***V. fluvialis***	***S.enteritidis***	***S.typhi***	***E.coli* (2)**	***P.aeruginosa***	***L. ivanovii***	***L. mono***	***E. faecalis***
Y 81	--	15	--	15	18	14	14	--	14
Y 89	--	15	--	14	14	13	11	--	13
Y 52	--	14	--	14	15	14	--	--	12
A 16	--	17	--	13	12	--	--	--	--

4.5 OPTIMIZATION OF GROWTH PARAMETERS

The chosen isolates were grown under submerged fermentation to determine optimum production. For optimum temperature, 25˚C to 40˚C were considered and for optimum pH the range was between 4 and 9. These parameters play an essential role during production as they influence the action of compounds such as enzymes during SM synthesis, and therefore directly impact product yields. When looking at temperature variations, isolate Y81 and A16 performed the best with maximum activity observed under 35˚C while Y89 performed best at 25˚C and Y52 performed the same in all studied temperatures. It can be concluded that all the isolates are mesophilic in nature and were able to produce antimicrobial compounds at relatively low temperatures with limited activity at 40˚C. As shown in **Figure 4.6**, isolate Y81 is the best performing isolate with the capacity to act against a large percentage of the test organisms.

The study on pH variations did not correlate with those of temperature. The isolate Y81, although it performed optimally under differing temperature, did not do as well at different pH values. Isolate A16 was observed to have favoured acidic as well as neutral conditions, Y52 performed ideally under an acidic environment, Y89 performed under a basic environment and Y81 showed activity in all pH scale ranges with optimum results obtained when cultured in an acidic environment (**Figure 4.11**).

TEMPERATURE VARIATIONS

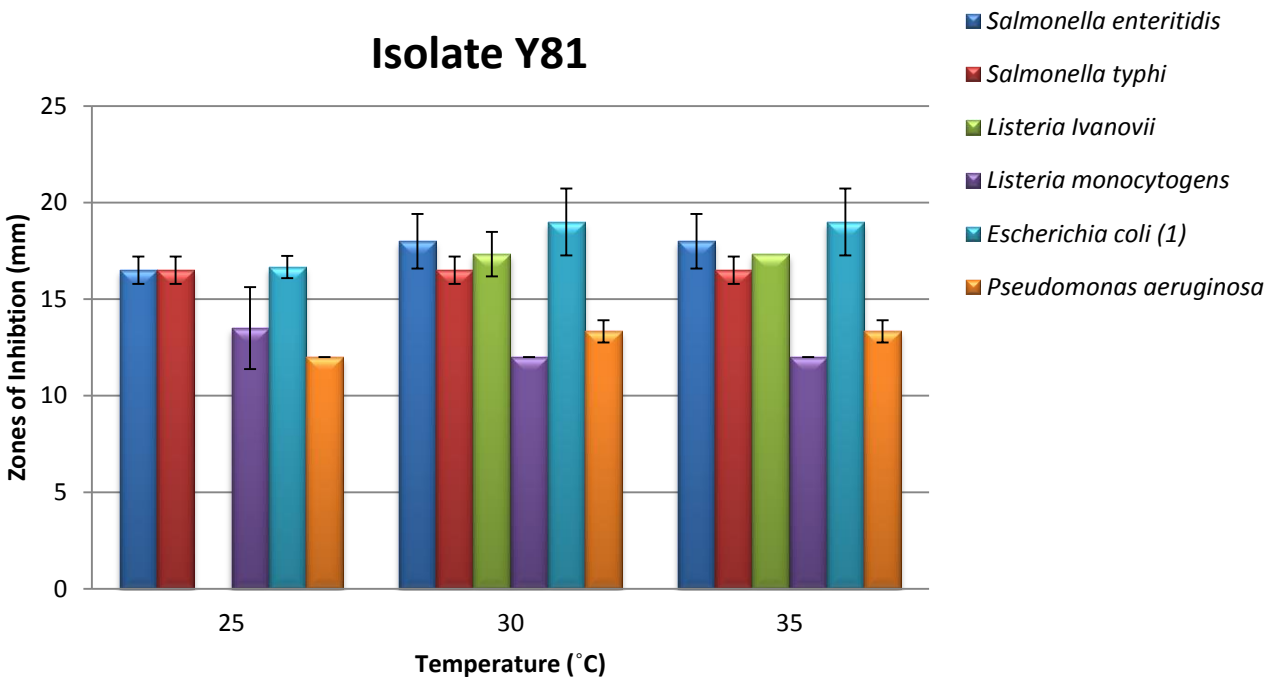

Figure 4. 2: Isolate Y81 showed the highest antimicrobial activity against an *E. coli* (1) strain followed by *S. enteriticus* and *L. ivanovii* and *S. typhi*. The best suited temperatures for production were 30˚C and 35˚C.

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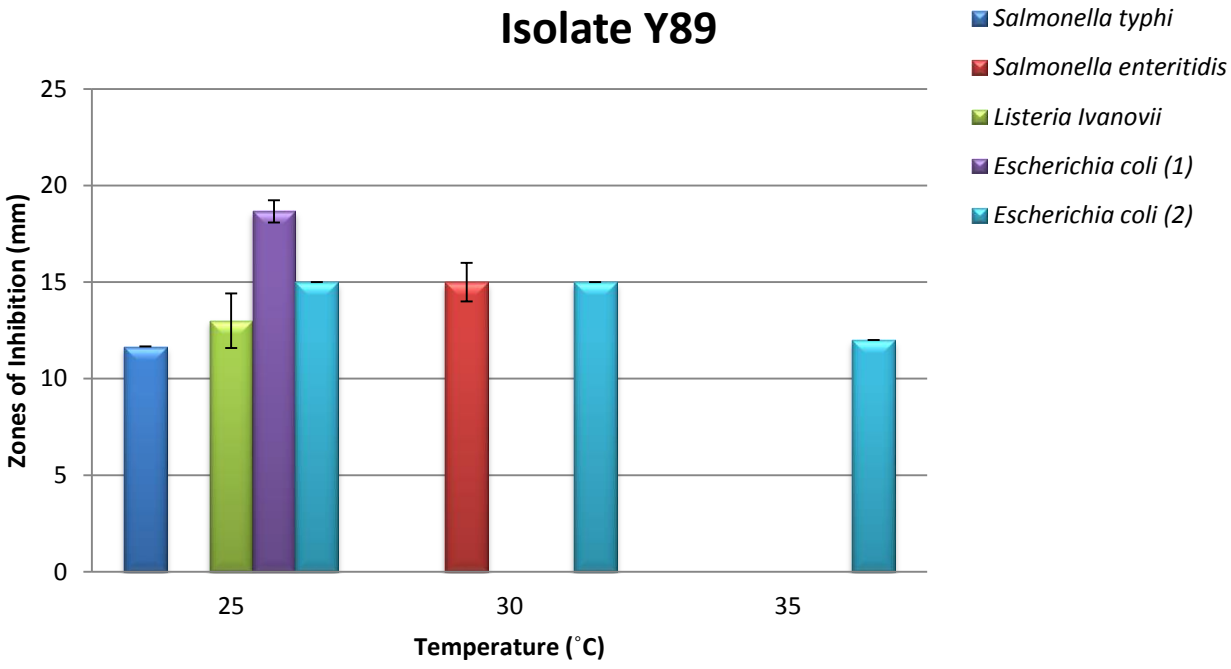

