ANTI-MYCOBACTERIUM PROPERTY OF
ANACARDIUM OCCIDENTALE

TAN WEI FEI

THIS DISSERTATION IS SUBMITTED TO FULFILL PARTIAL OF THE
REQUIREMENT TO OBTAIN A DEGREE IN BACHELOR OF SCIENCE
WITH HONOUR

INDUSTRIAL CHEMISTRY PROGRAMME
SCHOOL OF SCIENCE AND TECHNOLOGY
UNIVERSITI MALAYSIA SABAH

MAY 2008
**BORANG PENGESAHAN STATUS TESIS**

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**IJAZAH**: IJAZAH SARJANA MUDA SANS DENGAN KERJASAMA.

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DECLARATION

I declare that this dissertation is the results of my own independent work, except where otherwise stated.

13 MAY 2008

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ACKNOWLEDGEMENT

Firstly, I would like to express my thanks to my supervisor, Dr. How Siew Eng for all her guidance and supervision along the progress of this final year project.

Secondly, I would like to dedicate my appreciation to all postgraduate students in Natural Products Lab especially to Mr. Khoo Yau Liang, Ms. Ch’ng Ai Ying and Ms. Teoh Hong Hong for their assistances and advices.

Thirdly, I would like to thank my teamwork partner Ms Khow Pei Ling for his assistance. I would like to thank Ms. Phang Yuik Chen for giving me all the moral support and her opinion for my study during the whole progress of final year project. I would also thank my all the coursemates especially Yap Boon Keat, Wong Siek Kuan, and all final year project student which under supervision of Dr. How Siew Eng for their helps, supports and friendship.

I would also like to thank my parents and family and my relatives for giving me much needed support in terms of their love, financial support and helps.

Finally this final year project was dedicated to my grandfather Mr. Tan See Chow who once being a chronic TB patient and survived from the disease.
ABSTRACT

ANTI-MYCOBACTERIUM PROPERTY OF ANACARDIUM OCCIDENTALE

Tuberculosis (TB) disease is caused by *Mycobacterium tuberculosis*. Despite the development of anti-TB drugs, but still unable to treat persistent TB, there were 3 million deaths in the 1990s due to this disease. Therefore, TB remains a leading cause of mortality worldwide into the 21st century. In this study, leave extracts were obtained and subjected to agar-diffusion screening systems with acetate and glucose utilization of H8000 *Mycobacterium smegmatis mc²155* targeted on glyoxylate cycle and two-component signal transduction. The screening result showed that all the extract fractions showed inhibition against glyoxylate cycle with the n-butanol showed the most promising potential to be develop as anti-persistent drugs for treating persistent mycobacteria with cytotoxic effect. In two-component screening system, the dichloromethane extract and aqueous extract showed promising activity targeting two-component system.
ABSTRAK

CIRI-CIRI ANTI-MIKOBAKTERIA DARIPADA
ANACARDIUM OCCIDENTALE

# LIST OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DECLARATION</td>
<td>ii</td>
</tr>
<tr>
<td>VERIFICATION</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>iv</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>v</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF CONTENTS</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF FORMULA</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF PHOTOS</td>
<td>xiv</td>
</tr>
<tr>
<td>LIST OF SYMBOLS, UNITS AND ABBREVIATION</td>
<td>xv</td>
</tr>
<tr>
<td>CHAPTER 1 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Tuberculosis and <em>Mycobacterium tuberculosis</em></td>
<td>1</td>
</tr>
<tr>
<td>1.2 Objectives of the study</td>
<td>5</td>
</tr>
<tr>
<td>1.3 Scope of study</td>
<td>5</td>
</tr>
<tr>
<td>CHAPTER 2 LITERATURE REVIEW</td>
<td>6</td>
</tr>
<tr>
<td>2.1 Tuberculosis</td>
<td>6</td>
</tr>
<tr>
<td>2.2 <em>M. tuberculosis</em></td>
<td>7</td>
</tr>
<tr>
<td>2.3 Current drugs</td>
<td>9</td>
</tr>
<tr>
<td>2.4 Problem exist</td>
<td>13</td>
</tr>
</tbody>
</table>
2.5 Potential drug targets 15
2.6 TCA and glyoxylate cycle 19
2.7 Targeting the enzymes in the glyoxylate bypass 22
  2.7.1 Isocitrate Lyase 22
  2.7.2 Malate Synthase 24
2.8 Catalytic mechanism of malate synthase (MS) and isocitrate lyase (ICL) 26
  2.8.1 Catalytic mechanism of malate synthase 26
  2.8.2 Catalytic mechanism of isocitrate lyase 27
2.9 The importance of Mg$^{2+}$ ion 28
2.10 Signal transduction system in bacteria 29
2.11 Two component signal transduction system 29
  2.11.1 DevR-DevS 33
  2.11.2 KdpD-KdpE 33
  2.11.3 MprA-MprB 34
  2.11.4 MtrA-MtrB 35
  2.11.5 NarL-NarS 35
  2.11.6 PhoP-PhoR 35
  2.11.7 PrrA-PrrB 36
  2.11.8 SenX3-Reg-X3 37
  2.11.9 TrcR-TrcS 37
  2.11.10 IdeR 38
2.12 Anacardium Occidentale 39

