An investigation into the enzymatic activity of deep-sea actinobacteria in decolourising Crystal Violet dye

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Abstract

Crystal Violet (CV) decolourising deep-sea actinobacteria could provide a great source of novel redox biocatalysts that can be used in various applications such as removal of triphenylmethane dyes from contaminated wastewater and soil, degradation of aromatic environmental pollutants, biotransformation of antimicrobial agents and degradation of xenobiotics.

CV is a triphenylmethane dye that has various applications, including use in medical, research and industrial applications, but its release into the environment poses a threat to aquatic life as it has characteristics of a biocide. Only a limited number of microorganisms are able to decolourise and degrade CV, and one of these proposed mechanisms by which they do so is the catalytic effect of oxidoreductase enzymes, including peroxidases, polyphenol oxidases and laccases. Triphenylmethane reductase has also been reported to be involved in decolourising CV, but the reaction involving this enzyme has not been studied systematically.

Eleven deep-sea actinobacteria were investigated and found to decolourise CV by either biodegradation or biosorption. *Gordonia* sp. JC 51 was selected as a candidate for further study as it could decolourise CV efficiently and could tolerate high concentrations (1mM) of CV. A combination of spectral scan studies, dye decolourisation, biodegradation assays, enzymatic assays, SDS-PAGE, Native PAGE, TLC and LC/MS/MS methods revealed the mechanism involved in the decolourisation of CV. *Gordonia* sp. JC 51 decolourised CV via enzymatic and non-enzymatic mechanisms. However, true decolourisation of CV was performed via biodegrading enzymes. Triphenylmethane reductase and polyphenol oxidase was confirmed to be the enzymes involved. Leucocrystal Violet was identified as the metabolite produced. CV also was sequentially *N*-demethylated, oxidised and cleaved into smaller compounds such as Michler's Ketone. In conclusion, *Gordonia* sp. JC 51 has potential as a whole cell biocatalyst and should be investigated further.

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List of Abbreviations

BG	Brilliant Green
BTB	Biocatalysis and Technical Biology
BRENDA	Braunschweig Enzyme Database
BSA	bovine serum albumin
CS	Culture sonicated
CSH	Culture sonicated and heat treated
CV	Crystal Violet
CVCB	Crystal Violet Carbinol Base
2,6 DMP	2,6-Dimethoxyphenol
DDMPR	*N, N*-Dimethyl-*N',N*'-dimethyl-*N"*-methylpararosaniline
DDPR	*N,N*-dimethyl-*N',N'*dimethyl pararosaniline
DMPR	*N,N*-dimethyl-*N*'-methylpararosaniline
DPR	*N,N*-dimethylpararosaniline
DP	*p*-dimethylaminophenol
DB	*N, N*- dimethylaminobenzaldehyde
DMBP	[*N, N*-dimethylaminophenyl][*N*-methylaminophenyl] benzophenone
DDMPR	*N, N*-dimethyl-*N', N'*-dimethyl-*N*''-methyl pararosaniline
DMMPR	*N,N*-dimethyl- *N*'- methyl-*N*''-methyl pararosaniline
DMHPR	*N,N*-Dimethyl-*N'*-methyl-*N*"-hydroxymethylpararosaniline
DHMPR	*N,N*-Dimethyl-*N'*-hydroxymethyl-*N*'-methylpararosaniline

DDHMPR	*N*,*N*-Dimethyl-*N*',*N*'-dimethyl-*N*"-hydroxymethyl-*N*"-methylpararosaniline
DHMMPR	*N,N*-Dimethyl-*N*'-hydroxymethyl-*N*'-methyl-*N*"-methylpararosaniline
DDHPR	*N,N*-Dimethyl-*N' ,N'*-methyl-*N*"-hydroxymethylpararosaniline
GC/MS	gas chromatography‑mass spectrometry
3-HAA.	3-Hydroxyantranilic acid
HB	hydroxybenzaldehyde
HPLC	High performance liquid chromatography
HMMMPR	*N*-Hydroxymethyl-*N*-methyl-*N'*-methyl-*N"*-methylpararosaniline
ISP	International *Streptomyces* Project
LMG	Leucomalachite Green
LCV	Leucocrystal Violet
L-DOPA	L-3, 4-dihydroxyphenylalanine
LMMF	low molecular mass fraction
LC/MS	liquid chromatography‑ mass spectrometry
LC	Liquid Chromatography
LCV	Leucocrystal Violet
MMMPR	*N*-methyl-*N'*-methyl-*N*"-methyl pararosaniline
MPR	*N*-methylpararosaniline
MK	Michler's ketone
MK	Michler's Ketone
MMHPR	*N*-Methyl-*N*'-methyl-*N*"-hydroxymethylpararosaniline
MMPR	*N*- methyl-*N*'- methyl pararosaniline

MG	Malachite Green
NADH	Nicotinamide adenine dinucleotide, reduced form
NCBI	National Center for Biotechnology Information
OD	optical density
PPO	polyphenol oxidase
PR	Pararosaniline
PAGE	Polyacrylamide gel electrophoresis
Rt	retention time
SD	standard deviation of the mean
SDS-PAGE	Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
SDR	short-chain dehydrogenase/reductase
TMR	triphenylmethane reductase
TLC	Thin layer chromatography
TMR	Triphenylmethane reductase
UC	Untreated culture
YEME	Yeast extract Malt extract

1 Introduction

1.1 Background

Crystal Violet (CV) is a triphenylmethane dye extensively used for many applications, such as fabric and food dye, as a bacteriostatic agent in medical solutions, as a biological stain (especially for Gram staining of bacteria), for treating skin infections and for preventing fungal growth in poultry feed and as a pH indicator (Jones & Falkinham III, 2003; Bumpus & Brock, 1988).

Wastewater treatment facilities often struggle to completely remove synthetic dyes, including triphenylmethane dyes such as CV, from contaminated wastewater (Bumpus & Brock, 1988; Chen, et al., 2007a). This has led to the pollution of aqueous environments (Bumpus & Brock, 1988) and dumping of insufficiently treated chemical waste has been shown to result in CV being found in soil and river sediments (Chen, et al., 2007a; Nelson & Hites, 1980). An increasing concern is that some triphenylmethane dyes are potent clastogens and are possibly responsible for promoting tumour growth in some fish species (Chen, et al., 2007a; Cho, et al., 2003).

There are currently several chemical and physical methods available to treat dye-containing wastewater, including chemical oxidation and reduction, chemical precipitation and flocculation, photolysis, adsorption, ion pair extraction, electrolysis, electrochemical treatment and advanced oxidation (Chen, et al., 2007b; Chen, et al., 2008). Most of these methods have limited applicability, they are generally expensive, and many produce large quantities of sludge (Chen, et al., 2008). The use of microbial biodegradation by the use of biocatalysts (whole cell biocatalysts or enzymes) could provide an environmentally friendly and cost competitive alternative; it has therefore attracted much interest (Alhassani, et al., 2007; Chen, et al., 2008; Ren, et al., 2006).

The enzymes involved in the decolourisation of dyes are usually the oxidoreductases that form part of the lignin degrading pathway in fungi and possibly also lignin degrading actinobacteria. These enzymes include laccase, lignin peroxidase and manganese peroxidase. A number of decomposition pathways of CV have been identified but there is still some uncertainty as to the exact action each enzyme takes and whether other enzymes play a role in the decomposition process.

Actinobacteria are a group of Gram positive, aerobic and are often filamentous bacteria that have extensive applications such as the production of antibiotics and potential pharmaceutical drugs. Deep-sea actinobacteria, from places such as the Izu-Bonin, Japan and Mariana Trenches could be an interesting source of redox biocatalysts. Since these bacteria are derived from great depth it is assumed that they have the ability to tolerate harsh environmental conditions such as high salinity, high pressure, reduced oxygen levels, alkaline pH, low temperature and low nutrient availability. Culturable deep-sea actinobacteria could provide a valuable resource of enzymes that are accessible for use as potential biocatalysts. These bacteria could also be used as whole cell biocatalyst as they may have adapted to extreme conditions similarly to those likely to be found in waste water streams.

1.2 Problem Statement

The effective decomposition of Crystal Violet in the context of waste water treatment could potentially be facilitated by a biocatalytic process using suitable oxidoreductase enzymes. To develop a suitable process, it is generally more desirable to use whole-cell biocatalysts rather than purified enzymes, especially if exact degradation pathways and enzymes involved are still unclear. Deep-sea actinobacteria potentially present suitable candidates for such bio-catalysts, but there has been very limited study of such organisms and the corresponding degradation pathways to date.

1.3 Scope of Investigation

The present investigation is focussed on the identification of a suitable micro-organism to decolourise CV for potential use as a biocatalyst in waste water treatment. To this end a selection of 11 deep-sea strains were screened for the most effective candidate to decolourise CV. The candidate strain, *Gordonia sp*. JC 51 was investigated further in terms of the effect of culturing conditions on CV decolourisation, the identification of the responsible enzyme as well as of the CV degradation pathway. Development of the organism into a biocatalyst and the associated testing in an applied context were outside the scope of the investigation.

2 Literature Review

2.1 Overview

In order to investigate the suitability of certain deep-sea actinobacteria as whole-cell biocatalysts for Crystal Violet (CV) decolourisation and identify the underlying pathways, literature was reviewed to assess the state of knowledge on the following aspects:

- Characteristics of CV and other triphenylmethane dyes and known bio/chemical pathways for their decolourisation and degradation
- Review of enzymes identified to play a role in the decolourisation of CV and other triphenylmethane dyes
- Review of microorganisms that can produce enzymes active in the decolourisation of CV, with specific focus on actinobacteria
- Overview of methods to study the degradation pathways
- Background on development of biocatalyst

The collected information was then used to formulate the research objectives in the context of finding and developing a novel biocatalyst for CV decolourisation.

2.2 Crystal Violet and its decolourisation

2.2.1 The Triphenylmethane Dyes: Crystal Violet

Synthetic dyes have complex aromatic structures and are extensively used in industry (especially in the textile industry) (Kaushik and Malik, 2008). An estimated 10-15% of synthetic dyes manufactured are released into the environment via industrial effluents, which has caused environmental problems (Hamid and Khalil-ur-Rehaman, 2009). This is because some the dyes are toxic and recalcitrant; resistant to biodegradation and difficult to degrade in wastewater treatment plants (Hamid and Khalil-ur-Rehaman, 2009).

