Chapter Two

2. Extraction of the antimycobacterial compound from the Pseudomonas isolate

2.1 Introduction:

Infectious diseases have evolved over the decades resulting in the arrival of new bacterial strains that are unaffected by previously relied on antibiotics. The need for new compounds has spurred on the search for anti-mycobacterial compounds from natural sources. As a result the identification and isolation of antimicrobial activity has had a few guidelines that are customary for novel potential drug candidate research. These entail a rapid screening technique, which involves micro-organisms, enzymes for the detection of the activity and a simple extraction procedure for the antimicrobial compound (Omura, 1986). These standards have arisen due to the demand of the large scale production of bacterial secondary metabolites which have biological significance such as antimicrobial activity. With respect to the antibiotic industry, fermentation broth cultivation is the common route for optimising of the production of secondary metabolites. In order to ensure a good concentration when extracting the compound, the first step entails finding out the inducer which leads to the production of the desired product. Most bacteria are known to express secondary metabolites during the stationary phase of their life cycle. This is believed to be due to various factors such as oxygen and nutrient deficiency, which leads to the elicitation of bacterial stress response and consequently to an increase in production of secondary metabolites. Once the production of the compound can be linked to a particular stress response, extractions can then be attempted.

There are several methods that have been used to extract natural products and antimicrobial compounds from micro-organisms. The conventional way of extracting an antibacterial compound is to prepare a crude extract which is thereafter purified, analysed and tested against bacterial assays (Potterat and Hamburger, 2006). There are three main methods that are used to extract secondary metabolites namely, liquid –
liquid or solvent extraction, reverse phase micelles and solid phase extraction (Yarbrough et al., 1993; Fendenuik and Shand, 1998; Tingyue, 2000). The most commonly used and favoured method is the liquid – liquid extraction. This method uses the separation of two phases that arise when immiscible liquids such as organic solvents and water are mixed together (Tingyue, 2000). The different phases allow for the compound with a particular polarity to reside in the liquid with the corresponding polarity. The different phases can be collected and reduced to produce crude extracts which can then be tested for antimicrobial activity using bacterial assays.

The bacterial assays used to assess antibacterial that are commonly used are divided into three groups, namely, diffusion, dilution and bioautographic methods. The first assay, diffusion was generally used to determine the amount of an antimicrobial compound in a crude extract. It makes use of a disc, hole or cylinder as a reservoir for the extract which is directly in contact with agar that contains the test organism. The second assay, dilution assays, is used to determine the minimum inhibitory concentration of the extract, antibiotic or essential oil. There are two variations of this assay an agar and liquid form. With the agar assay, a fixed amount of the extract is mixed into the agar of nutrient plates and with respect to the liquid form, the turbidity of the culture serves as an indicator as to how effective the compound or extract concentration is (Rios, Recio and Villar, 1988). These assays are standard techniques used to characterise antibacterial extracts and would be modified in this study in order to characterise the activity of the extract obtained from the Pseudomonas isolate.

Given that the reason for the observed activity of the Pseudomonas isolate was not known, an investigation to identify if the activity was a result of nutrient competition or a secreted compound was done first. Once bacterial competition was eliminated as the reason for the activity, extractions that made use of organic solvents such as ethyl acetate, methanol, chloroform and acidified acetone were referred to. These solvents and methods were used to isolate various types of antimicrobial compounds such as bacteriocins, cyclic lipopetides and antimicrobial peptides from Bacillus and Pseudomonas species and other micro-organisms which produce secondary metabolites (Kuiper et al., 2003). The methods rely on the premise that different molecules have different charges and can be precipitated at a particular pH or made soluble in a specific organic solvent (with similar polarity). With respect to the
Pseudomonas strain that was isolated, several attempts to extract the activity were made and will be discussed further (section 2.2). For the purposes of this study the Pseudomonas isolate will be referred to as Pseudomonas αmb.

2.2 Materials and methods:

2.2.1. Culturing of bacteria:

The isolated bacterial strain (Pseudomonas αmb) and other bacterial isolates (Escherichia coli 32, Escherichia coli 29, Pseudomonas, putida dss2, Pseudomonas. putida 317, Micrococcus luteus NTNC 8983, Bacillus cereus EL39, Bacillus subtilis DLS) were propagated for experiments as follows: a single colony of the bacteria was inoculated into 20 ml of Luria broth (LB) and incubated at 37°C (30°C for P. putida dss2 and 317) on a shaker (180 rpm) overnight. For the growth of M. smegmatis mc²155, one colony was inoculated into 20 ml of 7H9 Middlebrook media supplemented (0.05% Tween 80, 2% glucose and 0.85 % NaCl) and incubated overnight at 37°C on a shaker (180 rpm). For M. luteus NTNC 8389, the exact same procedure was followed with the exception of the growth media i.e. tryptone soy broth (TSB) instead of LB.