CHAPTER 3 METHODOLOGY 42
3.1 Sample collection 42
3.2 Crude extract of \textit{A. occidentale} 42
3.3 Bioassay Fractionation 43
3.4 Agar-diffusion Screening System 46

3.4.1 Targeting the glyoxylate cycle 46
   a. Preparation of M9 minimal broth 46
   b. Preparation of \textit{M. smegmatis} seed culture 46
   c. Preparation of screening system 47
   d. Screening of plant extract 47

3.4.2 Targeting the two-component signal transduction system 50
   a. Preparation of the \textit{M. smegmatis} (H8000) low Mg$^{2+}$ ion environment (20µM) and high Mg$^{2+}$ ion environment (5mM) 50
   b. Preparation of screening system 51
   c. Screening of plant extract 51

CHAPTER 4 RESULTS 54
4.1 Crude Extract of \textit{A. occidentale} 54
4.2 Solvent-solvent Extraction 54
4.3 Agar-diffusion Screening System on Crude Extract and Extract Fractions of \textit{A. occidentale} 55
   4.3.1 Targeting the glyoxylate cycle 55
   4.3.2 Targeting the two-component signal transduction system 61

CHAPTER 5 DISCUSSIONS 67
5.1 The \textit{Anacardium occidentale} leave extract 67
5.2 Agar-diffusion Screening System Targeting the Glyoxylate Cycle 68