Crystal Violet (CV), also known as Genetian Violet and Basic Violet 3, belongs to the group of synthetic dyes called triphenylmethane dyes, which also includes Malachite Green (MG), Cotton Blue (CB) and Basic Fuschin (BF) (Jang et al., 2005; Shedbalkar, et al., 2008). It is used extensively in industries such as the textile, food and cosmetic industries. CV is toxic to

most microbes, is carcinogenic, mutagenic and recalcitrant (Parshetti, et al., 2011). It is often used a bacteriostatic agent in medical solutions, as a biological stain (especially for Gram staining of bacteria), for treating skin infections and for preventing fungal growth in poultry feed and as pH indicator (Jones & Falkinham III, 2003; Bumpus & Brock, 1988).

Triphenylmethane dyes are described as chemical structures that have a core structure containing at least three 6-Carbon ring structures that are joined together by a central carbon (Figure 2.1). They are twisted from the central carbon like blades of a propeller.

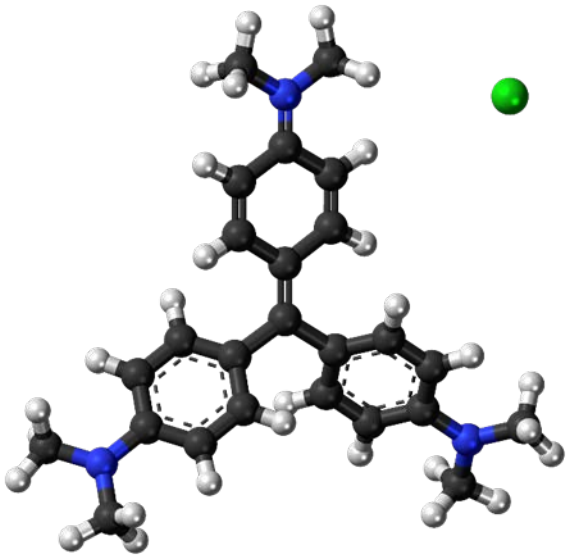


Figure 2.1 Crystal Violet as a chloride salt represented in a ball-and-stick model. Black balls represent carbon atoms, Blue balls represent nitrogen atoms, grey balls represent hydrogen atoms and green ball represent chloride anion. (Jynto, 2011).

2.2.2 CV as an antimicrobial agent

CV has the following properties, which makes it not easily biodegraded and decolourised. Firstly, it is known to have antimicrobial properties (Azmi et al., 1998). It inhibits the growth (Azmi, et al., 1998) of both fungi and bacteria. Secondly it binds to lipids, peptidoglycan in bacterial cell membranes, DNA and some proteins. Thirdly it permeabilises membranes. Fourthly it hampers cellular respiration (Azmi, et al., 1998), which means it prevents the metabolism of food to carbon dioxide, water and energy. Of the number of microorganisms currently available and known there are only a select few that can decolourise CV and tolerate it. Those that can tolerate CV have strong metabolic capabilities.

2.2.3 Decolourisation of CV

Depending on the area of research CV decolourisation could be defined differently. For example, in wastewater treatment CV decolourisation would be characterised as the removal of colour from the solution or water by adsorbing onto the biomass, and after turbidity is removed there should be no colour in solution (dos Santos et al., 2007). However, the insoluble fraction may still contain dye and therefore this phenomenon cannot be classified as true decolourisation.

In 'true' decolourisation the dye compound changes from a chromophoric compound to a non-chromophoric compound by chemical or biochemical attack. This could involve the attack of the chromophoric group of the dye by simple oxidation or reduction or cleavage of the chromophoric group resulting in break down products. All these mechanisms leading to a colourless compound or compounds.

Biodegradation is the removal of the dye from both the solution and the biomass through bio-chemical pathways. Biodegradation involves enzymes and the cells are active, whereas biosorption could occur in either living or dead cells (Kaushik and Malik, 2008). Biodegradation of CV or other triphenylmethane dyes involves oxidoreductase enzymes such as laccase, peroxidase and polyphenol oxidase, and the enzyme triphenylmethane reductase may also be involved (Jang et al., 2005)

2.2.4 CV decolourisation reactions

Four main reactions are reported in the literature to occur during the conversion of CV, resulting in degradation and/or decolourisation. These reactions are:

- the reduction of CV to Leucocrystal Violet,
- the hydroxylation or oxidation of CV to Carbinol of Crystal Violet,
- the demethylation of CV to *N, N*-Dimethyl-*N', N'*-dimethyl-*N*"-methylpararosaniline, and
- the cleavage of CV to Michler's ketone and p-dimethylaminophenol.

2.2.4.1 *The reduction of CV to Leucocrystal Violet*

The enzyme triphenylmethane reductase (TMR), from *Citrobacter sp.* strain KCTC 18061, catalyses the reduction of CV to Leucocrystal Violet (LCV) (Figure 2.2) using NADH as cofactor (Jang, et al., 2005). The cofactor NADH alone has also been shown to reduce triphenylmethane dyes that are similar in structure to that of CV (Tacal & Özer, 2007).

However, without the enzyme, large concentrations of NADH are required and reaction rates are much slower. In the reaction the NADH donates hydrogen (Jang, et al., 2005), which is inserted into the central carbon of CV. This results in the formation of the colourless compound LCV (Jang et al., 2005) and the oxidized NAD^+ cofactor.

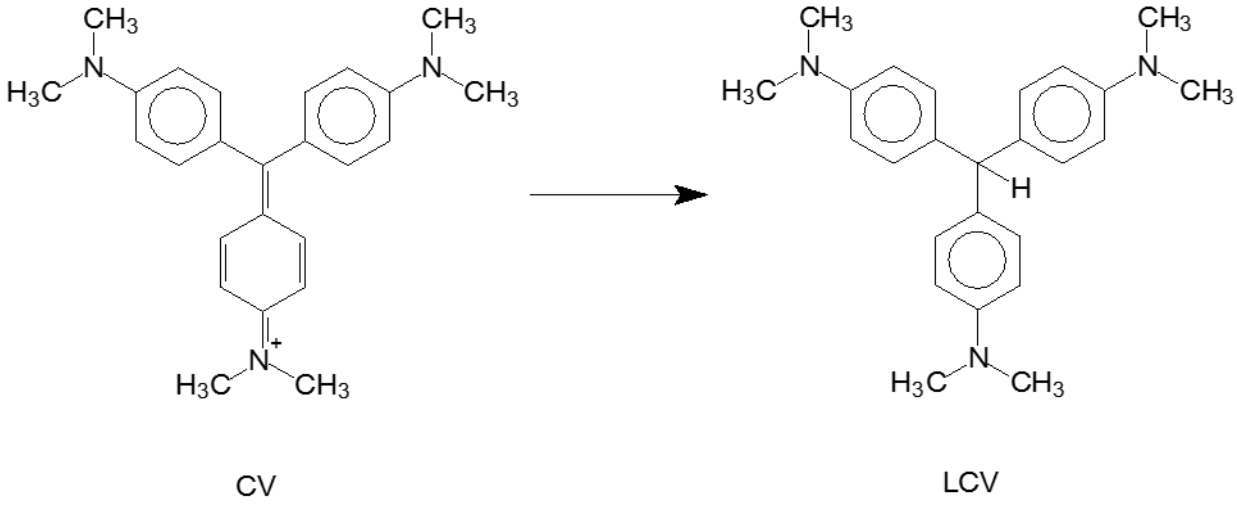


Figure 2.2 Reduction reaction of CV to LCV (Chen, et al., 2008; Jang, et al., 2005)

2.2.4.2 *The hydroxylation of CV to Carbinol Crystal Violet*

Hydroxylation is the process of adding an -OH group to a compound. It is the process of converting an organic compound to an alcohol. In the case of Crystal Violet, the hydroxylation of this compounds to the central carbon results in the formation of a tertiary alcohol. This compound is called Carbinol Crystal Violet (CCV).

In general, the type of enzymes that catalyse the hydroxylation of organic compounds are usually monooxygenases and peroxidases. Oxidases may also be involved. The chemical degradation reaction following this pathway involves the use of the alkaline NaOH at high concentrations.

The addition of the base NaOH, at stoichiometric concentrations, to CV results in the formation of Crystal Violet Carbinol Base (CVCB; also known as tris[4-(dimethylamino) phenyl] methanol) (Figure 2.3). The hydroxyl ion (OH-) attacks the central carbon atom of the dye (Goldacre & Phillips, 1949), which results in the formation of the decolourised product.

Therefore, a high pH will result in the decolourisation of this dye. To date there has been no enzyme reported to speed up the hydroxylation of the central carbon of CV. Based on the type of product produced in the hydroxylation reaction it is possible that the enzymes responsible could be a hydroxylase or monooxygenase.

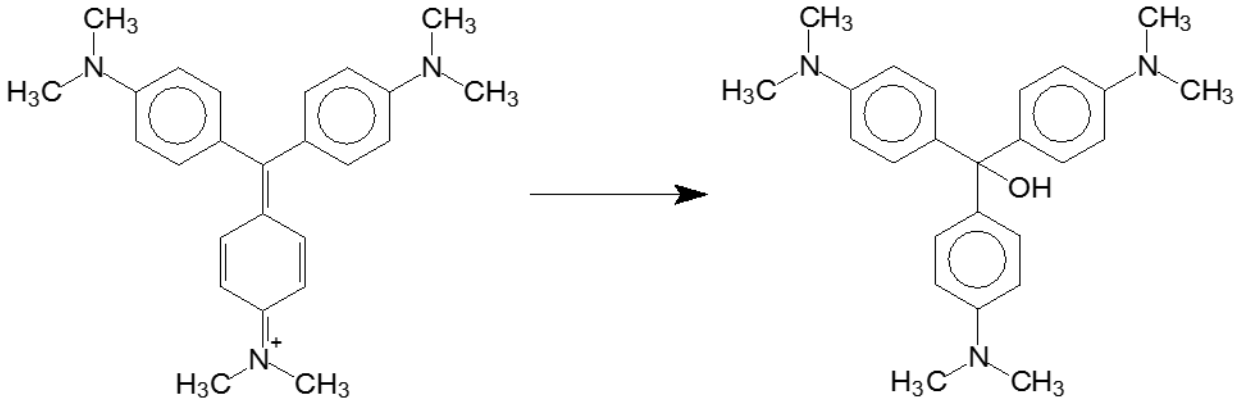


Figure 2.3 Hydroxylation reaction of CV to CVCB (Goldacre & Phillips, 1949).