2.2.2 Assessment of the inhibitory activity of the Pseudomonas isolate and crude extracts:

In order to assess the inhibitory activity of the isolate an indicator plate assay was done using M. smegmatis mc²155 as the indicator strain. Mycobacterial indicator plates (IPs) were made using 7H10 Middlebrook media which was seeded with 10% mycobacterial cultures (OD₆₀₀ₙₐₐ of 0.6). The Pseudomonas αmb cultures, were streaked onto M. smegmatis mc²155 IP’s and incubated overnight at 37°C and viewed for clear zone formation. For the testing of crude extracts, a modified indicator plate method, where wells were made in the indicator plates by punching holes in the agar with a Pasteur pipette was used. The crude extracts were added to the wells of the modified IP’s and were incubated at 37°C overnight.
2.2.3. Confirmation that the production of an antimycobacterial activity by the *Pseudomonas amb* was unique:

Several *Pseudomonas putida* strains, namely 317, 236, 207 and dSS2 (obtained from the culture collection of Dr. D. Lindsay) were streaked onto *M. smegmatis mc²155* indicator plates (IP) (7H10 seeded with 10% bacterial culture) and screened for similar inhibitory activity to that of the *P. amb* strain.

2.2.4. Extraction of antimycobacterial activity:

Based on our assumption that the isolate was secreting a compound that produced the inhibitory activity, it was hypothesised that the active compound could be present in the supernatant. Thus, the initial attempt to extract the activity was done by separating the bacterial cells from culture via centrifugation and the syringe filtered supernatant. The cell supernatant was tested in a bioassay (2.2.3); however, no activity was observed (data not shown). We then attempted to use ethyl acetate to extract experiments the anti-mycobacterial compound. The methods used were adapted from Wratten et al 1977.

2.2.4.1 Determination of the appropriate bacterial culturing method for the extraction of the antimycobacterial activity:

We attempted to extract the compound from a few sources, including bacterial cells from both broth and agar plates, as well as from spent agar and culture supernatant. In order to determine which method was the most effective, at extracting the bioactivity, the results from the crude extracts were standardised for this comparison as follows:

\[
\text{Wet cell mass (g)} \times \text{Volume tested (µl)}
\]

Final volume of extract (µl)
a) Ethyl acetate extraction from broth culture (Wratten et al 1977):

A 300ml *Pseudomonas* culture (O.D$_{600nm}$ of 1.2) was precipitated via centrifugation (6000 rpm for 10 minutes) and the wet cell mass was recorded. Approximately 300ml and 200ml of ethyl acetate were added to the supernatant and wet cell mass respectively. The emulsions were incubated at 4°C for 5 days. The organic phases were removed and the wet cell mass extract was filtered with cellulose filter paper (grade 1) to remove cell debri. The extracts were reduced via rotary evaporation (50°C - 60°C). A 50µl aliquot of each extract was tested in a bioassay using *M. smegmatis* mc$^2$155 indicator plates (control- working extract and ethyl acetate alone). The extracts were also streaked onto LA plates to ensure that the extract were free of *Pseudomonas* cells. The plates were incubated overnight at 37°C.

b) Ethyl acetate extraction of antimycobacterial activity from agar:

One hundred 7H10 plates and 7H10 *M. smegmatis* mc$^2$155 IP’s respectively were inoculated with the *Pseudomonas isolate* culture (incubated for 2 days at 37˚C). The total wet cell mass of the bacterial cells was recorded. Approximately 300ml of ethyl acetate was added to the bacterial cells and the agar (liquidised agar) respectively. The flasks were incubated at 4°C for 5 days. The organic phases of the extracts were filtered via cellulose filter paper and reduced via rotary evaporation (50- 60°C). The extracts were tested in the *M. smegmatis* IP bioassay mentioned previously.

c) Effect of air availability on the production of the antimycobacterial compound:

A single colony inoculation of *P. amb* in LB (50ml) was done in 3 different volume Erlenmeyer flasks, i.e. a 50ml, 100ml and 250ml respectively. The cultures were incubated for 4 weeks at 37°C (180rpm). The bacterial cells were precipitated (8000rpm for 15 minutes) and the wet cell mass recorded. The wet cells were suspended in 100ml of ethyl acetate at 4°C and equilibrated for 7 days. The ethyl acetate was filtered (cellulose filter) to remove cell debri and reduced via rotary-evaporation (60°C). 100µl of each extract was tested for bioactivity using the bioassay.
d) **Large scale extraction from *Pseudomonas* isolate using bacterial cells propagated on 7H10 agar plates**