5.3 Agar-diffusion Screening System Targeting the Two-component Signal Transduction System.

CHAPTER 6 CONCLUSION 75

REFERENCES 76

APPENDIX A 83

APPENDIX B 84

APPENDIX C 85
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table No.</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Components added in top layer of screening agar</td>
<td>47</td>
</tr>
<tr>
<td>4.1</td>
<td>The weight of each extract fraction and yield.</td>
<td>55</td>
</tr>
<tr>
<td>4.2</td>
<td>Effect of <em>A. occidentale</em> crude extract, petroleum ether, dichloromethane, n-butanol and aqueous extract fractions on the growth of <em>M. smegmatis mc²155, H8000</em> targeting the glyoxylate cycle.</td>
<td>56</td>
</tr>
<tr>
<td>4.3</td>
<td>Effect of <em>A. occidentale</em> crude extract, petroleum ether, dichloromethane, n-butanol and aqueous extract fractions on growth of <em>M. smegmatis mc²155, H8000</em> targeting the two-component signal transduction system.</td>
<td>62</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Commonly used first line anti-TB drugs</td>
<td>11</td>
</tr>
<tr>
<td>2.2</td>
<td>Commonly used second line anti-TB drugs</td>
<td>12</td>
</tr>
<tr>
<td>2.3</td>
<td>Structures of new anti-TB drugs</td>
<td>19</td>
</tr>
<tr>
<td>2.4</td>
<td>Glyoxylate cycle, a part in Tricarboxylic Acid (TCA) cycle in <em>Mycobacterium</em></td>
<td>22</td>
</tr>
<tr>
<td>2.5</td>
<td>Structure of 3-nitropropionate, 3-bromopyruvate and itaconic acid</td>
<td>25</td>
</tr>
<tr>
<td>2.6</td>
<td>Reaction of ICL and MS and structures of the reactants and products</td>
<td>27</td>
</tr>
<tr>
<td>2.7</td>
<td>Chemistry of the two-component system</td>
<td>31</td>
</tr>
<tr>
<td>2.8</td>
<td>Phosphorylation in a two-component system</td>
<td>32</td>
</tr>
<tr>
<td>2.9</td>
<td>The leaves of <em>A. occidentale</em></td>
<td>39</td>
</tr>
<tr>
<td>2.10</td>
<td>The true fruit of <em>A. occidentale</em></td>
<td>40</td>
</tr>
<tr>
<td>3.1</td>
<td>Summary of bioassay fractionation.</td>
<td>45</td>
</tr>
<tr>
<td>3.2</td>
<td>Summary of the agar-diffusion system targeting the glyoxylate cycle.</td>
<td>49</td>
</tr>
<tr>
<td>3.3</td>
<td>Summary of the two-component based screening system.</td>
<td>52</td>
</tr>
<tr>
<td>3.5</td>
<td>Summary of the methodology</td>
<td>53</td>
</tr>
<tr>
<td>4.1</td>
<td>Labeling of photo 4.1</td>
<td>57</td>
</tr>
<tr>
<td>4.2</td>
<td>Labeling of photo 4.2</td>
<td>58</td>
</tr>
<tr>
<td>4.3</td>
<td>Labeling of photo 4.3</td>
<td>59</td>
</tr>
<tr>
<td>4.4</td>
<td>Labeling of photo 4.4</td>
<td>60</td>
</tr>
<tr>
<td>4.5</td>
<td>Labeling of photo 4.5</td>
<td>63</td>
</tr>
<tr>
<td>4.6</td>
<td>Labeling of photo 4.6</td>
<td>64</td>
</tr>
<tr>
<td>4.7</td>
<td>Labeling of photo 4.7</td>
<td>65</td>
</tr>
<tr>
<td>4.8</td>
<td>Labeling of photo 4.8</td>
<td>66</td>
</tr>
<tr>
<td>Formula No.</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>------------</td>
<td>--------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>3.1</td>
<td>The percentage of yielding formula.</td>
<td>43</td>
</tr>
<tr>
<td>Photo No.</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>4.1</td>
<td>Effect of <em>A. occidentale</em> crude extracts on growth of H8000 <em>M. smegmatis</em> mc²155 targeting glyoxylate cycle.</td>
<td>57</td>
</tr>
<tr>
<td>4.2</td>
<td>Effect of <em>A. occidentale</em> petroleum ether and dichloromethane extracts fractions on the growth of H8000 <em>M. smegmatis</em> mc²155 targeting glyoxylate cycle.</td>
<td>58</td>
</tr>
<tr>
<td>4.3</td>
<td>Effect of <em>A. occidentale</em> n-butanol extract fraction on growth of H8000 <em>M. smegmatis</em> mc²155 targeting glyoxylate cycle.</td>
<td>59</td>
</tr>
<tr>
<td>4.4</td>
<td>Effect of <em>A. occidentale</em> aqueous extract fraction on the growth of H8000 <em>M. smegmatis</em> mc²155 targeting glyoxylate cycle.</td>
<td>60</td>
</tr>
<tr>
<td>4.5</td>
<td>Effect of <em>A. occidentale</em> crude extracts on the growth of H8000 <em>M. smegmatis</em> mc²155 targeting two-component signal transduction system.</td>
<td>63</td>
</tr>
<tr>
<td>4.6</td>
<td>Effect of <em>A. occidentale</em> petroleum ether and dichloromethane extracts fractions on growth of H8000 <em>M. smegmatis</em> mc²155 targeting two-component signal transduction system.</td>
<td>64</td>
</tr>
<tr>
<td>4.7</td>
<td>Effect of <em>A. occidentale</em> n-butanol extract fraction on growth of H8000 <em>M. smegmatis</em> mc²155 targeting two-component signal transduction system.</td>
<td>65</td>
</tr>
<tr>
<td>4.8</td>
<td>Effect of <em>A. occidentale</em> aqueous extract fraction on growth of H8000 <em>M. smegmatis</em> mc²155 targeting two-component signal transduction system.</td>
<td>66</td>
</tr>
</tbody>
</table>
**LIST OF SYMBOLS, UNITS AND ABBREVIATION**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
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<td><em>Mycobacterium</em></td>
</tr>
<tr>
<td>$wv^{-1}$</td>
<td>weight over volume</td>
</tr>
<tr>
<td>$vv^{-1}$</td>
<td>volume over volume</td>
</tr>
<tr>
<td>%</td>
<td>percent</td>
</tr>
<tr>
<td>$s$</td>
<td>second</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>$\mu$L</td>
<td>microliter</td>
</tr>
<tr>
<td>$^\circ$C</td>
<td>degree Celcius</td>
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<tr>
<td>mgmL$^{-1}$</td>
<td>miligram per milliliter</td>
</tr>
<tr>
<td>$\mu$gmL$^{-1}$</td>
<td>microgram per milliliter</td>
</tr>
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<td>miliMolar</td>
</tr>
<tr>
<td>$\mu$M</td>
<td>microMolar</td>
</tr>
<tr>
<td>$Mg^{2+}$</td>
<td>Magnesium Ion</td>
</tr>
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<td>millimeter</td>
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<td>$\mu$m</td>
<td>micrometer</td>
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<tr>
<td>nm</td>
<td>nanometer</td>
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<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
</tr>
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<td>BCG</td>
<td>Bacille Calmette-Guerin</td>
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<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<td>AIDS</td>
<td>Acquired Immuno Deficiency Syndrome</td>
</tr>
<tr>
<td>$^\circ$</td>
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</tr>
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</tr>
<tr>
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<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>MXF</td>
<td>moxifloxacin</td>
</tr>
<tr>
<td>DOT</td>
<td>Directly Observed Therapy</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>Magnesium sulphate</td>
</tr>
<tr>
<td><em>A. occidentale</em></td>
<td><em>Anacardium occidentale</em></td>
</tr>
<tr>
<td>No.</td>
<td>number</td>
</tr>
<tr>
<td>-----</td>
<td>--------</td>
</tr>
<tr>
<td>ICL</td>
<td>Isocitrate Lyase</td>
</tr>
<tr>
<td>MS</td>
<td>Malate Synthase</td>
</tr>
</tbody>
</table>
Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (*M. tuberculosis*). It is an illness of the respiratory system caused by the *M. tuberculosis*. The mycobacterium can be spreaded easily by coughing and sneezing. A person will be infected by inhaling the airborne *M. tuberculosis*.