2.2.4.3 *The demethylation of CV to N, N-Dimethyl-N', N'-dimethyl-N"-methylpararosaniline*

Lignin peroxidase from *P. chrysosporium* was reported to catalyze the *N*-demethylation of CV to *N, N, N', N', N", N*"-pentamethylpararosaniline (also known as *N, N*-Dimethyl-N',N'-dimethyl-*N*"-methylpararosaniline; DDMPR or Methyl Violet) (Figure 2.4) and subsequently to *N, N, N'*, *N*"-tetramethylpararosaniline and *N, N', N*"-tetramethylpararosaniline (Bumpus & Brock, 1988). This sequential *N*-demethylation of CV occurred only when H_2O_2 was supplied as co-substrate (Bumpus & Brock, 1988).

Similarly, *Pseudomonas putida* caused the N-demethylation of CV to *N, N*-Dimethyl-*N', N'*-dimethyl-*N*"-methylpararosaniline (Chen, et al., 2007a). It was able to degrade CV via a stepwise demethylation process in which mono- to hexa-demethylated CV species were generated (Chen, et al., 2007a). The enzyme involved in this reaction by this bacterium was not determined.

In a chemical process, a TiO_2-mediated photocatalytic system (which involved TiO_2 and UV irradiation) was used to catalyse the *N*-demethylation of CV to *N, N*-Dimethyl-*N', N'*-dimethyl-*N"*-methylpararosaniline as well as several other intermediates (Chen, et al., 2007b).

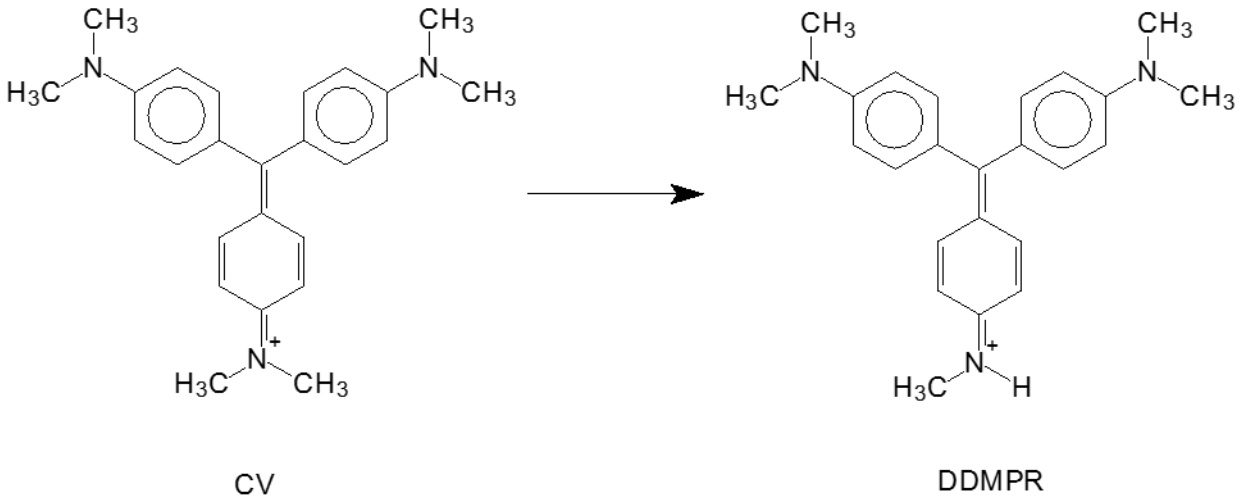


Figure 2.4 Demethylation reaction of CV to DDMPR (Bumpus & Brock, 1988; Chen, et al., 2007a; Chen, et al., 2007b)

2.2.4.4 *The direct cleavage of CV to Michler's ketone and p-dimethylaminophenol*

CV can be broken down to its phenolic monomer called p-dimethylaminophenol and dimer Michler's ketone. *Nocardia corallina* (currently known as *Rhodococcus rhodochrous*) (Yatome, et al., 1993) and *Stenotrophomonas maltophilia* LK-24 (Kim, et al., 2002) biotransformed CV to Michler's ketone and p-dimethylaminophenol (Figure 2.5). In the studies the enzymes involved were not determined and further characterised. The enzyme involved in the decolourisation of CV by *Nocardia corallina* was intracellular. No further characterisation of this enzyme was conducted in the study.

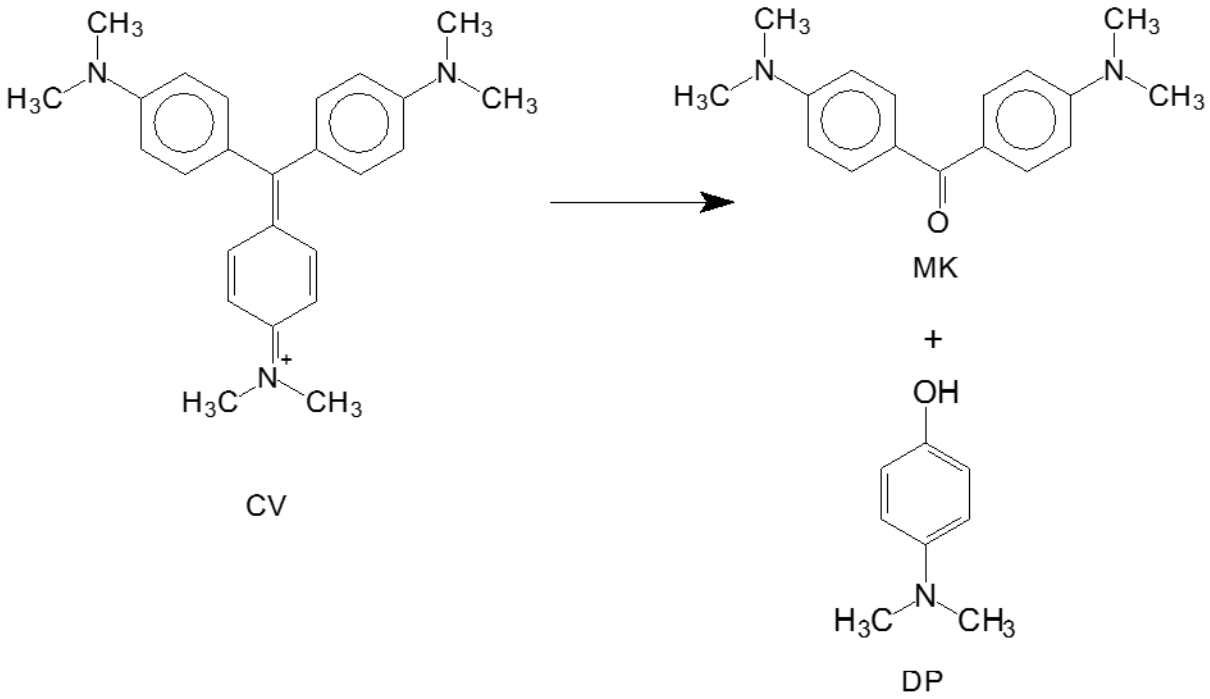


Figure 2.5 Cleavage reaction of CV to MK and DP (Yatome, et al., 1993; Kim, et al., 2002)

2.2.5 Biodegradation pathways of Crystal Violet

CV can be attacked via two main pathways which involve either the destruction of the chromophore structure of the dye or by N-dealkylation process. The biodegradation of CV by *Shewanella sp.* and *P. putida*, respectively, are examples of these two-main degradation pathways (Chen et al. 2008).

2.2.5.1 *Destruction of the chromophore structure*

Chen, et al. (2008) determined the biodegradation pathway of CV by the *Shewanella sp.* strain NTOU1. The authors proposed that this bacterium could reductively split CV into Michler's ketone (MK) and *N, N*-dimethylaminophenol (DP in Figure 1.6). MK is then further split into DP and *N, N*- dimethylaminobenzaldehyde (DB) and demethylated into [*N, N*-dimethylaminophenyl] [*N*-methylaminophenyl] benzophenone (DMBP). The DP is further degraded to 4-hydroxybenzaldehyde (HB in Figure 2.6). The enzymes involved in each reaction step were not determined.