Ninety eight 7H10 agar plates were spread with 0.1ml of *P. putida* culture (OD$_{600nm}$ of 1.885) and incubated for 2 days at 37°C. The bacterial cells were scraped off the plates using a spatula and the wet cell mass was recorded. The wet cells were mixed with 300ml of ethyl acetate were incubated at 4°C for 7 days. During the week the extract was shaken gently and a glass pipette was used to separate cells (to prevent clumping) to ensure maximum exposure of the cells to the ethyl acetate. Thereafter the extracts were filtered to prevent the bacterial cells from being present in the crude extract using a grade A, sterile cellulose filter (autoclaved). The ethyl acetate was reduced via rotary evaporation (50°C). A 50ul aliquot of the extract was tested as mentioned in 2.2.3.

e) **Optimised ethyl acetate extraction technique:**

For the ethyl acetate extraction procedure, glassware that had been cleaned with 70% isopropanol and baked at 300°C was used to avoid detergent contamination. An aliquot of 0.1ml of a *P. putida* culture (OD$_{600nm}$ of 1.885) was spread plated onto 500 7H10 plates (dried for 2 days in 37°C incubator). The plates were incubated for 3 days at 37°C. The bacterial cells were scraped off the plates and the wet cell mass recorded (8g). The cells were mixed with 300ml of ethyl acetate in a glass flask and were incubated at 4°C for 5 days. During the week the extract was shaken gently and a glass pipette was used to separate cells (to prevent clumping) to ensure maximum exposure of the cells to the ethyl acetate. Thereafter the extracts were filtered using autoclaved a grade 1A, sterile cellulose filterpaper to prevent the bacterial cells from being present in the crude extract. The ethyl acetate was removed via rotary evaporation (50°C). The precipitate was resuspended in 3ml. A 20ul aliquot of the extract was tested as mentioned in 2.2.4a and c.

2.3 Testing of the crude extract against environmental strains:
2.3.1 Determination of colony forming units of overnight bacterial cultures:

In order to standardise the number of bacterial cells used to make an indicator plate among the various bacterial strains, the cfu/ml of each bacterial strain after an overnight incubation was determined. Standardisation of the indicator plate allowed for comparison among all strains of bacteria tested.

All the respective bacterial cultures were grown in 20ml of LB (in a 50ml falcon tube) that was inoculated from a single colony. The strains were propagated by being placed on a shaker (200rpm) overnight in the 30°C \((Pseudomonas putida ~317 \text{ and } Pseudomonas putida~ dss2)\) and in the 37°C \((Mycobacterium smegmatis, Bacillus subtilis DLS, Bacillus EL39, Micrococcus luteus \text{ and } E. coli 29 \text{ and } E. coli 32)\) incubators respectively. The time of inoculation and time of removal from the incubator was recorded for each strain (see Appendix C, Table13). A serial dilution \((10^{-1} \text{ to } 10^{-10})\) of the respective cultures was done where 50µl of each dilution was spotted onto an LA plate. The plates were incubated overnight in the appropriate incubator.

2.3.2 The effect of dilution of the crude extract on the zone of inhibition amongst the respective bacterial strains:

It was assumed that since the \(Pseudomonas\) isolate was found on an \(M. smegmatis\) plate, the inhibitory activity produced could be specific to mycobacteria. In order to investigate this further the crude extract was assayed against several environmental strains. The extract was diluted in different ratios and the inoculum used to seed the respective indicator plates was standardised. The following cultures were grown \((\text{O.D}_{600nm} \sim 0.6-0.7)\) namely: \(P. putida ~317, \text{ dss2}; E. coli 29, 32, 49; Bacillus cereus DLS, 39, EL39\) and the \(Pseudomonas\) isolate. The LA was seeded with 10% of the bacterial cultures. The extract was tested using the modified IP method (previously mentioned in 2.2.2) as follows- some of the extract was diluted to a final volume of 20µl, i.e 1:1, 1:3 (ratio of crude extract: ethyl acetate) and 20 µl of undiluted extract, and undiluted ethyl acetate was also tested. Dilution of the crude extract would help to
determine if the extract activity is enhanced since a high concentration of the compound would cause aggregation of the antimycobacterial compound thus leading to a decrease in the inhibition shown. The plates were incubated overnight at the respective temperatures 30°C for the P. putida 317 and dss2 strains, and 37°C for the remaining strains.