TB is the most common cause of death especially among the third-world countries, and the first trace of the infection can be backtracked to 3000 years ago in the Egyptian Empire (Black, 2002). Before 1900, there was no effective treatment of TB and this caused approximately one-third of population in US died because of TB before reaching adult stage (Black, 2002). Even now, TB continues to be a devastating pathogen throughout the world, particularly in developing nations (Jamshidi & Palsson, 2007).

Before the issue of the first anti-TB drugs at 1944, the only way to combat *M. tuberculosis* was improving the body immune system by improving sanitary and
providing adequate nutrition. After two decades, the drugs for treatment of TB were developed. These include p-aminosalicylic acid, isoniazid, pyrazinamide, D-cycloserine, ethambutol, ethionamide, rifampicin and along with the development of BCG (Khasnobis et al., 2002). Despite the development of anti-TB drugs, there were 3 million deaths in the 1990s due to this disease. Therefore TB remains a leading cause of mortality worldwide into the 21st century (Smith et al., 2004).

The high number of mortality was due to the development of Multi-Drug Resistant TB (MDR-TB) due to long term treatment by a cocktail of first line and second line anti-TB drugs. The persistent phase of infection that is recalcitrant to conventional anti-TB drugs (Smith et al., 2004), and the HIV related tuberculosis (Burman & Jones, 2001). Therefore there is a need to develop new anti-TB drugs.