Figure 4. 3: Isolate Y89 showed optimum production at 25^0C, with maximum antimicrobial activity against *E. coli* (1 and 2).

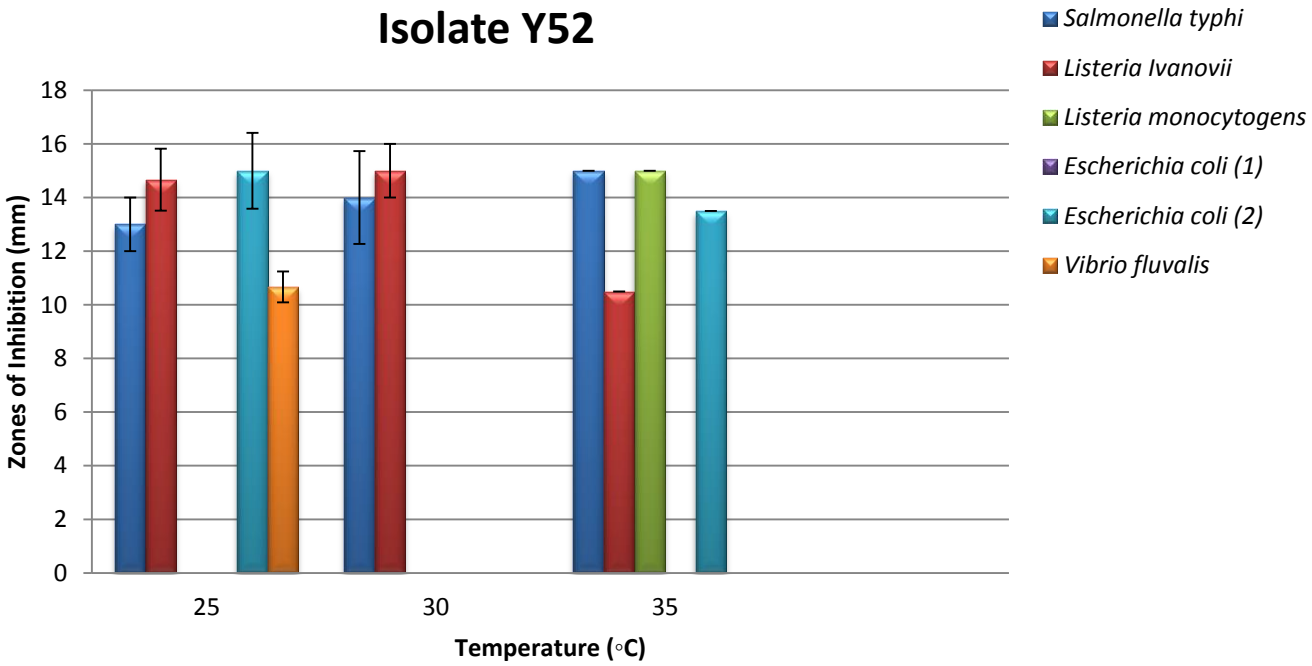

Figure 4. 4: The antimicrobial activity shown by isolate Y52 did not differ drastically between the test organisms.

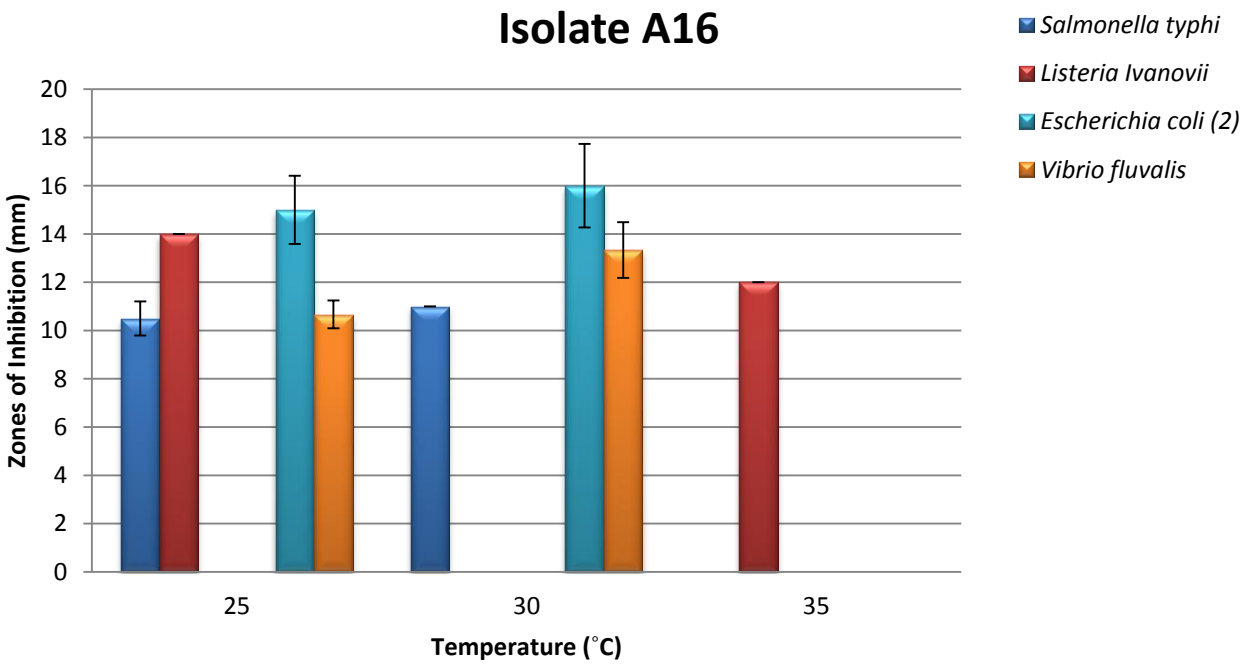

Figure 4. 5: As compared to other isolates, A16 acted on the least number of Gram-positive and Gram-negative bacteria.