2.4. Results:

2.4.1 Confirmation of specificity of the antimycobacterial activity of the bacterial isolate

Since various Pseudomonas species are capable of producing secondary metabolites due to nutrient stress, i.e. nutrient competition, we considered that other Pseudomonas isolates could possibly react in a similar manner. Thus in order to be certain that the antagonistic activity (clear zones of approximately 2mm in width around the Pseudomonas growth) that was observed was specific to our isolate several environmental Pseudomonas strains were streaked onto M. smegmatis IP’s and observed for inhibitory zones. The inhibitory activity was only observed on the indicator plate which was streaked with our isolate (Fig. 3A). Isolates that were streaked onto plates C, D and E (Fig. 3) showed poor growth on the 7H9 medium in comparison to those streaked on A and B. However, P. putida dss2 (Fig 3B) did grow well and did not show any zone inhibition. This implied that the inhibitory activity was unique to our isolate and may therefore reflect the presence of a secreted product.

Figure 3: Streak plates of Pseudomonas isolate and other Pseudomonas strains on M. smegmatis mc2155 IP’s. The streak plates are in the following order: A: Pseudomonas isolate, B: P. putida dss2; C: P. putida 317, D: P. putida 236 and E: P. putida 207. The plates were observed for the appearance of clear zones around the bacterial growth. Clear zones were only observed with our isolate (A) whereas no zones were observed with the other isolates (B-E).
2.4.2 Extraction of antimycobacterial activity:

Since we assumed that the activity seen was due to the secretion of a compound we hypothesised that the compound would reside in the supernatant in the broth culture. However extraction from the supernatant was not possible, hence ethyl acetate was introduced since the probability that the compound is non-polar and thus would be identified in a polar medium (supernatant).

2.4.2.1 Determination of the appropriate bacterial culturing method for the extraction of the antimycobacterial activity:

Figure 4: Affect of media on appearance of zones. Bioassays were done using different media in the indicator plate. Luria agar (LA) served as a nutrient rich medium (plate on the left) whilst 7H10 acted as a low nutrient medium (plate on the right). From the figure the difference in inhibitory activity on the 7H10 IPs are much clearer to that of the LA IP. The inhibitory response seen could be initiated by nutrient stress or bacterial competition.

Since relatively large areas of inhibition were seen on the M. smegmatis mc²155 7H10 IP’s when the Pseudomonas isolate was streaked on them, we considered that the compound might have diffused into the agar in large concentrations. With general extraction methods a large amount of cell mass is needed in order to obtain a high concentration of the inhibitory compound to be extracted (Wratten et al., 1977). We hypothesised that mass cultivation of the Pseudomonas isolate in broth culture and on more agar plates might yield a greater cell mass and therefore facilitate a better extraction of the compound. We also considered that the inhibitory compound could be associated with the cell wall of the bacteria. Thus the compound was extracted from the cells and culture medium respectively with the 7H10 plates without the M.
*smegmatis* mc²155 present (control) using ethyl acetate. Table 3 above shows the different values for the respective cell, supernatant and agar extracts where z.o.i of 5mm is equal to the width of the well. From Table 3 it can be seen that more cells were attained using the 7H10 agar plates (control) in comparison to 7H9 broth. Despite this, the respective cell and agar extracts attained from the 7H10 plates (7mm and 5mm respectively) did not differ significantly (1mm difference) from the broth culture cell and supernatant extracts (6mm and 6mm respectively). Also, the possibility that *M. smegmatis* mc²155 enhanced the production of the compound seemed unlikely, since both the 7H10 plates and 7H10 IP’s produced a z.o.i of 5mm from the agar. Only a faint z.o.i was seen from the broth supernatant and cell extracts as well as the cells from the 7H10 plates (control). The latter had a marginally greater radius (by 1mm) as can be seen in Table 3. Even though the broth culture produced a slight z.o.i, the extraction from the bacterial cells obtained from the 7H10 plates was favoured since the clear z.o.i had been maintained for more than 3 days whilst the indicator strain recovered after two days with the broth culture extracts.

Table 3: Comparison of the crude extracts obtained from the different ethyl acetate extraction methods.

<table>
<thead>
<tr>
<th></th>
<th>Broth culture</th>
<th>Growth on agar plates</th>
<th>Growth on IP’s</th>
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<tr>
<td></td>
<td>Sp Cells cells</td>
<td>Agar Cells Agar</td>
<td></td>
</tr>
<tr>
<td>Wet cell mass (g)</td>
<td>2.7 2.7</td>
<td>3.6 3.6</td>
<td>4.9 4.9</td>
</tr>
<tr>
<td>Final volume (µl)</td>
<td>8000 5000</td>
<td>4500 11000</td>
<td>7000 10000</td>
</tr>
<tr>
<td>Volume tested (µl)</td>
<td>50 50</td>
<td>50 50</td>
<td>50 50</td>
</tr>
<tr>
<td>Standardisation unit*</td>
<td>0.71 0.03</td>
<td>0.04 0.02</td>
<td>0.03 0.02</td>
</tr>
<tr>
<td>z.o.i radius (mm)</td>
<td>6 6</td>
<td>7 5</td>
<td>6 5</td>
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</table>