Although there are many conventional anti-TB drugs that can be used for the treatment, but the drawback of these drugs are the side effects of them (Winstanley, 1995) and the drugs only target actively growing bacteria in cell process such as cell wall biogenesis and chromosome replication. There are still persistent bacteria left inside the body. These bacteria will eventually become active and more resistant to the anti-TB drugs that used to treat the bacteria before (Smith et al., 2004). Therefore study has to be done to treat the persistent bacteria to ensure the successful of the TB treatment.

Isocitrate Lyase (ICL) and Malate Synthase (MS) are two key enzymes in the glyoxylate shunt (Bentrup et al., 1999; Smith et al., 2003). It is potential to inhibit the two
enzymes to prevent growth of the *Mycobacteria* (Bentrup *et al.*, 1999; Smith *et al.*, 2003). This is due to the fact that the *Mycobacteria* need acetate or fatty acids as their carbon source employed in the glyoxylate bypass for the biosynthesis of cellular material (Bentrup *et al.*, 1999; Smith *et al.*, 2003). The key enzymes of this bypass are isocitrate lyase and malate synthase (Bentrup *et al.*, 1999).

ICL helps to cleave isocitrate to succinate and glyoxylate and the malate synthase is an enzyme that condenses glyoxylate with acetyl coenzyme A (acetyl-CoA) to yield malate (Bentrup *et al.*, 1999). Muñoz-Elías & McKinney (2005) reported that the growth of *M. Tuberculosis* were disturbed when the ICL were inhibited in mice. Smith *et al.* (2003) reported that the disturbance of growth of *M. tuberculosis* was observed when MS was inhibited. The inhibition of these two enzymes holds a key to inhibit the *mycobacteria* growth (Vivek, 2006).

Two-component system is a signal transduction system in a cell that response to external stimulus (Parkinson, 1994). This system has a specific sensor kinase and a response regulator protein. The sensor kinase detects a signal from the environment, autophosphorylates at a specific histidine residue using energy from ATP hydrolysis and transmits a phosphoryl group to the response regulator. The response regulator is thus activated and this DNA-binding protein will bind to DNA to regulate transcription. To complete this regulation, this system has to be terminated by a phosphatase. In some bacteria, this reaction is carried out by the sensor kinase itself and some even has a third
protein for this termination. Some antibacterial agents were identified to inhibit the two-component signal transduction system (Barrett et al., 1998).

Cashew nut (Anarcadium occidentale) is a heart like shaped fruit widely grown in Africa and West Indies. In Nigeria about 5000 - 7000 tones are produced annually and mainly as an export crop. There was limited information in the nutritional composition, utilization and physicochemical properties of the cashew nut leaves (Aremu et al., 2006).

Researches had shown that the fruit juice produce by A. occidentale contains anti-tumor agents against BT-20 breast cancer, the anacardic acids found in the fruit juice of A. occidentale exhibit moderate cytotoxic activity against both BT-20 breast and HeLa epithelioid cervix carcinoma cells (Kubo et al., 1993). A research done by Mota et al. (1985) found out properties of anti-inflammatory actions of tannins isolated from the bark of A. occidentale. Furthermore, Kubo et al. (1994) found out that anacardic acids, 2-methylcardols, and cardols isolated from various parts of the A. occidentale exhibited tyrosinase inhibitory activity.

A previous study in our group by Ch’ng (2007) & Teoh (2007) showed inhibiting effect of the crude extract, n-butanol and aqueous extracts of A. occidentale against Mycobacteria. In this study, the Mycobacteria used was M. smegmatis, this was due to the mycobacteria was not hazardous to human, easy to grow and contain the similarity pathway of metabolism and two-component signal transduction system to M. tuberculosis (Etienne et al., 2005).
1.2 The objective of this study were:

- To prepare extracts and fractions of *A. occidentale*.
- To screen the extracts and fractions of *A. occidentale* against *M. smegmatis mc²155, H8000* targeting the glyoxylate cycle and the two-component signal transduction system using an agar-diffusion screening system.

1.3 Scope of Study:

Leaves sample of the *A. occidentale* was extracted to obtain the crude extract. Then the crude extract of the plant was evaluated for its biological inhibitory activity through an agar-diffusion screening system.