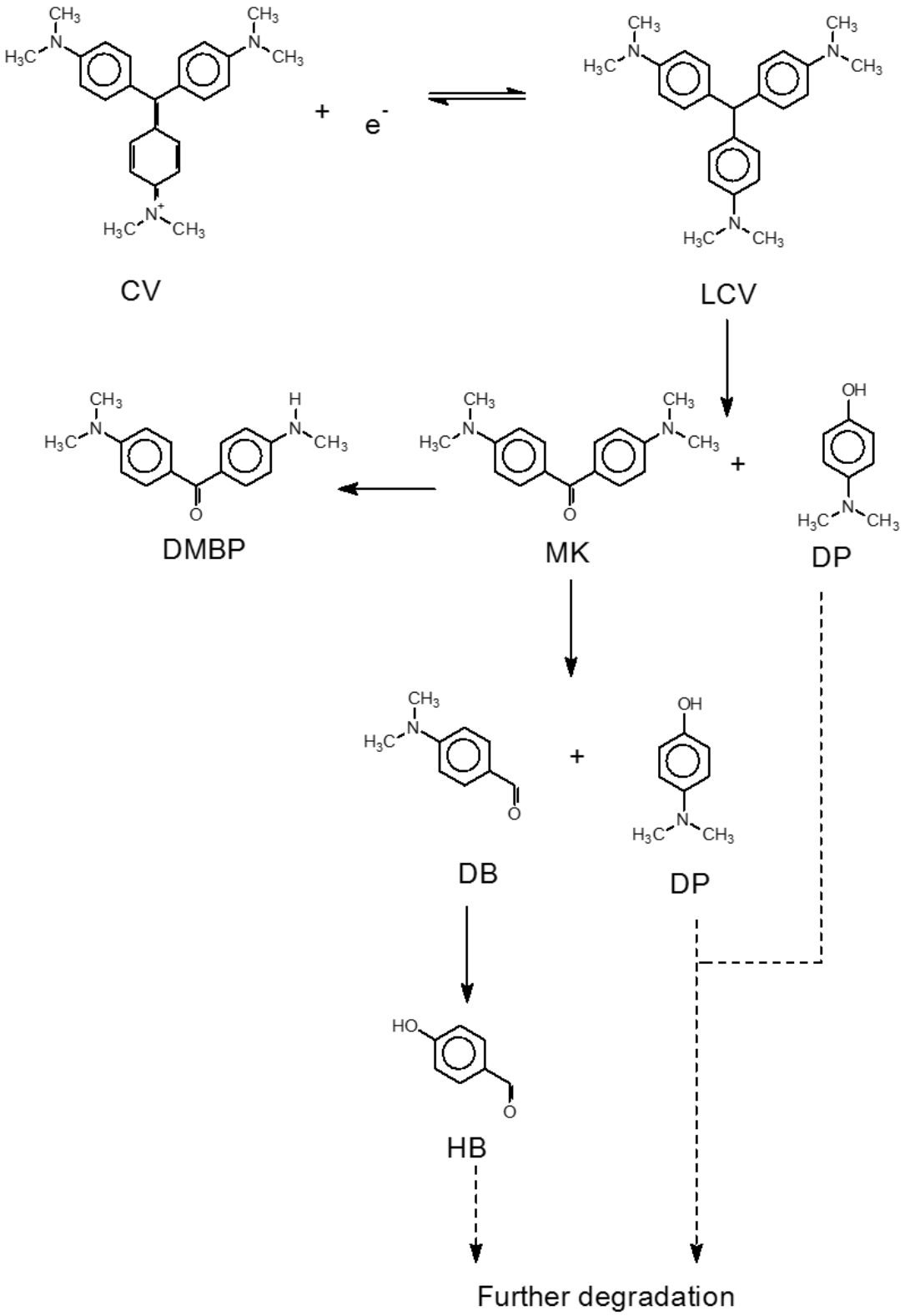


Figure 2.6 The pathways of CV degradation by *Shewanella sp.* NTOU1 under static anaerobic conditions (Chen, et al., 2008)

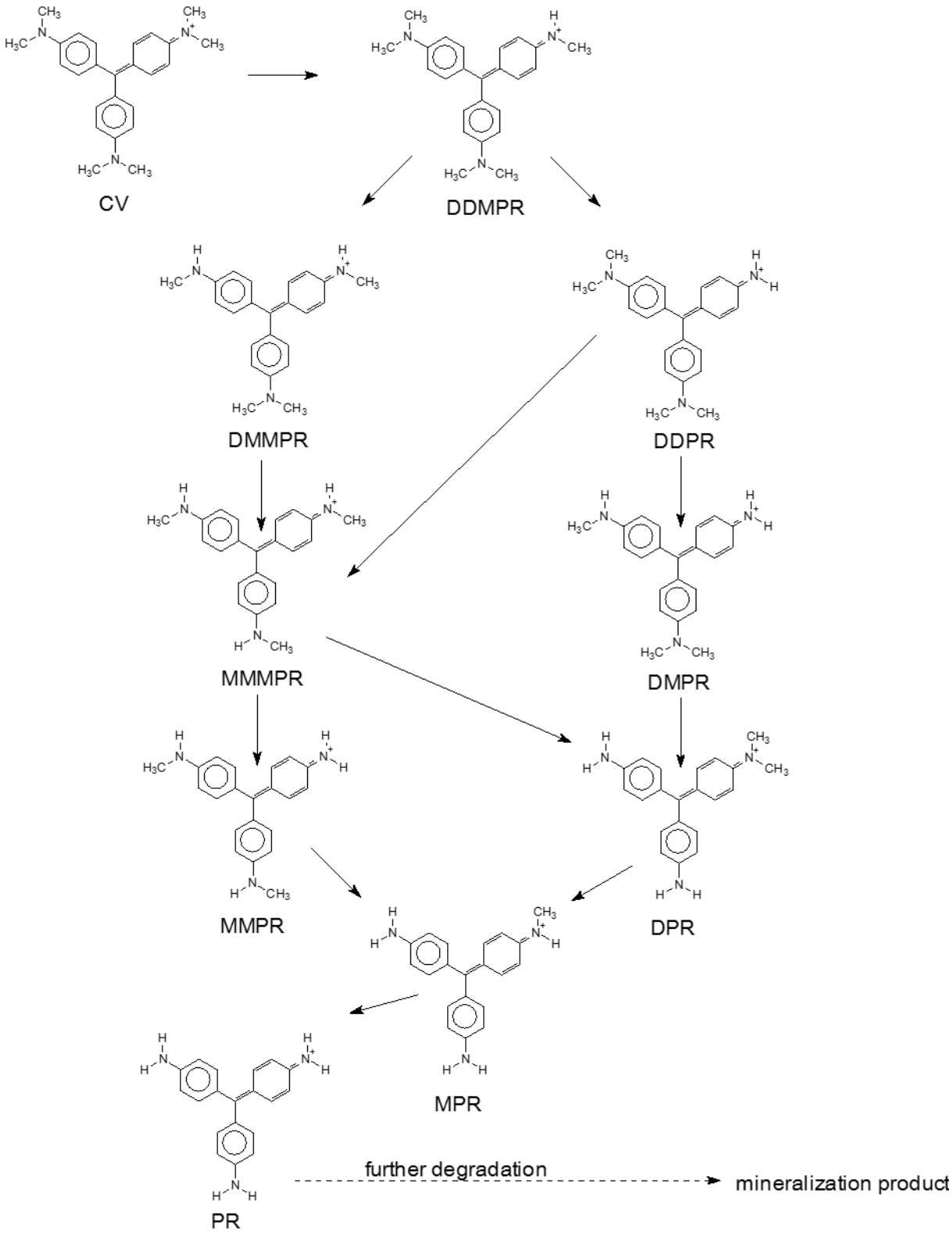


Figure 2.7 The CV demethylation pathways in *Pseudomonas putida* (Chen, et al., 2007a)

2.2.5.2 *N-dealkylation of CV*

Chen, et al. (2007a) reported the biodegradation of CV by *P. putida*. The mechanism by which this organism degraded the CV was a step-wise demethylation process which resulted in mono-, di-, tri-, tetra-, penta-, and hexa-demethylated CV species (Figure 2.7). The enzymes involved in each reaction step were not investigated.

2.3 Enzymes Involved in Biodegradation

There has been an increased level of interest in the use of enzymes as alternatives to physicochemical treatment methods in decolourisation of dyes from wastewater (Saratale, et al., 2011). Oxidoreductases have been the main enzymes involved in the decolourisation of triphenylmethane dyes such as CV (Khan & Husain, 2007; Kaushik & Malik, 2009; Pricelius, et al., 2007). These enzymes are of great interest because they do not need expensive cofactors and they have broad substrate specificity.

Oxidoreductases are enzymes that catalyse oxidation and reduction reactions which generally involve electron transfer, oxygen insertion, hydride transfer, hydrogen extraction, proton abstraction or other steps between donor and acceptor molecules (Xu, 2005). Two half reactions take place simultaneously involving at least two substrates (one donor and one acceptor), which are activated or transformed (Xu, 2005).

Oxidoreductases are the largest class of enzymes, but a limited number are available as industrial enzymes. There is currently a great interest in catalytic oxidative transformations, because of the need to replace chemical oxidations (which use heavy metal salts) with cleaner catalytic alternatives such as those using hydrogen peroxide or oxygen as oxidants and catalysed reactions that produce specific chemicals, decreasing waste generation and avoiding enantiomeric imbalance (van Deurzen, et al., 1997). The use of oxidoreductases has the potential to meet these two goals. Oxidoreductases that are currently used in industry have shown great promise as they are specific, energy-saving and biodegradable (Xu, 2005).

Oxidoreductases have various applications and have been described in several review papers (Xu, 2005; Torres et al., 2003; Ogawa and Shimizu, 2002; Schmid et al., 2001; Urlacher and Schmid, 2006; Li et al., 2002, Duran and Esposito, 2000; Burton, 2003; Kirk et al., 2002; Matsuda et al., 2009). Representatives of oxidoreductases have demonstrated the ability to

decolourise or degrade synthetic dyes (Kaushik and Malik, 2008; Saratale et al., 2011; Azmi et al., 1998; Banat et al., 1996, dos Santos et al., 2007 and McMullan et al. 2001), detoxify organic pollutants (Torres et al., 2003), treatment of wastewater and contaminated soil (Duran and Esposito, 2000), large-scale production of valuable chemicals (Ogawa and Shimizu, 2002).

Within the oxidoreductases class of enzymes there exist four main subgroups which are oxidases, peroxidases, oxygenases and dehydrogenases/reductases. Numerous studies have demonstrated the fact that oxidoreductases (especially those playing a role in lignin degradation) have the ability to decolourise and/or biodegrade synthetic dyes, either directly or indirectly.

Hamid and Khalil-ur-Rehaman (2009) described, in a review, the ability of lignin peroxidase, manganese peroxidase and horseradish peroxidase to decolourise, degrade or/and precipitate azo dyes in the presence of hydrogen peroxide.

Several reports have shown the ability of laccases to decolourise/degrade triphenylmethane, azo and anthraquinone dyes (Shedbalkar, et al., 2008). Laccase has been demonstrated to decolourise/degrade dyes efficiently when redox mediators are used (Dubé, et al., 2008; Kokol, et al., 2007; Nyanhongo, et al., 2002) and when combined with cellobiose dehydrogenase (Ciullini, et al., 2008). Polyphenol oxidase has also been shown to have the ability to decolourise/degrade dyes such as textile reactive dyes (Khan & Husain, 2007).