Key: supernatant (sp), zone of inhibition (z.o.i), * standardisation unit calculated from the formula in section 2.2.4.1. A z.o.i of 5mm = width of the well.
2.4.2.2 Effect of air availability on the production of the antimycobacterial compound:

Since a z.o.i from the broth cultures was observed in previous experiments, the possibility that the compound could be produced due to another factor in broth culture was investigated. Taking into consideration that many secondary metabolites can be produced under oxygen stress, we proposed growing the same volume of culture in flasks containing different air volumes during propagation. The weight of the wet cell mass was negligible (0.57g for the 50ml, 0.44g for the 100ml and 0.50g for the 250ml). From the Fig 5 above, we can see that the z.o.i of the crude extracts from the broth cells are minimal. However, it seems that the flask which contained most air space i.e the 250ml flask did not produce any z.o.i at all. The same experiment was done using 7H9 media in larger volumes of culture, but no significant z.o.i were seen (data not shown). Thus the preferred route for extraction of this compound was to extract the antimycobacterial compound from the bacterial cells grown on 7H10 agar.

![Figure 5: Ethyl acetate extraction of antimycobacterial activity from broth culture.](image)

The wells are labelled in accordance to the extracts from the respective volume flasks. All flasks contained a 50ml culture of the *Pseudomonas* isolate.

2.4.2.3 Large scale ethyl acetate extraction on *Pseudomonas* isolate cells grown on 7H10 using plates
A large scale ethyl acetate extraction adapted from Abrahams (2004) was attempted on cells grown on 7H10 plates. The bacterial cells appeared to have changed in appearance after the incubation period. The colour of the bacterial cells had changed from a pale yellow to a yellow-brown colour which was similar to the media. The extract was an intense yellow colour. The zone of inhibition observed (Fig 6B: z.o.i radius = 9mm) showed two concentric zones of inhibition present. This suggests that two activities could have been present in the crude extract. In order to determine if this was the case a large-scale extract was tested against *M. luteus* NTNC 8389. *M. luteus* NTNC 8389 is a bacterium that is found in the same phylogenetic tree as *M. smegmatis* (Muto and Osawa; 1987) and is more susceptible to inhibitory compounds than *M. smegmatis*.

From Fig 7 it can be clearly seen that there are two zones present on the respective IP’s plates. The *M. smegmatis* IP has a large clear zone in comparison with the *M. luteus* IP (B) which is much smaller whilst the secondary turbid zone is smaller in the *M. smegmatis* IP in comparison to the *M. luteus* IP. The size of the zones appeared to be inverted when comparing the size between the two plates even though the same extract was used. These observations suggest that the activity of the compounds could be specific against the different bacterial species.
2.4.3 Testing of crude extract against environmental bacteria:

The testing of the extract against environmental bacterial strains was done using IP’s that were standardised in accordance to their cfu/ml. Dilution of the crude extracts made a difference especially with *M. smegmatis* which showed the largest zone of inhibition with undiluted 100% crude extract (13 mm) and no inhibition zone at the most diluted. However, with *M. luteus*, it appears that dilution of the extract from 100- 50% did increase the size of the z.o.i but only marginally (by 3mm) and further dilution showed a decrease in the inhibitory activity (by 2mm). With respect to the gram negative strains *E. coli* 29, *E. coli* 32 and gram positive strain *B. cereus*, the 25% dilution of the crude extract showed no inhibitory activity against bacteria, whilst *Bacillus subtilis* showed 1mm which is negligible. Amongst most bacterial strains, with the exception of *B. subtilis*, the greatest zone of inhibition was shown at the 100% undiluted crude extract. The strongest inhibition zone was observed in *P. putida* 317 at this dilution (16mm).
Figure 8: Diluted crude extract against different bacterial strains. In picture A- Top row: E. coli 32, E. coli 29, P. putida dss2, P. putida 317. Bottom row: M. smegmatis mc²155, M. luteus, Bacillus subtilis EL39, Bacillus cereus DLS. The numbers on the plate represent the percentage of the crude extract present in the wells- (1) 100%, (2) 50%, (3) 25%, (4) ethyl acetate. Picture B is a representation of the sizes of the zones of inhibition against different bacterial strains using diluted crude extract. The percentage dilution is shown in the key above (i.e. 25%, 50% and 100%-undiluted). All samples were diluted in ethyl acetate. The radius for each well was measured and plotted in accordance to the respective strains.