The crude extract with positive activity using bioassay-guided fractionation was partitioned into petroleum ether, dichloromethane, n-butanol and aqueous extracts fractions. The partitioned fractions were evaluated for its biological activities using an agar-diffusion screening system.

The study focuses on the biological activities of inhibition against persistent latent TB infection targeting on glyoxylate cycle and the two-component signal transduction system in *M. smegmatis mc²155, H8000*. 
CHAPTER 2

LITERATURE REVIEW

2.1 Tuberculosis

TB can be acquired by the inhalation of droplet nuclei of respiratory secretions or particles of dry sputum containing tubercle bacilli. TB often infects the lungs of the host, but in some cases, TB can also infect bones, urogenital tract, meninges, lymphatic system, and peritoneum. These infections are classified as extrapulmonary tuberculosis (Black, 2002).

When a person inhales *M. tuberculosis*, the *mycobacterium* will undergo a rapid expansion under limited host immune respond in the lungs before the host body immune system detects the invading bacteria. After a period of time when the host immune system finally realize the bacteria infection, the host immune system will eventually responds and limits dissemination of infection by developing granulomas or ‘tubercles’ around the infection site (Bentrup & Russell, 2001).
There are two types of pulmonary tuberculosis; the primary tuberculosis and secondary tuberculosis. Primary tuberculosis consists of three stages of infections. Primary tuberculosis is due to the first infection of an unsensitized host. At the stage one of the primary tuberculosis disease, most of the inhaled mycobacterium will be phagocytized by the neutrophils and white blood cells initially and macrophages later. The stage two of the disease occurs when some of the bacilli survive and replicate slowly in the cell that phagocytized them. Eventually the cell will die off and more bacilli will produce. At the third stage, the overwhelming bacilli in the lungs caused the immune system to respond by secrete more fluid in the lungs. These fluids will eventually surround the infection site and solidify to become chronic granulomas or tubercles (Black, 2002; Sheffield, 1994).

Secondary tuberculosis infection occurs when the host has some surviving bacilli left in the body and host resistant is impaired due to immunosuppression from any cause, including malnutrition, alcoholism, malignant disease, silicosis, diabetes and acquired immune deficiency syndrome (AIDS). These infections are mainly occurring in the post-primary tuberculosis patients, old folks and drug addict (Sheffield, 1994).

2.2 M. tuberculosis

*M. tuberculosis* is a member of *Mycobacteriaceae*. The *mycobacterium* was first described by Robert Koch in 1905 (Kanai, 1991; Snewin, 2001). The complete genome
sequence and annotation of *M. tuberculosis* strain was published in 1998 (Cole *et al.*, 1998).

*M. tuberculosis* is a large slender or slightly-curved rod bacterium, its shape can be around 1-4 μm in length and 0.3-0.6 μm in breadth. It is a gram-positive bacterium and can grow optimally at 37 °C, pH ranging from 6.4-7.0 is also a suitable condition for growth. But it has a typically slow replication rate ranging from 14-20 hours under optimum conditions. Furthermore *M. tuberculosis* growth tends to make serpentine and cord-like pattern of bacillary arrangement due to a parallel orientation of mutual contact. The bacteria itself can adapt themselves to microaerophilic environment or in the host body hostile environment by changing their metabolic machinery and continue surviving (Kanai, 1991; Manabe & Bishai, 2000).

The notable characteristic of *M. tuberculosis* is its “acid-fastness” characteristics, it is due to when the mycobacteria once stained, they resist to decolourization by mineral acids (Kanai, 1991; Snewin, 2001). There is also a very characteristic feature that appears in *M. tuberculosis* but not other *Mycobacteria*, the characteristic is the feature of the cell wall of *M. tuberculosis*. In the insoluble cell wall core are chemically composed of three covalently linked macromolecules, they are highly cross-linked peptidoglycan, arabinogalactan (AG) and mycolic acids. The *mycobacteria* peptidoglycan is distinct with other bacteria peptidoglycan due to the muramic acid residues are N-glycolylated with glycolic acids and the cross-linking bonds are found between two residues of diaminopimelic acids as well as between diaminopimelic acid and D-alanine residues.
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