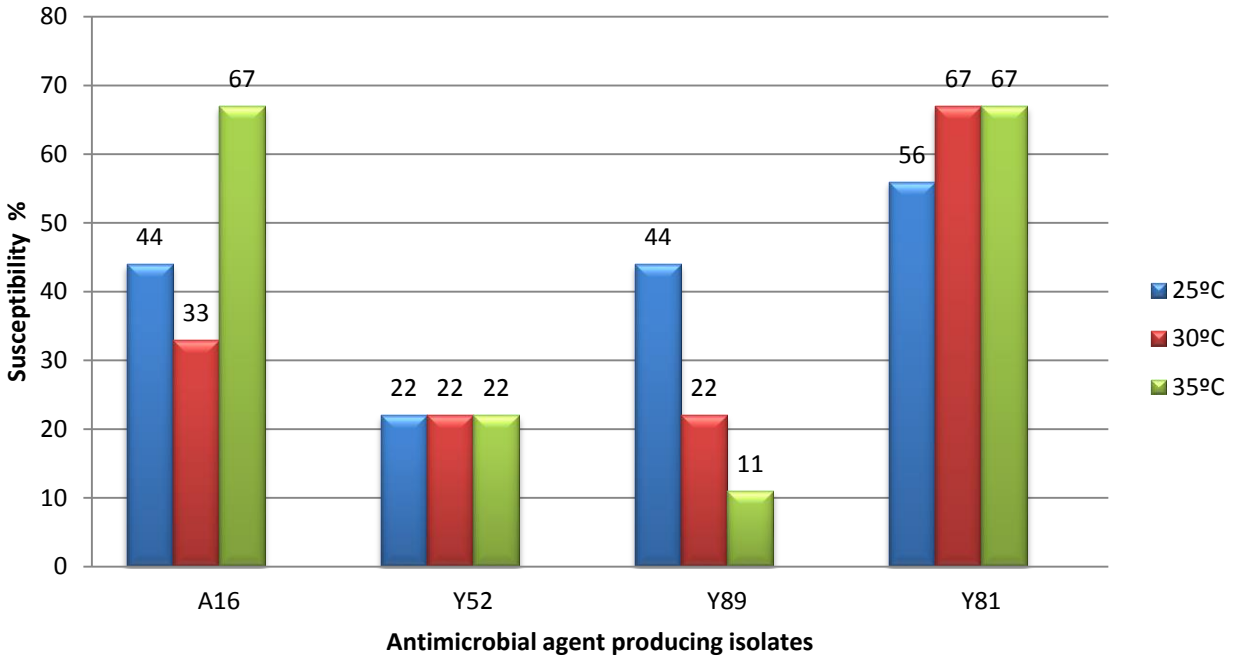

Figure 4. 6: Antibiotic production was observed on all studied temperature ranges, except for 40˚C. The optimum production temperature for all isolates was 35˚C with the exception of isolate Y89 while the isolate Y52 performed the same in all ranges.

pH VARIATIONS

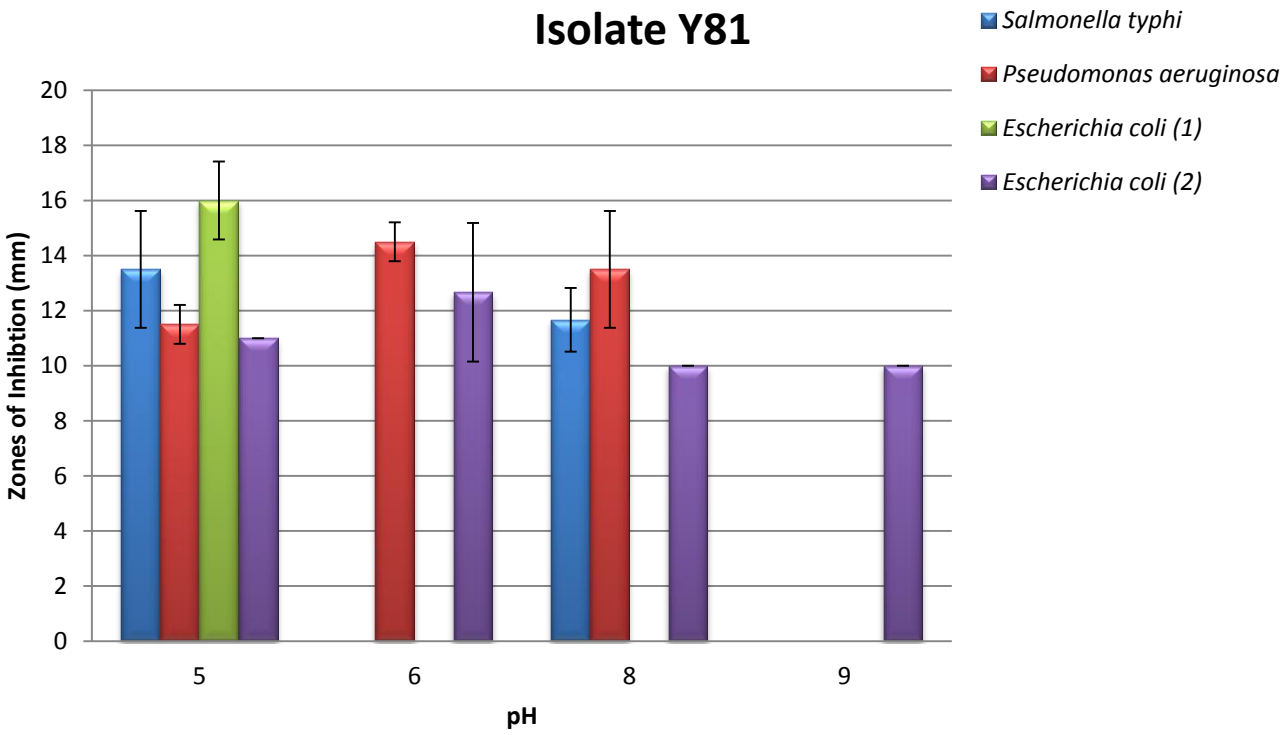

Figure 4. 7: Bacterial inhibition occurred at a basic pH, with the highest inhibition being against the Gram-negative E. coli.