2.5 Discussion:

An environmental bacterial strain was isolated from soil by Dr L.K Lukusa. The soil sample was obtained from Mpumalanga (assisted by a mycotoxin laboratory). The bacterial isolate was shown to exhibit an inhibitory activity against M. smegmatis mc²155. Comparison of the 16S rDNA sequence (BLASTn on NCBI website) of the isolate revealed that it was related to several Pseudomonas putida and Pseudomonas fluorescens strains. These strains are known to produce compounds e.g. biosurfactants, are used in bioremediation, biodegradation and exhibit pathogenic traits (see Appendix B, Table11). Since Pseudomonas strains possess the necessary machinery to produce various types of secondary metabolites (refer to chapter one)
and our strain appeared to have an inhibitory affect on *M. smegmatis*, we hypothesised that this strain (designated *Pseudomonas αmb*) could possibly be producing an antimycobacterial compound.

An observation of the streak plate antimycobacterial assays showed that the z.o.i were seen in close proximity to the *Pseudomonas* colonies and that they appeared more prominent on the 7H10 *M. smegmatis* indicator plates (IP’s) in comparison to LA *M. smegmatis* IP’s. From this observation we speculated that the bacteria could be secreting a substance into the environment in response to nutrient competition between the bacteria. Abrahams (2004) believed that inhibitory compounds are produced in close proximity to the cells and in some cases are bound to the cells. This arrangement allows for a slow release of the compound enabling the organism to repel the competitor without exhausting the resources required for its production (Abrahams, 2004). Thus, extraction of the compound from *Pseudomonas αmb* was thought to be possible if the bacteria had favourable conditions to produce the antimycobacterial compound.

With respect to *Pseudomonas* spp the production of certain compounds can occur at a specific growth phase of the bacterium or occur due to changes in temperature, pH, oxygen, carbon or nitrogen sources (Raaijmakers, Bruijn and de Kock. 2006; Santa Anna L.M et al. 2001). For instance, *P. aueruginosa* produces rhamnolipids in the exponential phase (Santa Anna L.M et al. 2001) whilst their production of CLPs in response to nutritional (carbon nitrogen) or temperature and pH stresses (Raaijmakers, Bruijn and de Kock, 2006) Other secondary metabolites, such as lipopeptides Sch 419558 and Sch 419559 from *Pseudomonas fluorescens*, are produced by fermenting the bacteria in liquid culture (Yang et al. 2004). Hence extraction of the compound from the supernatant of a stationary phase bacterial culture was first attempted. However, this particular *Pseudomonas* isolate (αmb) did not show the presence of the antimycobacterial activity in the supernatants.

The failure of these attempts prompted the use of liquid-liquid extraction using ethyl acetate from broth culture. Organic solvents extraction was used as some antimicrobial compounds have been extracted with the use of organic solvents (see Appendix B, Table 12) (Tingyue, 2000) and if the compound of interest was not
soluble in water, antimicrobial activity from the bacterial culture supernatants would not be seen. Hence, the organic solvent ethyl acetate was thought to enhance the solubility of the compound should the antimycobacterial compound be hydrophobic (Isken and de Bont, 1998) The broth culture ethyl acetate extracts showed poor evidence of the presence of the antimycobacterial compound. This was surprising since most secondary metabolites such as CLPs, biosurfactants and bacteriocins were extracted from the supernatants using organic solvents (Silva et al., 1987). However, perhaps the reason that the extracts were not active was due to a low concentration of the active compound. As previously mentioned there are various factors that influence the production of antimicrobials.