Azo reductases catalyse the reductive cleavage of azo groups (-N=N-) with the presence of NADH and have been reported to degrade azo-type dyes (Jadhav, et al., 2007). A cytochrome P450 system was reported to mediate both the reduction and N-demethylation reaction of methyl violet (Shedbalkar, et al., 2008).

The enzymes reported to be involved in the decolourisation and degradation of triphenylmethane dyes are triphenylmethane reductase (Jang et al., 2005; Gao et al., 2014; Stolze et al., 2012; Kim et al., 2008; Jang et al., 2007; Fu et al., 2012; Huan et al., 2011 and Li et al., 2009), laccase (Yang et al., 2015; Shanmugam et al., 2017), peroxidase (Nouren and Bhatti, 2015) and lignin peroxidase (Bumpus and Brock, 1988). This is summarised in Table 2-1.

Table 2-1 Enzymes reported to be involved in CV decolourisation or biodegradation

Enzyme	Microorganism	Reference
Laccase	*Cyathus bulleri*	(Vasdev, et al., 1995)
Triphenylmethane reductase	*Citrobacter* sp.	(Jang, et al., 2005)
Lignin peroxidase	*Phanerochaete chrysosporium*	(Bumpus & Brock, 1988)
	Cyathus bulleri	(Vasdev, et al., 1995)
	Rhodococcus quingshengii JB301	(Li, et al., 2014)
NADH/NADPH-dependent oxygenase	*Aeromonas hydrophila* strain DN322	(Ren, et al., 2006)

2.3.1 Triphenylmethane reductase

Triphenylmethane reductase was isolated from a *Citrobacter sp.* strain KCTC 18061 and characterised as an enzyme that catalyses the reduction of triphenylmethane dyes (Jang, et al., 2005; Kim, et al., 2008) to their leuco forms (examples are shown in Table 2-2). The triphenylmethane reductase first reported by Jang, et al. (2005) was a homodimer with the molecular mass of 31 kDa. It was shown to be NADH-dependent and to have optimal enzyme activity at pH 9 and 60°C (Jang, et al., 2005).

Kim, et al. (2008) determined the crystal structure of this enzyme and found that it has structural similarity to the short-chain dehydrogenase/reductase (SDR) family of proteins. These authors determined the potential molecular mechanism for triphenylmethane reduction (Table 2-2) (Kim, et al., 2008). Triphenylmethane reductase catalyses the NAD(P)H-dependent reduction of the triphenylmethane dyes as a homodimer in solution and has substrate specificity that is dependent on the chemical structures of these dyes.

Since the availability of the tmr gene sequence by Jang et al. (2005) several studies have shown that triphenylmethane reductase has been found in members belonging to Pseudomonas (Huan et al., 2011; Li et al., 2009). An *Escherichia coli* DH5a harbouring plasmid pKV11, pKV29, pKV36 or pIK405, which contain the triphenylmethane reductase gene, tmr, decolourised CV to LCV (Stolze, et al., 2012). These plasmids were originally from bacteria isolated from a wastewater treatment plant and belonged to the genera *Comamonas*

and *Delftia* (Stolze, et al., 2012). Based on these studies it has been shown that bacteria with the tmr gene genotype has the phenotype of both CV decolourisation and CV resistance. The tmr gene from *Citrobacter sp.* has been overexpressed in transgenic *Arabidopsis* plants (Fu et al., 2012) and has been used to make mutants proteins using site-directed mutagenesis to study NADH-binding residues(Jang et al., 2007), active site and NADPH binding(Kim et al., 2008) to determine kinetics of mutants against the wild-type TMR, used to make fusion proteins containing the tmr gene with a N-terminal domain of ice nucleation protein to provide attachment to the surface of an *E.coli* host leading to engineered or designer TMR whole cell biocatalyst (Gao et al., 2014).

Table 2-2 The reactions of triphenylmethane reductase and lignin peroxidase with triphenylmethane dye as substrates

Entry	Substrate	Enzyme	Cofactor/ Co-substrate	Products	Reference
1	Crystal Violet	Triphenylmethane reductase	NADH	Leucocrystal Violet	(Jang, et al., 2005)
2	Malachite Green	Triphenylmethane reductase	NADH	Leuco-malachite Green	(Kim, et al., 2008)
3	Cotton Blue	Lignin peroxidase	H_2O_2	Triphenylmethane and sulphonamide	(Shedbalkar, et al., 2008)
4	Crystal Violet	Lignin peroxidase	H_2O_2	*N,N,N',N",N"*-pentamethylpararosaline *N,N,N',N"*-tetramethylpararosaniline *N,N',N"*-tetramethylpararosaniline	(Bumpus & Brock, 1988)

2.3.2 Lignin Peroxidase

The enzyme lignin peroxidase, from *Phanerochaete chrysosporium* was reported to be responsible for catalysing the N-demethylation of CV (Table 2.1) to *N,N,N',N",N"*-pentamethylpararosaline (also known as *N,N*-Dimethyl-*N',N'*-dimethyl-*N",N"*-methylpararosaniline) and subsequently to *N,N,N',N"*-tetramethylpararosaniline and *N,N',N"*-

tetramethylpararosaniline (Bumpus & Brock, 1988). This sequential *N*-demethylation of CV occurred only when H_2O_2 was supplied as co-substrate (Bumpus & Brock, 1988), see Table 2-2.

2.3.3 Laccase

Fungal Laccase has been reported to decolourise Malachite Green (MG) (Yang et al., 2015) and CV (Shanmugam et al., 2017). The laccase could *N*-demethylate (remove methyl groups from nitrogen) MG as well as hydroxylate MG followed by cleavage to monomers and dimers. Laccase was reported to reduce CV to LCV and subsequently produce benzene substituted derivatives.

Both these studies represent laccases obtained from fungal sources. It has been found that microorganisms producing laccases does not always show the decolourisation of triphenylmethane dyes. This could be that most laccases may require mediators for decolourisation to occur. It could be that in most cases triphenylmethane dyes may be inhibitors to the enzymes. As the laccase activity towards triphenylmethane dyes is non-specific, the presence of laccase may or may not result in the decolourisation of triphenylmethane dyes.

Laccase and aminopyrine N-demethylase were suggested to be involved in the degradation of CV by *Agrobacterium radiobacter* MTCC 8161 and in combination produced N-demethylated, oxidatively cleaved products, resulting in pararosanilines, benzophenones, aminophenols, aminobenzaldehydes and phenols (Parshetti, et al., 2011)

2.4 Microorganisms involved in the decolourisation of Crystal Violet and other triphenylmethane dyes

2.4.1 General

Crystal Violet is both an antifungal and antibacterial agent and would greatly effect the growth of both fungi and bacteria. There are only limited microorganisms that are able to tolerate the effects of CV and are able to decolourise the dye. CV affects Gram positive bacteria more than Gram negative bacteria.

CV decolourising actinobacteria reported were *Rhodococcus* (Li et al., 2014), *Streptomyces*, *Nocardia* and *Mycobacteria*. Members of Bacillus of the Gram-positive bacteria have also been describe as being CV decolourising. Most CV decolourising tests involving bacteria were from Gram negative bacteria. These bacteria reported belong to members of *Comamonas*, *Delftia*, *Agrobacterium* (Parshetti et al., 2011), *Bulkholderia* (Cheng et al., 2012), *Shewanella* (Chen et al., 2008), *Pseudomonas* (Chen, 2007), *Strenotrophomonas* (Kim et al., 2002), and *Aeromonas* (Pan et al., 2012).

Several types of microorganisms, bacteria, yeast and fungi, have been reported to have the ability to decolourise (the removal of colour) or biodegrade (the breakdown of the compound) CV (Table 2-3). Table 2-4 shows the extent decolourisation percentage of CV by several bacteria under various conditions. The following paragraphs describe examples of bacteria that are able decolourise CV and various other dyes.

A *Bacillus cereus* strain DC11 was reported by Deng, et al. (2008) to be able to decolourise triphenylmethane dyes, anthraquinone and azo dyes. This bacterium was able to decolourise 90% of 20 μM CV within 24 h, 96% of 55 μM Malachite green (MG) within 3 h and 90% of 25 μM Brilliant green within 24 h.

Ren, et al. (2006) reported a broad-spectrum dye decolourising *Aeromonas hydrophila* strain able to decolourise more than 90% of 50 mg/l of triphenylmethane dyes: CV, Basic Fuschin, Brilliant Green (BG) and MG within 10 h under aerobic conditions. This bacterium was also able to utilise CV as a sole carbon and energy source for cell growth.

A *Citrobacter* sp. isolated from the soil of an effluent treatment plant in the textile and dyeing industry was reported to be able to decolourise several recalcitrant dyes (An, et al., 2002). This bacterium was able to decolourise more than 90% of CV and more than 80% of Gentian Violet, MG and BG, at 100 μM, within 1 h (An, et al., 2002). An, et al. (2002) observed that decolourisation of these dyes was in the extracellular fluid (culture supernatant) and suggested that it was due to enzymatic degradation.

Table 2-3 Microorganisms reported to decolourise or biodegrade Crystal Violet

Microorganisms	Reference
Actinobacteria	
Rhodococcus quingshengii JB301	(Li, et al., 2014)
Mycobacterium avium, M. intracellulare, M. scrofulaceum, M. marinum, M. chelonae	(Jones & Falkinham III, 2003)
Mycobacterium sp.	(Azmi, et al., 1998)
Corynebacterium sp.	(Azmi, et al., 1998)
Nocardia corallina (now *Rhodococcus rhodochrous*)	(Yatome, et al., 1993)
Other Bacteria	
Shewanella sp. NTOU1	(Chen, et al., 2008)
Pseudomonas putida	(Chen, et al., 2007a)
Aeromonas hydrophila strain DN322	(Ren, et al., 2006)
Stenotrophomas malto	(Kim, et al., 2002)
Citrobacter sp.	(An, et al., 2002)
Kurthia sp.	(Sani & Banerjee, 1999)
Bacillus subtilis IFO 13719	(Yatome, et al., 1991)
Fungi	
Phanerochaete chrysosporium	(Bumpus & Brock, 1988)
Coriolus versicolor (*Trametes versicolor*)	(Yesilada, 1995)
Cyanthus bulleri, C. stercoreus, C. striatus	(Vasdev, et al., 1995)
Laetiporus sulphurous	(Yesilada, 1995)
Funalia trogii	(Yesilada, 1995)
Yeast	
Rhodotorulae rubra, R.sp.	(Kwasniewska, 1985)

Jones and Falkinham III (2003) demonstrated the ability of several waterborne pathogenic mycobacteria to tolerate high concentrations of CV and MG. The resistance of these mycobacteria to these dyes was partially due to their ability to decolourise CV and MG. The decolourised products lacked antimicrobial activity. Small quantities of MG and its reduced, decolourised metabolite were found in the lipid fraction of one mycobacterial strain that was grown in the presence of MG. The mechanism for strain resistance to the dye was by sequestering the dyes in cell surface lipid. The decolourisation activity was determined to be membrane-associated, as specific decolourisation activity was five times higher in the membrane fraction than in the crude extract. It was also determined that a protein was responsible as no decolourisation activity was detected after treatment of the membrane

fraction with heat and proteases. The decolourisation activity was stimulated by ferrous ions and inhibited by dinitrophenol and metyrapone (Jones & Falkinham III, 2003).