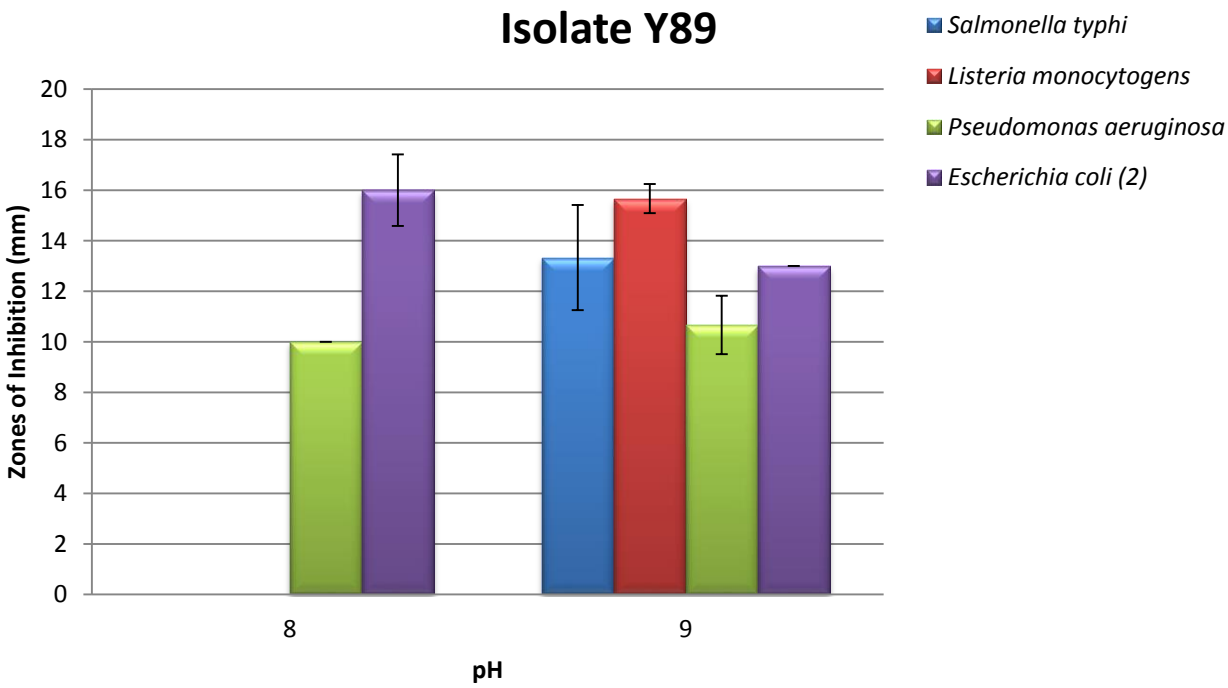

Figure 4. 8: A basic environment was most conducive for antimicrobial compound production, with optimum activity at pH 9.

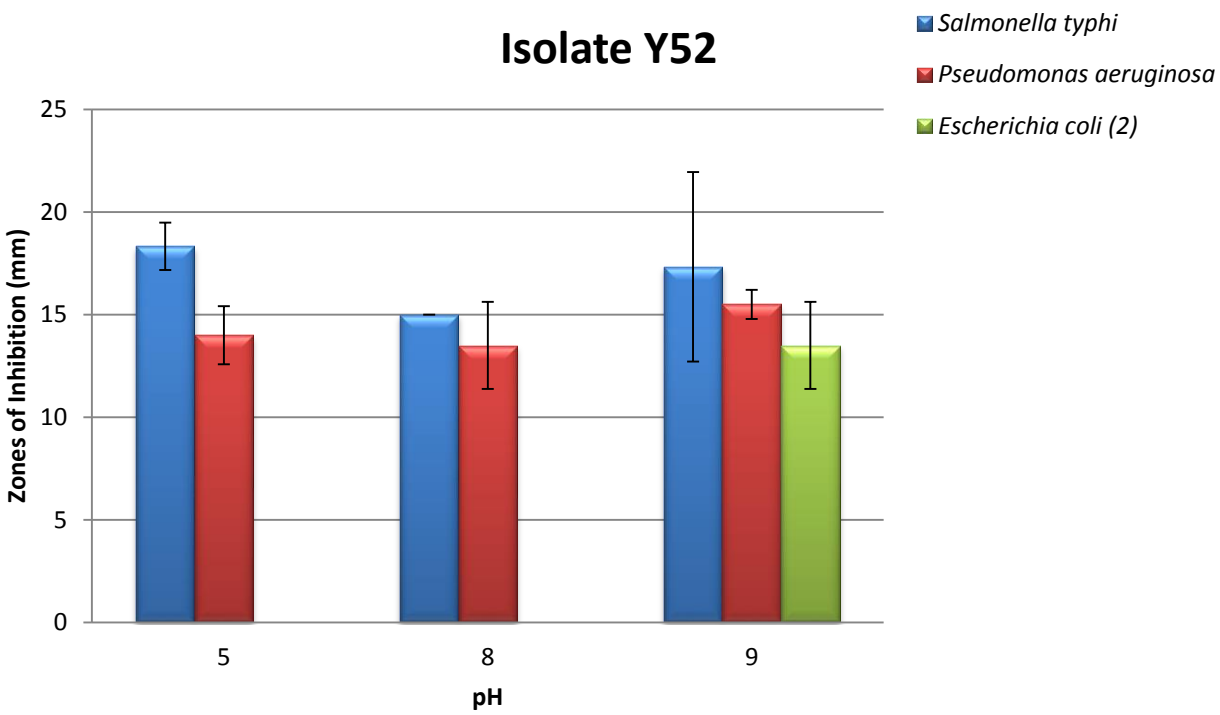

Figure 4. 9: Isolate Y52 showed versatility as antimicrobial production was observed both in acidic and basic media.