Since the inhibitory activity was primarily observed on the 7H10 M. smegmatis indicator plates, it was thought that the activity could be a response to the presence of M. smegmatis. Thus, M. smegmatis indicator plates as well as non-indicator plates were used to extract the activity. The ethyl acetate extraction on the agar and bacterial cells revealed that there was no inhibition from the agar extracts scraped from the either set of plates. This suggested that the response seen on the M. smegmatis IP’s could be possibly due to nutrient stress from the low nutrient media (7H10). However there was another possibility that the ethyl acetate was not the most efficient extraction solvent to use, thus methanol-chloroform and acidified acetone methods for extraction was attempted. The results from the three extractions were compared and it was found that acidified acetone was easily contaminated with fungal spores. The methanol-chloroform extracts did show presence of the inhibitory activity but the ethyl acetate extracts appeared to produce consistent results and was deemed logistically better (in terms of time and equipment available) method to use at the time (data not shown). Even though the negative control (ethyl acetate only) showed no inhibitory activity towards M. smegmatis, the effect of the organic solvent on the bacterium was a concern. Organic solvents have been shown to affect bacterial cells in the following ways: it can lead to inhibition of replication, production of biosurfactants, phenotypic changes and even tolerance towards the solvents (Carla et al., 2004). Interestingly, mycobacteria have been shown to tolerate concentrations of 50% of DMF, ethanol or butanol (Carla et al., 2004) despite their bacterial wall consisting of mycolic acids which make the cell wall prone to hydrophobic antibiotics (Chatterjee, 1997).
Since 7H10 media was a minimal nutrient media, it could have initiated a stress response due to nutrient deprivation. However, if this was purely the case then the activity would also have been seen with the same intensity (to that of the cell extracts off the 7H10 agar) in broth culture. The 7H9 broth media has the similar constituents to the 7H10 agar; however, the z.o.i produced from the ethyl acetate extractions differed with respect to the type of zone produced (i.e. clear versus turbid). An explanation of the discrepancy could be due to the difference between broth and agar culturing of bacteria. Mclean et al. (1997) noted that there were changes in bacterial phenotypes with respect to bacteria grown in agitated broth media versus an agar medium. They found that *Pseudomonas* planktonic cells were rod-shaped and regular whilst on agar the cells (resembling cells found in biofilms) were long and filamentous (Yang et al., 2007; Mclean et al., 1997). Although we did not look at the shape of the bacterium in the different culturing techniques the colonies of our strain had changed from a yellow colour to a yellow–brown colour when grown on 7H10 agar. These colours were reiterated in the crude extracts.

Changes in phenotype can be induced by quorum sensing (QS) molecules known as N-acylhomoserine lactone (AHL) (Arevalo-Ferro et al., 2005). The accumulation of the molecules leads to the activation of different regulation systems, including those responsible for antibiotic production (Zhang and Pierson, 2001), e.g. class IIa bacteriocins production is regulated by QS systems (Drider et al., 2006). For instance in *Pseudomonas putida* IsoF, proteomic analysis revealed that QS down regulated the expression of outer membrane proteins during biofilm formation (Arevalo-Ferro et al., 2005) which lead to phenotypic changes. However, this phenotypic change can only take place if the cell density is high enough because only then is QS induced (Arevalo-Ferro et al., 2005, Juhas, Eberl and Tummler, 2005). This could explain why we were able to see activity clearly from crude extracts that were from the bacterial cells that were grown on agar. However with respect to our *Pseudomonas* strain the wet cell mass of the agar and broth cultures showed a difference of merely 1g. This suggests that perhaps the cell density rather than the cell number influences the production of the compound. Cell density seems to be the trigger for the production of pyoluteorin which is a polyketide synthesised antibiotic by *Pseudomonas fluorescens* Pf5 (Brodhagen, Henkels and Loper, 2003). As cell density is related to the mass of
the bacterial cells over the volume of cells, this would imply that the bacterial cells would be in close proximity to each other, which would be similar to cells aggregating.

Cell aggregation has been known to be sign of switching on of secondary pathways which lead to the production of antibiotics. During the late exponential phase or early logarithmic phase, cell division stops, leading to cell aggregation (Yarbrough et al., 1993). Aggregation of bacterial cells seems to imply the formation of biofilms which has been shown to play a role in the production of antibiotics and antimicrobial compounds (mentioned above). With respect to the bacterium *Pseudomonas alteromonas luteoviolacea*, antibiotic production of violacein ceased in broth culture but was present on surfaces, which suggested that biofilm formation was essential for the production of secondary metabolites (Yang et al., 2007). This could explain the lack of inhibitory activity seen from crude extracts that were from the *Pseudomonas* amb broth cultures. These cultures were incubated for a few days, after which aggregation of the cells did not occur. However, a 4 week old broth culture did yield active crude extracts but the activities were weak (radius <4mm). Larger volumes of broth cultures were attempted, however, no active crude extract were obtained from these attempts which reaffirmed that the bacteria were producing the compound when grown on agar only. Although broth culture could yield more bacterial cells, we were still able to obtain better activity from crude extracts obtained from cells grown on agar, which implies that the agar provided a surface for biofilms to form and could be the trigger for switching on the metabolic pathway needed to produce the antimycobacterial compound. However, one of the major problems with assessing the activity of the crude extracts, is that the variability amongst the various attempts.