Table 2-4 Extent of Crystal Violet decolourisation shown by various bacteria

Microorganisms	**Dye concentration (μM)**	**Conditions**	**Decolourisation (%)**	**Time (h)**	**Reference**
Shewanella sp. NTOU1	122.6	30°C static	100	28	(Chen, et al., 2008)
Pseudomonas putida	60	pH 7.5/ 37°C	~80	~168 (1 week)	(Chen, et al., 2007a)
Stenotrophomas malto	120	37°C 150 rpm	80	30	(Kim, et al., 2002)
Aeromonas hydrophila strain DN322	~122	30°C Microaerophilic (0.01-0.5 mg/l oxygen)	96	6	(Ren, et al., 2006)
Citrobacter sp.	100	pH 7.5/37°C 250 rpm	93	1	(An, et al., 2002)
Kurthia sp.	20	30°C 200 rpm	85	0.5	(Sani & Banerjee, 1999)
Nocardia corallina (now *Rhodococcus rhodochrous*)	2.3	pH 7.0/25°C Shaking	~80	1.5	(Yatome, et al., 1993)

Chen et al., 2008 reported the pathway for the decolourisation and degradation of CV by *Shewanella*. The bacteria demonstrated the decolourisation of CV to LCV and the cleavage to MK to p-dimethylaminophenol. MK was either N-demethylated or further cleaved to p-dimethylaminophenol and p-dimethyaminobenzaldehyde. The p-dimethylaminobenzaldehyde was further transformed to 4-hydroxybenzaldehyde. This study demonstrated that it is possible to convert the violet trimer to a colourless trimer. The colourless trimer could then be degraded to a dimer and monomer. The dimer could further be degraded to two monomers. The monomers are further modified and transformed and finally mineralised (converted to

CO_2, water and other low molecular weight compounds such as NH_3). The products were identified and analysed by GC/MS.

Chen et al. (2007) reported the N-demethylation of CV by *Pseudomonas*. The bacterium was able to sequentially remove the methyl groups from the tertiary amine side-groups. The process involves converting tertiary amines to secondary amines to finally primary amines. All the *N*-demethylated intermediates were colour compounds. The fungal N-demethylation of CV was found to involve the enzyme lignin peroxidase. The enzymes involved by this bacterium was not described in the literature paper. Chen et al., (2007b) reported the photocatalysed N-demethylation of CV.

Li et al., 2014 demonstrated that *Rhodococcus* was able to decolourise and degrade both Crystal violet and Methyl Violet. Both dyes underwent the reduction of the dye to their leuco-derivatives, which were LCV and LMV respectively. The products detected were Leuco-N-demethylated forms of the dyes, MK and its N-demethylated derivatives. This indicates that the bacterium performs several types of reactions, which include *N*-demethylation, reduction and cleavage. The enzymes detected were lignin peroxidase and DCIP-NADPH reductase. No laccase or tyrosinase was detected by this bacterium. It should not be excluded that the conversion of CV to LCV may be the involvement of the enzyme triphenylmethane reductase.

Stolze et al., 2012 found that that an *E. coli host* cloned with the tmr gene could decolourise CV and converted it to LCV and subsequently MK and p-dimethylaminobenzaldehyde, which was determined by LC/MS/MS analysis. The researchers proposed that CV was reduced to LCV by triphenylmethane reductase and subsequent catalysis involved other enzymes belonging to the host's own degradative enzymatic system. Based on this study it can be suggested that the simple conversion of CV to LCV provides the bacterial resistance to the harmful effects of the dye. LCV is more susceptible to microbial attack than CV, possibly because the conjugated system has been disrupted (colour is removed), that the double bond system attached to the central carbon is removed (enable cleavage at the single bonds), that the compound is not ionic (no binding to negative charged DNA and other negatively charged cellular components) and insoluble in water (the compound can either sediment to the bottom or be at the membrane where it can be directly attacked).

2.4.2 Actinobacteria

Actinobacteria are a group of bacteria that have been classified according to a system proposed by Stackebrant et al. (2007). This hierarchic classification system is used to differentiate and identify the different actinobacteria according to their 16srDNA. These bacteria are primarily soil inhabitants but have been widely distributed across the world. One of the main features of these bacteria is their ability to degrade organic matter and play an important role in the mineralisation of organic material.

Members of actinobacteria have demonstrated their metabolic diversity in the degradation of aromatic compounds (Phale et al., 2007), decolourisation and degradation of textile dyes (McMullan et al., 2001) and triphenylmethane dyes (Li et al., 2014; Azmi et al., 1998), production of extracellular peroxidases (Mercer et al., 1996) and have been proposed as a source of oxidative enzymes (Le Roes-Hill et al., 2011). Even marine actinobacteria have been demonstrated to provide a source of novel secondary metabolites (Fiedler et al., 2005). Actinobacteria also have been used to for synthesis application such as the production of antibiotics, antioxidants and various other bioactive compounds. Current known actinobacteria belonging to *Rhodococcus* (Martínková, et al., 2009), *Gordonia* (Shen, et al., 2006), *Williamsia* (Thompson, et al., 2005) and *Pseudonocardia* (Vainberg, et al., 2006) have demonstrated strong degradative abilities.

2.4.2.1 *Rhodococcus*

There are several review papers that have described *Rhodococcus* and its biodegradation potential (Bell et al., 1998; Larkin et al., 2005 and Martinkova et al., 2009). A member of *Rhodococcus* was also demonstrated to both decolourise and degrade the triphenylmethane dyes, CV and Methyl Violet (Li et al., 2014), which will be discussed in detail later. One of the enzymes that was involved was lignin peroxidase, but there could be other enzymes also involved that was not screened for such as the enzyme triphenylmethane reductase. In bioinformatic databases such as NCBI it is clear that triphenylmethane reductase is found in members of *Rhodococcus*. Since triphenylmethane reductase is involved in the catalysis of CV to LCV and that was the product produced by the *Rhodococcus*. It may be the enzyme involved. There have been no reports of deep-sea *Rhodococcus* having the ability to decolourise or degrade CV.

2.4.2.2 *Gordonia*

Arenskotter et al. (2004) reviewed about the metabolically diverse genus *Gordonia*. There have been no reports on the ability of members of *Gordonia* to decolourise and degrade CV.

2.4.2.3 *Williamsia*

In 2004, Stach and colleagues were the first to describe *Williamsia maris* DSM 44693 (Stach et al., 2004). The bacterium was isolated from sediments obtained from the Sea of Japan. There are no reports on the ability of *Williamsia maris* in decolourising or degrading CV.

2.4.2.4 *Pseudonocardia*

Members of *Pseudonocardia* have demonstrated their ability to degrade various chemicals, including environmental pollutants. There has been no published works that has described the ability of members of *Pseudonocardia* being able to decolourise CV.

2.4.3 Deep-sea actinobacteria as a source of industrial biocatalyst

Bull et al., 2000 reviewed search and discovery strategies for Biotechnology. In the review the bio-prospects of deep-sea actinobacteria were discussed, which highlighted many advantages of using deep-sea actinobacteria as a source of biocatalysts.

Ferrer et al., 2007 discusses the benefits of mining enzymes from extreme environments. Demirjian et al., 2001 describes how enzymes can be obtained from extremophiles. Bull et al., 2005 discusses how marine actinobacteria can be used and the challenges that are faced using them. Beloqui et al., 2008 described the trends used in Industrial Microbiology.

Actinobacteria are regarded as the most economically and biotechnologically valuable prokaryotes because many commercially known antibiotics, antitumor agents and industrial enzymes come from actinobacteria (Lam, 2006). About 60% of the earth is covered by the oceans which extend to depths of more than 2000 m, most of which are untapped as resources of microbial diversity (Colquhoun, et al., 1998). Marine environments are far different from terrestrial ones and it is hypothesized that marine actinobacteria have different characteristics than their terrestrial counterparts. They may, therefore, also produce different types of bioactive compounds (Lam, 2006). This possibility reflects the genetic and metabolic diversity of marine actinobacteria which is still largely unknown (Lam, 2006). Thus, deep-sea actinobacterial metabolites such as enzymes could be a valuable resource (Table 2-5), and deep-sea actinobacteria are largely unexplored in terms of their oxidative enzymes.

Trincone (2010) reviews the potential biocatalysts originating from sea environments, emphasizing the importance of these enzymes and providing examples to encourage the bioprospecting for new biocatalysts from sea environments for future application.

Actinobacteria in general are known for their ability to mineralise organic matter and the deep-sea is considered a major sink for global carbon, which contain sediments of recalcitrant dissolved organic matter that are 4-6000 years old. Based on Bull et al., 2005 it is possible that deep-sea actinobacteria contribute in mobilising this ancient organic matter. The deep-sea actinobacteria and their catabolic oxidoreductases involved may be extremely valuable as industrial biocatalysts.

It is possible that enzymes (including whole-cell biocatalyst) obtained from the deep-sea may be valuable. Deep-sea actinobacterial enzymes could, for example, have enhanced turnover rates, can be active at cold temperatures, could have high-affinity towards substrate, and have interesting biotransformation and bioremediation properties (Table 2-5).