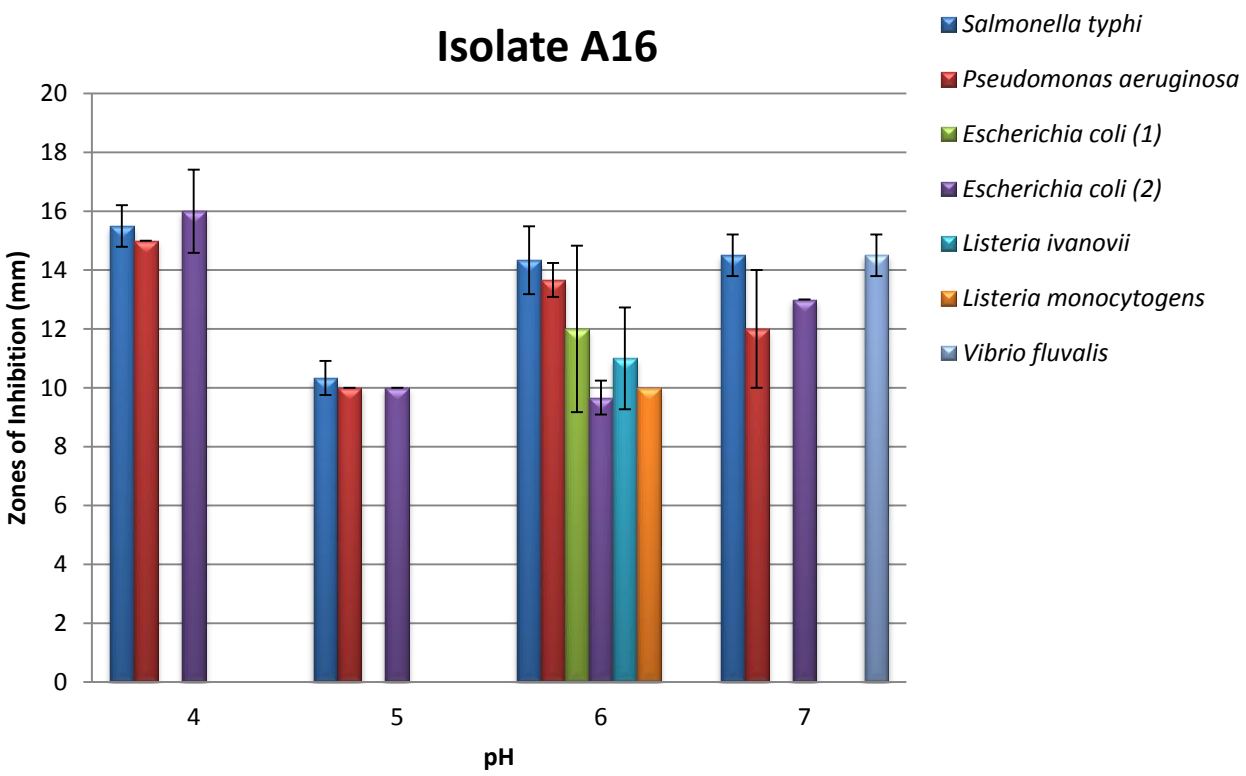

Figure 4. 10: The production of antimicrobial compound by isolate A16 was optimum under acidic conditions

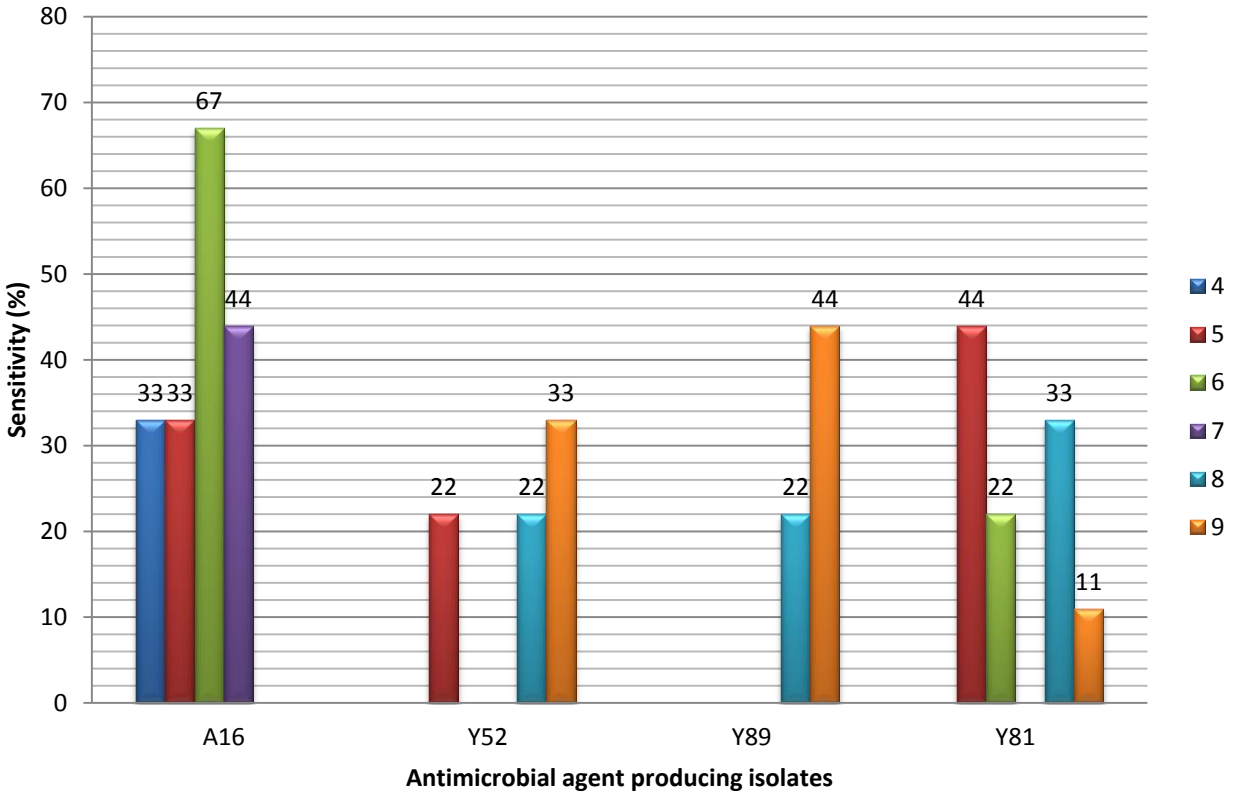

Figure 4. 11: Isolate A16 best performed under different pH range, with optimum activity at pH 6. Basic environments were also favourable to productions as indicated by isolate Y89.