Even though there was consistency in the preparation of the extracts there was no guarantee that extracts would be active. In fact, only 10% of all extracts showed a distinct z.o.i when tested on an *M. smegmatis* IP. According to Yarbrough et al, (1993) each extract has its own constituents which might vary from another considerably. This was quite evident when trying to standardise crude extracts in terms of the bacterial cell wet mass and the z.o.i radius. It was concluded that standardisation of different crude extracts was not possible and the amount of bacterial cells did not necessarily mean an increased yield from the crude extract.
With respect to *Pseudomonas*, there are various QS systems that are partly responsible for the inducing the production of antimicrobial compounds. The AHL molecules serve as triggers for biosynthesis of phenazines, CLPs and quinolone compounds which could be the case with this *Pseudomonas* pαmb strain. Identification of what triggers the production of the compound might give clues to the nature of the molecule. Another way to do this is to look at the range of bacteria strains that the crude extract is active against. The bacterial range of inhibitory activity of the crude extract was investigated against some gram negative and positive bacteria which included *E. coli* 29 and *E. coli* 32, *P. putida* dss2 and *P. putida* 317, *B. subtilis* EL39 and *B. cereus* DLS and *M. smegmatis* mc2155 and *M. luteus* NTNC 8983. Two strains in particular that were chosen because they were well characterised. The *P. putida* dss2 strain is a bioremediation strain (NCBI accession no.: GL1CO DQ 304687) and *M. luteus* which is in the same phylogenetic tree as *M. smegmatis* and both have high GC content of approximately 75% (Muto and Osawa, 1987).

In terms of the inhibitory activity noted, the most significant appeared to be against the *P. putida* 317 but only at the 100% concentration and none at 25%. At 50 percent it seems that the *B. subtilis* DLS is affected the most by the crude extract. The gram negative *Pseudomonas* strains are affected by the crude extract at the 50% dilution and undiluted. The variance in the size of the z.o.i amongst the different dilutions seems to imply that the inhibitory activity of the compound is perhaps concentration dependent. From the data it can be presumed that at the 25% dilution the concentration of the compound is too low to produce a large zone of inhibition.

On the other hand, the activity of the 100% undiluted extract, was also lower than or the same as the 50% diluted crude extract. Taking into consideration that the undiluted crude extract is more concentrated, it is possible that the compound of interest is aggregating. Studies looking at lipopetides have found that these compounds have a tendency to aggregate or form oligomers due to their tails have highly hydrophobic properties. A loss of bactericidal activity was witnessed against gram positive *B. subtilis* and gram negative *P. aeruginosa* due to aggregation of the tails in the lipopeptide PA-K3X7W (Strauss and Hancock, 2006). Since the hydrophobic tails are responsible for the mode of action of the peptides, aggregation
would lead to the decrease in sites available for the insertion of the tails into the cell membrane (Strauss and Hancock, 2006). However, concentration of the compound may not always be the reason for the relative zone sizes that were noted amongst the bioassays. With respect to algae antimicrobial crude extracts, the sizes of the zones were not concurrent with the concentration of the compound that resided within the extracts. The differences were attributed to factors such temperature, diffusibility of the agar and polarity which affected the zone size (Vlachos, Chitchley and von Holy, 1997).

Even though this may be the case, it is evident that the extracts are quite specific to the organisms that it has an effect on. We proposed the possibility that there might be various compounds acting on the specific gram negative and positive bacteria which gave rise to the difference in inhibition zones. There are many pseudomonas strains that produce more than one molecule when stressed. For instance, *Pseudomonas putida* PCL1445 produces two CLP molecules putisolvin I and putisolvin II which are found in the supernatant of this bacterial strain (Dubern *et al.*, 2005) and in *Pseudomonas aeruginosa* various pyocins (Pyo Ib, Pyo Ic, Pyo II, Pyo III and Pyo IV) were isolated from a crude extract (Korzybski T, Z. Kowszyk- Gindifer and W. Kuryłowicz., 1967). Hence, there is a possibility that there could be more than one molecule. However, the only way to determine if this is the case is to purify the active components from the crude extract and use mass spectrometry to determine a fragmentation pattern of the active and non-active extracts. This would then create a fragmentation finger print of the crude extracts, thus enabling us to identify the molecular masses that are unique to our active extracts (chapter three).

We observed that after a five day incubation of the IP’s which were tested with the crude extracts, the inhibition zones had diminished. This could be due to various reasons, namely, degradation of the antimycobacterial compound (in this case the compound would then be bacteriostatic) or the development of resistance by *M. smegmatis*. That said, the appearance of such zones is also found with other antibiotic compounds. In the case of chlorophenicol, the z.o.i diminished between 18-36 hours after incubation when tested against *Clostridium perfringens*. The drug was believed to be bacteriostatic, however such an assumption needs to be confirmed via broth studies which was done and their assumption confirmed at a later stage (Louie *et al.*, 2006).
1976). With respect to our crude extract, once the compound is purified, *in vitro* studies can be done in future to determine if the action of the compound is bacteriostatic or bactericidal. Alternatively the diminished zone size could be the result of the development of resistance towards the compound of interest.