Deep-sea actinobacteria can be a valuable source of biocatalysts such as novel industrial redox biocatalysts (either as whole cells or as enzymes). A search strategy for discovering potential producing strains involved the use of CV decolourisation activity assays. As discussed in previous sections, CV decolourisation has been used as a method for isolating lignin degrading enzymes such as laccase, peroxidases and polyphenol oxidase, and may also be used to discover other oxidoreductases such as triphenylmethane reductase (Jang et al., 2005). However, no research has been conducted, which involve the isolation and characterisation of actinobacterial triphenylmethane reductases. Most studies on biological dye decolourisation/degradation have concentrated on white rot fungi and only a limited extent on actinobacteria (Niladevi & Prema, 2008). Deep-sea actinobacteria are a largely untapped resource and could provide novel biocatalysts (either whole cells or enzymes) for industrial or environmental applications such as dye decolourisation and biodegradation.

Table 2-5 Different deep-sea habitats, their defining conditions and bioprospecting opportunities (Bull, et al., 2000)

Habitat	Defining conditions	Bioprospecting opportunities
Ocean trenches	High pressure	Novel and improved biocatalysts and chemistry
Deep-seas, polar seas, cold seeps	Low temperature	Cold-active biocatalysts; bioremediation; surfactants; bioantifreeze
Sea water	Low nutrient concentration	High-affinity catalysts and ligands
Hydrothermal vents	High temperature, metals	Thermostable and solvent-stable biocatalysts; biohydrometallurgy
Sediments, epibioses, and symbioses	High nutrient concentration; defence mechanisms	Novel bioactive chemicals; sensing, signalling, and defence chemicals; consortia for enhanced turnover rates
Saturated brines	High salinity	Halotolerant biocatalysts; novel metabolites
Hydrocarbon seeps	Hydrocarbon	Bioremediation, biotransformation
Deep-sub sea floor sediments	Anaerobic	Anaerobic biotransformation

Deep-sea actinobacteria were selected because existing actinobacteria play a role in the mineralisation of organic matter and they have both economical and industrial importance. Deep-sea actinobacteria may produce better biocatalyst than their terrestrial counterparts and may produce more robust enzymes suitable in industry. These bacteria are assumed to play a role in mobilising the carbon found in the sea-floor.

Such deep-sea bacteria can be found in Pacific Ocean sediments, such as obtained from Izu-Bonin and Japan Trenches at great depths (Bull et al., 2000). At these depths the oxygen levels are extremely low, temperatures are low, sunlight low, high water activities, low nutrient levels, high salt conditions and high pressures. To survive these bacteria have adapted with unique properties that could be valuable in terms of biocatalyst applications and processes. Low nutrient levels mean that these bacteria have strong degradative abilities and can degrade possible recalcitrant compounds as a form of carbon and energy source. There is potential for various interesting compounds to be discovered from marine bacteria, which

range from antibiotics, antivirals, anticancer agents and various other valuable bioactive compounds.

Biocatalysts from marine microorganisms have novel and unusual properties, and the reason for the interest in these marine enzymes (biocatalysts) is the fact of their activity and stability under extreme conditions (Bull et al., 2000). For example, it was found that some deep-sea bacteria their enzyme production increased by high pressure. Another property was found was solvent tolerance as several high tolerant bacteria were able to degrade crude oil and polyaromatic hydrocarbons.

CV-decolourising deep-sea actinobacteria could thus provide a valuable resource of interesting redox biocatalysts that can be manufactured in industry and applied in various degradative applications.

2.5 Methods used to uncover the possible mechanism of dye decolourisation

Dye decolourisation tests have been used as a method for detecting oxidoreductases such as those belonging to the lignin degradation pathway (laccases, peroxidases and polyphenol oxidases). Examples of the screening of actinobacterial strains using dye decolourisation have been demonstrated in the studies by Le Roes-Hill et al. (2011) and Kirby (2006). Usually the enzymes such as laccase are first screened for and then applied and/ or tested for the ability to decolourise dyes. Four out of five CV decolourising actinobacteria (from *Streptomyces* spp. and *Nocardia* spp.) produced both peroxidase and laccase activity (Le Roes-Hill, et al., 2011). In these actinobacterial studies lignin degrading enzymes and enzymes involved in catalysing phenolic residues were searched for. The technique has also been used to search for enzymes such as laccase and peroxidases. It has been found that enzymes that catalyse CV could be Cytochrome P450 monooxygenase, triphenylmethane reductase and triphenylmethane oxygenase.

2.5.1 Dye decolourisation tests on solid media format

Dye decolourisation tests monitor the removal of the dye substrate. In solid plate assays the dye is incorporated into the medium and the colonies of the inoculated microorganisms are monitored for the appearance of a clear halo around the colony. The size of the clearing

around the colony demonstrates the intensity or concentration of the enzymes. However, a problem with diffusion could prevent accuracy in measurements of the halo. The colour of the colony should also be monitored as it determines the mechanism of decolourisation.

Biosorption, which is a form of decolourisation, involves the attachment or binding of the dye to the cell wall of the cells. The mechanism leads to the clearing around the cell, but the cell biomass will have the colour of the dye. Biodegradation on the other hand is the breakdown of the dye. It is visible as a form of decolourisation where there is a clear halo around the colony and the colony retains its original pigment.

2.5.2 Decolourisation tests in liquid format

Decolourisation in liquid systems can be monitored in several ways. Visual observation of the decolourisation can be performed. Positive results are when the liquid changes from the colour of the dye to the colour without the dye present. Negative results would be considered when the dye colour remains. For more quantitative analysis the culture can be separated from the medium. The supernatant can be removed at various time points during decolourisation. The liquid samples can be tested through spectrophotometry (kinetic, fixed point or spectral scans), visually, thin layer chromatography (TLC), LC/MS/MS as well as other methods.

2.5.3 Two-point decolourisation analysis

For fixed point analysis the sample is taken at two points, more specifically the initial and the end point. The photo-absorbance differences are then monitored to see if there is a decrease in absorbance. The dye usually has an absorbance maximum, which is used to monitor the decrease in absorbance. Various methods have been used to display the results. Either the absorbance is shown or there is further calculation to determine the percentage or ratio. To extend this, a spectral scan can be performed. This will determine if the decolourisation is truly decolourisation and not just a shift to another colour peak within the visible light spectrum. True decolourisation will result in the reduction of the maximum absorbance peak of the dye and the appearance of a peak within the UV range of the spectrum. Complete mineralization however will show no peaks throughout the spectrum.

2.5.4 Methods to follow substrate decolourisation and product appearance

In this study the chemical screening of the biocatalytic products of CV decolourisation, which contain a mixture of unknown products or metabolites from growth and fermentation is required to identify reaction pathways. The liquid fraction (supernatant) will contain the

remaining dye substrate and the products that are produced, provided these are not further consumed (taken into the cell) by the microorganism. The products are separated, purified and identified using UV/Visible spectra, thin layer chromatography and liquid chromatography/mass- spectrometry/ mass-spectrometry (LC/MS/MS).

2.5.4.1 *TLC*

With thin layer chromatography one can monitor the disappearance of the dye compound (substrate) and the appearance of the product using normal visible light as a detection method. One can further extend the visualization techniques by making use of UV light, iodine vapours or any other established methods that are described in the literature if the researcher does not know which products will be produced. The use of UV/Vis spectra can give a clue as to whether the product can be detected in the UV spectrum.

2.5.4.2 *LC/MS/MS analysis*

If unable to determine the products by the methods described above, LC/MS/MS can be used. The liquid sample mixture, which contains the products and the residual substrate, is prepared for the LC/MS/MS. The LC separates the mixture according to their hydrophobicity or other property. Each peak, which represents a compound is then separated according to size. This is then further fragmented into smaller compounds, which can help in the identification of the compound. Currently the use of databases such as MASSBANK and other methods and software can be used to aid in the identification of the unknown products.

2.5.4.3 *Bio-desorption*

Biosorption is the binding of the dye to the cell. However, studies have revealed that this is not just a non-biodegrading mechanism. Some cells make use of this binding to make the dye accessible to its membrane or intracellular enzymes so that they are able to break down the dye. So, when the dye binds it is then attacked by the intracellular and membrane bound enzymes. In order to study these products that are bound to the cell a desorber is used such as a solvent. An example would be the use of ethanol to remove dye (and possibly its coloured/decolourised products). The solvent extract can then be analysed by TLC, HPLC and LC/MS/MS.

2.5.4.4 *The use of cell-free enzyme samples for the detection of dye decolourising enzymes*

The dye decolourisation can alternatively be detected using NATIVE-PAGE. The cell protein fractions are separated by the PAGE (polyacrylamide electrophoresis) and then subjected to dye solution in a buffer. A decolourised band would demonstrate the positive result for the sole enzyme responsible. A second PAGE could be performed where the gel is stained with Coomassie Blue. Or the current gel is de-stained and then re-stained with Coomassie Blue. SDS-PAGE could be included in the analysis methods to detect the size of the enzyme.

After decolourisation tests samples can be taken and used to detect which enzymes are responsible. These enzymes could be the lignin degrading enzymes laccases, peroxidases and polyphenol oxidases. The question that should be asked is whether these enzymes are present initially?

There are three possibilities that should be considered when screening for enzyme activities. They are:

1. The enzyme could be detected before exposure of CV and not after.
2. The enzyme is not there before exposure but present after
3. The enzyme is there irrespective of exposure

For the first possibility the products of CV could be inhibitory and interfere with the enzyme assay. For the second possibility is that the enzyme is induced. For the third possibility the enzyme is not affected by the absence or presence of CV. This could be an enzyme involved in the metabolism of the microorganism.

Examples of the different methods used to detect CV decolourisation or biodegradation metabolites are listed in Table 2-6 overleaf.