4.6 PLASMID CURING

In determining the influence and/or role played by plasmids in antibiotic production, the producer isolates were cured of their plasmids using Sodium dodecyl sulphate (SDS) and Ethidium Bromide (EtBr) as curing agents at differing concentrations. To determine the success of the curing process under different concentrations, the results were viewed using a 0.7% agarose gel under UV light. The Quick Load® 1 kb Extended DNA ladder was used as a reference to determine the size of the plasmid(s) contained in each isolate. The plasmids in all isolates were found to be 48.5 kb in size with the exception of isolate Y89 which was 20 kb. **Figure 4.12** shows Y52 (left) and A16 (right), where sample Y52 (A) was cured by a concentration of 1 mg/ml SDS. Isolate A16 (1, 2, 3 4, 5, 6, 7 and 10) were cured by 1 mg/ml SDS, 5 mg/ml SDS, 7 mg/ml SDS, 125 µg/ml EtBr, 6.5 µg/ml EtBr, 5µg/ml EtBr and 11 mg/ml SDS, respectively with lane 11 being the uncured sample. In **Figure 4.13**, the represented isolates Y81 (left) and Y89 (right) were cured using the same concentrations and chemicals. For isolate Y81, lane M is the uncured sample and lanes A, B, G and J represent 1 mg/ml SDS, 5 mg/ml SDS, 7 mg/ml SDS and 10 mg/ml SDS, respectively.

After the curing process, antimicrobial activity was determined using he agar-well diffusion method. There were notable changes in both the antimicrobial activity of the isolates as well as their physical characteristics. The antimicrobial activity after curing was observed to have increased (**Figure 4.14**) as can be seen by the zones of inhibition. Plates 1 (Y81), 2 (A16) and 3 (Y89) were cured by EtBr. B11, H11 and F11 are isolated cured by a concentration of 7 µg/ml and B12, H12 and F12 by 11µg/ml. The peculiar appearance of the grown colonies (**Figure 4.15**) only occurred after plasmid curing meaning that the curing agents also had an impact in the growth of the selected bacterial cells. The colony colour was light yellow to a cream colour, which appeared moist.

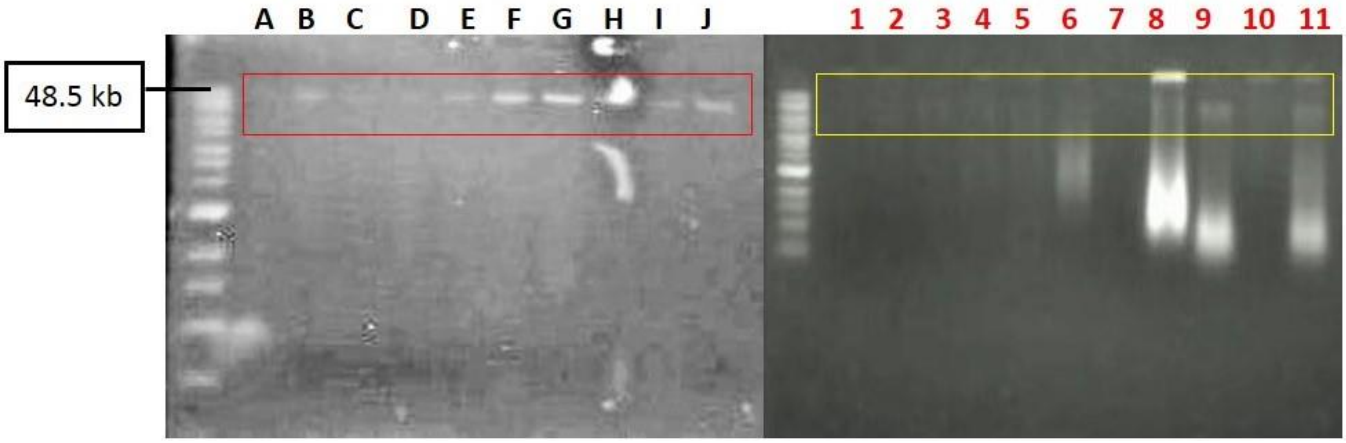

Figure 4. 12: Y52 (left) and A16 (right). The plasmid size for both isolates was 48.5 kb with a few curing agent concentrations able to cure the plasmids. Y52 (J) is the non-cured strain with Y52 (A) being an SDS (1 mg/ml) cured strain.

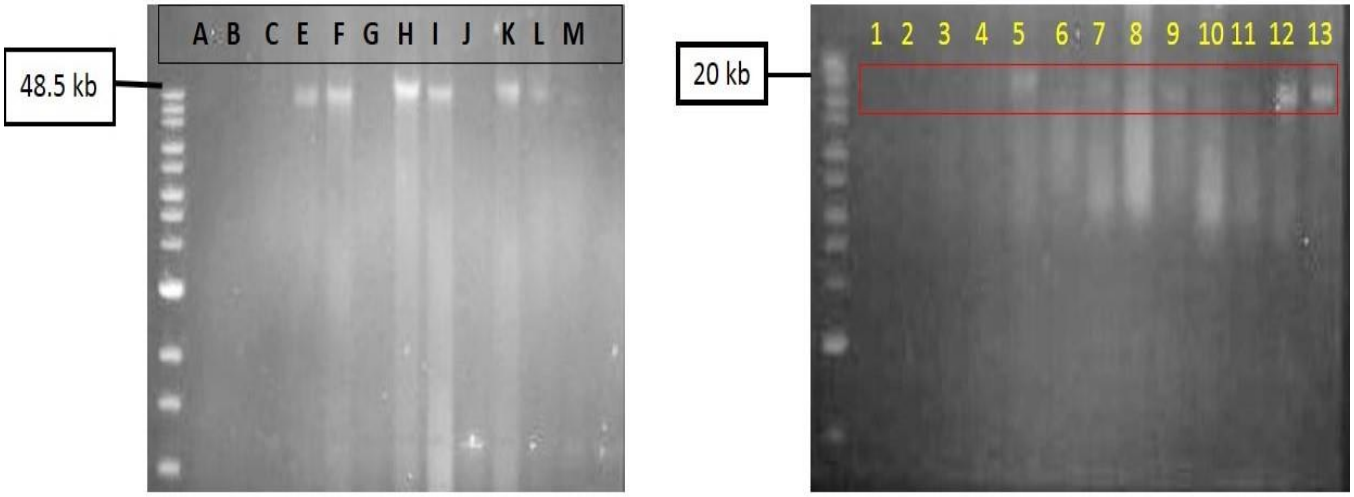

Figure 4. 13: Y81 (left) and Y89 (right). Both isolates were cured by SDS and EtBr at different concentrations. Y81 (M) and Y89 (13) represent the non-cured strains.

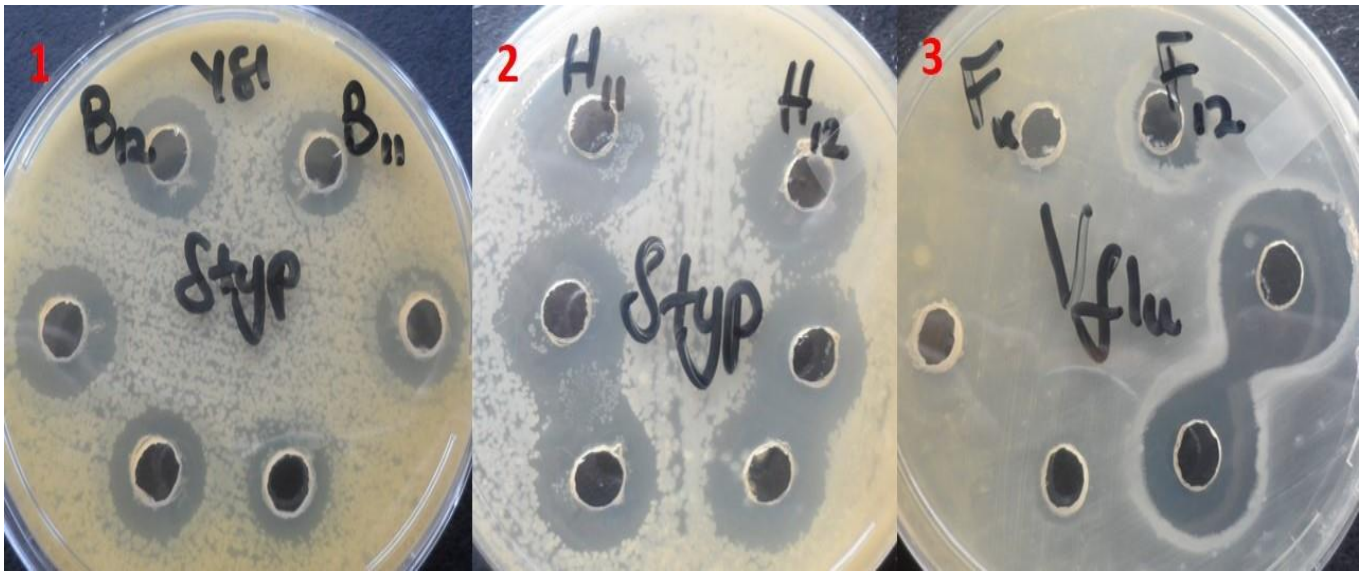

Figure 4. 14: After the curing process, antimicrobial activity was enhanced and showed more distinct and clear zones of inhibition. Y81 (1), A16 (2) and Y89 (3) represents the following; B11, H11 and F11 (EtBr - 7 µg/ml) and B12, H12 and F12 (EtBr - 11µg/ml). Y81 and A16 were assayed against *Salmonella typhi* and Y89 against Vibrio fluvalis.

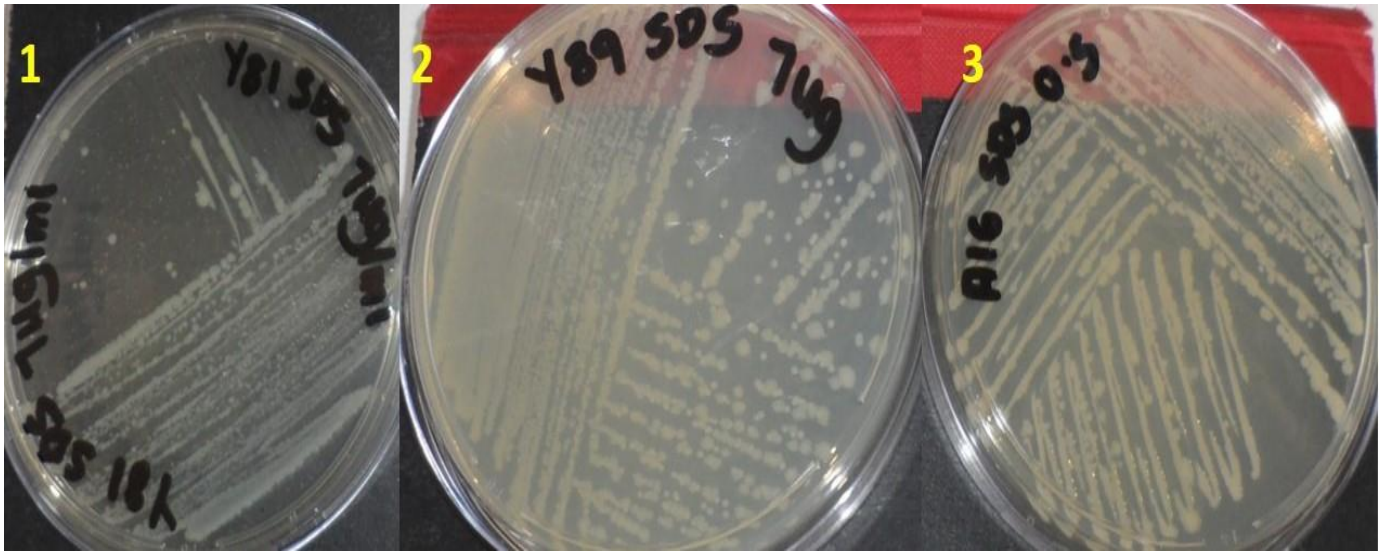

Figure 4. 15: The physical characteristics of the cured strains also showed a notable difference when compared to the non-cured strains. The colonies appeared to be pale in colour and moist.

CHAPTER FIVE

5.1 GENERAL DISCUSSION AND CONCLUSION

It comes as no surprise that most pathogenic bacteria are resistant to a number of antibiotics, in 2004 estimated at 70%, as they have evolved over the years of their existence on earth. Most pathogenic bacteria have been exposed to a range of naturally occurring antibiotics which has resulted in resistance genes as they have acquired survival mechanisms (Demain and Sanchez, 2009). This therefore is threatening the efficiency of available medications and is a major concern as infectious diseases are amongst the leading causes of death (Projan, 2003). In the modern day and age, antibiotics play a crucial part in the day-to-day activities. Many invasive medical procedures would be life threatening without the use of antibiotics and international travel and trade would be plagued by infectious and untreatable diseases (Outterson, 2014). A report by O'Neill, 2014 suggests that by the year 2050 the death toll, due to resistance, would amount to 10 million individuals each year resulting in negative impacts on economic growth.

In addition to resistance, the need for these new antibiotics is also pressed by the lack of progress in discovery and identification as a large majority of antimicrobials used in the present time are those discovered in the 1940s and 1960s (Lewis, 2013). The increasing threat has become more concerning than ever as recently, a "superbug" resistant to numerous antibiotics emerged in the United States in 2016. The isolated *Escherichia coli* strain encoded mcr-1 and $bla_{\text{CTX-M}}$ resistance genes located on the IncF plasmid (McGann *et al.*, 2016). There is, however, some glimpse of hope as new compounds such as teixobactin have been discovered. Teixobactin is a product of uncultured bacteria which showed activity against *S. aureus, Bacillus anthracis, Mycobacterium tuberculosis and Clostridium difficile* without any detectable resistance. It is specifically active against Gram-positive bacteria and myobacteria and acts as an inhibitor that disrupts the synthesis of peptidoglycan (Ling *et al.*, 2015). Discoveries such as the prior mentioned may be the answer to combating resistance as well as ending the use of toxic substances, such as colistin, as last resorts.

The isolated *Pseudomonas* species belong to two distinct clades where *Pseudomonas* species (Y81, Y52 and A16) falls in the same clade as *Pseudomonas plecoglossicida* and *Pseudomonas* species (Y89) is in close relation with *Pseudomonas* sp. Co-11a. Although *Pseudomonas* sp. Co-11a is associated with a number of *Pseudomonas* species, *P. plecoglossicida* is grouped under *Pseudomonas putida* Gram-negative bacteria which are associated with bioremediation and biocontrol functions (Nelson *et al.*, 2003; Otenio *et al.*, 2005). *P. putida* is reported as an opportunistic pathogen which is susceptible to antimicrobial compounds; however, there has been evidence of its ability to produce antimicrobial compounds (Bhattacharya *et al*., 2015). The first reported strain with the ability to produce an antimicrobial compound active against a wide range of bacteria, including multi-resistant bacteria, was isolated from marine sponge *Mycale Microsigmatosa* (Marinho *et al*., 2009). The production of antimicrobial metabolites was determined by the agar-well diffusion method, with the optimum production observed after a five day incubation period. Taking into consideration the habitat from which the isolates were obtained, the production of these antimicrobial compounds may be associated with defence. From the obtained results the isolates which were active against Gram-negative bacteria, with the exception of isolate A16, did not show reproducibility as the re-screening process no longer yielded the initial results and activity was lost. There were, however, a selected few that showed reproducible antimicrobial activity against Gram-positive. The varying activity observed between the two classes of target bacteria is directly influenced by the cell wall characteristic in each class.

Due to the antibiotic production observed from the selected strains, optimized fermentation conditions were studied. The effects of pH (4–9) and temperature (25°C–40°C) on antimicrobial metabolites production were conducted by inoculating a 24 hour old culture in nutrient broth.

The optimum temperature for the isolates is as follows; Y89 (25°C) with the highest antimicrobial action against *E. coli*, Y81 (30°C–35°C) with maximum inhibition against *E. coli* and *S. enteriticus*, Y52 (25°C–35°C) with good inhibitory activity against a majority of the test organisms and A16 which showed varying antimicrobial activity. Overall, the optimum temperature for production was 35°C, which inhibited the growth of 67% of all test organisms. These results, therefore, showed that the selected strains were strictly mesophilic by nature as neither isolate produced any compound beyond 35°C. This was also observed on antimicrobial production of a related species *P. putida*, where activity was observed when the isolates were grown at room temperature (Marinho *et al*., 2009). The optimum pH varied from acidic to basic but never neutral, as might have been expected when comparing with key antibiotic producers *Streptomyces* (Narayana and Vijayalakshmi, 2008). The importance of studying these parameters is due to the involvement of enzymes in secondary metabolite production. A number of crucial enzyme classes control and/or play an important function in the catalysis of reactions which result in SM synthesis and pH impacts significantly on their activity (Guimarães *et al.*, 2004).

Plasmids carried by bacterial cells, in most cases, encode genes which are associated with resistance against specific drugs (Svara and Rankin, 2011). They are one of the key targets in understanding mechanisms of resistance and how it can be eliminated. It is also important to study other metabolic activities that they might play part in, such as the production of SM. In this case, if a plasmid is responsible for the production of an important compound it is crucial to understand how much of a role it plays as this may influence production. One such method which assists in determining the role of plasmids in host cells is curing. There are a number of curing techniques which include using heat, radiation and chemicals. The chemical route was used in the current study, with SDS and EtBr as curing agents of choice. Using curing agents is mainly to promote plasmid segregation, and so far there is no universal agent specifically chosen for curing plasmids and as such studies are based on trial and error (Zaman *et al.*, 2010; Letchumanan *et al.*, 2015).

Both chemical agents showed curing abilities and thus allowed for the determination of the role played by plasmids in antibiotic production.

The plasmid size of all isolates, with the exception of Y89 (20 kb), was 48.5 kb. Although not all concentrations were effective in curing the plasmids, a conclusion could be deduced that plasmids were not involved in antibiotic production. After the curing process, the antimicrobial activity was determined using the agar-well diffusion method which gave unexpected results such as the increase in activity as opposed to reduced or halted activity against test organisms. The inhibition against test strains increased, giving clearer zones when compared to those observed prior to curing. One reason that may explain this occurrence is the mutation of genes by the chemical agents which might have altered with antibiotic production as was seen in studies on actinobacteria by Ogura *et al.* (1986) and Pudi *et al.* (2016). The exclusion of plasmid in antibiotic production therefore affirms that the genes coding for these compounds are chromosomal borne and cannot be acquired through horizontal gene transfer.

5.2 CONCLUSION

The results of the present study identified that the isolated *Pseudomonas* species showed activity against human pathogens, which is a significant finding. The group *Pseudomonas putida* is associated with bioremediation and biocontrol, two fields with no link to human pathogenicity. Taking note of this ability for antimicrobial production, the environment can play a major role in preparing bacterial strains to adapt to extreme settings where they may need to fight off predation and/or a need to withstand harsh environments. The Hogsback woodland is a diverse environment with interesting microbial life that might open a gateway to novel findings in the scientific world. It is an interesting venture to target such a location, which has not been scientifically impaired with, as there might still be interesting findings to discover.

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APPENDIX

TEST ORGANISMS

Gram-negative Bacteria	Gram-positive bacteria
Salmonella enteritidis	*Listeria Ivanovii*
Vibrio fluvialis	*Pseudomonas aeruginosa*
Salmonella typhi	*Listeria monocytogens*
Escherichia coli [*E. coli* (2)]	*Enterococcus faecalis*
Escherichia coli ATCC 3695 [*E. coli* (1)]	

PLASMID CURING

Extraction buffers

Buffer E	2 mM EDTA; 40 Mm Tris-Acetate
Lysing buffer	1 x TAE buffer
Phenol-Chloroform solution	1:1, vol/vol