2.5.4.5 *Bioinformatics*

The use of bioinformatics in the search for novel biocatalysts is based on the collection of data, storage and mining of databases to provide a better understanding of all the information available to date and from which experiments in screening and searching of actual experiments could be improved and or tested. It is also possible to conduct virtual experiments that are computational based using these databases and from which only essential experiments can be conducted. The current bioinformatics databases provide information on

DNA (genomes, genes), RNA and protein sequences, proteomes, macromolecular structures, biotransformation's, metabolic pathways (metabolomes), biodiversity and systematics (Bull, 2000). The current availability of whole-genome sequence of various actinobacteria provides data that are useful in the development and screening of potential biocatalysts. The annotation of genes and their functional identification provide a list of all the proteins or enzymes that they encode. The combination of whole genome sequence and tools in bioinformatics potentially enable rapid searches for specific enzyme encoding genes.

Table 2-6 The different metabolites produced during biodegradation/decolourisation of Crystal Violet by various microorganisms and the methods used to determine them.

Microorganism	Enzyme	Method	Metabolites	Ref.
Rhodococcus quingshengii JB301	Lignin peroxidase	LC-MS	Leucocrystal Violet; methyl violet; Leucomethyl Violet; *N,N*-dimethyl-*N*'- methyl-*N*''-methyl pararosaniline; *N,N*-dimethyl-*N',N'*-dimethyl pararosaniline; [*N*-methylaminophenyl][*N*-methylaminophenyl]benzophenone; [*N,N*-dimethylaminophenyl][*N*-methylaminophenyl]benzophenone; [*N,N*-dimethylaminophenyl][aminophenyl]benzophenone; Michler's Ketone	Li, et al. 2014
Shewanella sp. NTOU1	Not determined	GC-MS	Leucocrystal violet; Michler's ketone; *N,N*-dimethylaminophenol; [*N,N*-dimethylaminophenyl][*N*-methylaminophenyl]benzophenone; *N,N*-dimethylaminobenzaldehyde, 4-hydroxybenzaldehyde	(Chen, et al., 2008)
Pseudomonas putida	Not determined	HPLC-ESI-MS	*N,N*-dimethyl-*N',N'*-dimethyl-*N*''-methyl pararosaniline; *N,N*-dimethyl- *N*'- methyl-*N*''-methyl pararosaniline; *N,N*-dimethyl-*N',N'*-dimethyl pararosaniline; *N*- methyl-*N*'- methyl-*N*''-methyl pararosaniline; *N,N*-dimethyl- *N*'- methyl- pararosaniline; *N*- methyl-*N*'- methyl pararosaniline; *N,N*-dimethyl pararosaniline; *N*- methyl pararosaniline; pararosaniline	(Chen, et al., 2007a)

Microorganism	**Enzyme**	**Method**	**Metabolites**	**Ref.**
Citrobacter sp. Strain KCTC 18061P	Triphenylmethane reductase	TLC, UV-Vis Spectrophotometry	Leucocrystal violet	Jang, et al. 2005
Stenotrophomonas maltophilia LK-24	Not determined	TLC, GC-MS	Michler's ketone and p-dimethylaminophenol	Kim, et al. 2002
Nocardia corallina (*Rhodococcus rhodochrous*)	Not determined	TLC, GC-MS, UV-Vis Spectrophotometry	Michler's ketone and p-dimethylaminophenol	Yatome, Yamada, et al. 1993
Phanerochaete chrysosporium	Lignin peroxidase	TLC, HPLC, MS	*N,N,N',N',N''*-pentamethylpararosaniline; *N,N, N', N''*-tetramethylpararosaniline; *N, N', N''*-trimethylapararosaniline	Bumpus & Brock, 1988

2.6 Biocatalytic processes towards CV decolourisation

2.6.1 Biocatalysis

Biocatalysis involves the use of biological catalysts (biocatalysts) (de Carvalho, 2011) to speed up organic chemical reactions and can be used in both industry and the environment. Biocatalysts are enzymes that are derived from biological systems such as bacteria, fungi, plants, animals and other living sources. When enzymes are used in an intact cell such as living bacteria or fungi they are then referred to as whole cell biocatalysts. The biocatalysts can be in the form of enzymes or whole cells. Enzymes could be prepared as broken cell preparations, partially purified or purified enzymes. Whole cells can be used as cultures in growth media or washed cells in desired solutions. Biocatalysts can be immobilised to increase stability and provide for its re-use. Usually pure enzymes are the preferred choice of biocatalyst, but when the purification of the enzyme is expensive and difficult then whole cell biocatalysts are selected instead.

Biocatalysis has various applications and potential in the chemical industry (Thomas et al., 2002) and especially with regard to obtaining sustainable industrial chemistry (Wohlgemuth, 2010). It is possible to find novel biocatalysts using database mining (Wackett, 2004) and screening environmental samples for desired activities.

Currently one of the global problems faced today is environmental pollution. For industries to be sustainable they have to make optimal use of renewable resources, decrease their impact in global warming and introduce clean or cleaner products and processes. Industries being sustainable also provides better marketing strategy, improve market interest and is favourable in both an environmental and economical point of view. Biocatalysts can replace chemical catalyst in various processes provided that the biocatalyst is ideal. Initially processes were developed to suit the biocatalyst conditions, however it is now possible to search for, modify and design biocatalysts suitable for the process.

Industries in various sectors have made use of biocatalysts either as enzymes or as whole cells. Exploiting biology for biocatalysts is currently one of the many industrial targets and also include manufacturing of drugs, biomaterials, crop protecting agents and food and feed ingredients. Industrial biocatalysis has developed into a major sector, because of its applications ranging from stereo- and regioselective transformations, processing of materials

like pulp, paper and leather, detergent additives and the biotreatment of wastes and toxic chemicals.

One of the main advantages of developing enzymes as industrial catalysts is the fact that they are considered more cleaner than chemical catalysts. Increasing use of biocatalyst in industry is dependent on the discovery of new natural enzymes and the modification or de novo design of the catalyst from existing known activities. There is also the use of ‘extremozymes’ that are enzymes that can function at extreme conditions and are derived from extremophiles which are microorganisms that can survive extreme environmental conditions.

2.6.2 Whole cell biocatalyst

Wachtmeister and Rother (2016) provide a review article discussing the use of whole cells as biocatalysts. Based on this article there were four main advantages of using whole cell biocatalysts.

One of the advantages of using whole cells is that it is the most cost effective form of biocatalyst (Wachtmeister and Rother, 2016). The preparation is simple, because no cell lysis and enzyme purification steps are required. Another advantage was that by using the whole cell the enzyme would be protected by harmful surrounding conditions (Wachtmeister and Rother, 2016). Also, when whole cells are used the microorganism provides its own cofactor regeneration system (Wachtmeister and Rother, 2016). When cofactors are required for the reaction the most cost-effective approach would be to use whole cells instead of isolated enzymes (Wachtmeister and Rother, 2016). With the use of whole cells, the addition of cofactors is not required (Wachtmeister and Rother, 2016). In general, it is found that reactions catalysed by oxidoreductases that require cofactors such as NADH and NADPH are performed using whole cells instead of isolated enzymes. Another advantage of using whole cell biocatalysts is when multi-step biocatalysis is needed. It is possible that bacteria can be designed to express a number of enzymes in a degradation pathway (Wachtmeister and Rother, 2016). Entire pathways from native hosts can be used to completely mineralise compounds (Wachtmeister and Rother, 2016).

There are also disadvantages when using whole cell biocatalysts, but there are a few strategies that are currently available that can be used to overcome these limitations. The use of immobilisation techniques enables whole cells to be recovered, recycled and to improve stability. Usually one of the main problems faced using whole cells is the mass transfer limitation of the substrate across the cell membrane. There are several solutions available to

solve this problem. The one solution is to permeabilise the cell wall by using surfactants, organic solvents or chelating agents. Other solutions include: the coexpression of membrane transporters and using cell surface display techniques.

For example, for cell surface display techniques involves a fusion protein of the enzyme and membrane anchors that are designed and expressed in a host, which then displays the enzyme on the surface of the cell. This technique results in a designer biocatalyst. For this strategy the enzyme does not require purification. The fusion protein of the enzyme and inner membrane anchors are developed (in an expression host), which is subsequently induces a phage protein. This results in the formation of pores in the membrane that results in the efflux of cellular contaminants and increased mass transfer of substrates and products.

2.6.3 Biocatalyst engineering

Illanes et al. (2012) reviewed the various approaches in improving biocatalysts. The strategies used, which can be divided into three sections such as structural strategies, environmental engineering approaches and physical stabilisation.

For the improvement of an enzyme biocatalyst can be achieved through the use of directed evolution and rational design. For environmental engineering approaches (biotransformation approaches) involves the optimisation of all physicochemical parameters of the reaction media. This includes the solvents used (water-miscible, nonaqueous, water-immiscible, anhydrous systems, supercritical fluids, gas phase and reversed micelles), salts, semisolid systems.

For physical stabilisation, which include the use of immobilisation and nanobiocatalysis. There are two main immobilisation systems, which are carrier-bound (immobilisation using an inert matrix) and carrier-free (immobilisation without support). Carrier-bound immobilisation techniques available are covalent immobilisation, non-covalent immobilisation, immobilisation by entrapment and membrane retention. Carrier- free immobilisation techniques available are CLEs cross-linked solution enzyme, CLECs cross-linked enzyme crystals and CLEA cross-linked enzyme aggregate.

2.6.4 Dye decolourisation using biocatalysis: A route for discovery of novel biocatalysts

There are currently several chemical and physical methods available to treat dye-containing wastewater, including chemical oxidation and reduction, chemical precipitation and

flocculation, photolysis, adsorption, ion pair extraction, electrolysis, electrochemical treatment and advanced oxidation (Chen, et al., 2007b; Chen, et al., 2008). These dye decolourisation technologies available usually are unable to clearly remove all the dye from wastewater and may result in sludge or may require a high energy input and therefore may be expensive to use.

Most oxidoreductases are used within cells as whole cell biocatalysts especially when isolation of the pure enzyme is costly, when expensive cofactors are required or when they involve the use of complex enzyme systems or membrane bound enzymes. In this case of using whole cells containing dye decolourising/degrading enzymes, biological reactor systems can be used to treat dye containing wastewater. Examples are aerobic activated-sludge or rotating biofilm reactors, aerobic-anaerobic packed-bed reactors, aerobic-anaerobic fluidized-bed reactors, aerobic-anaerobic sequential batch or continuous-flow reactors and anaerobic batch reactors (Banat, et al., 1996).

Oxidoreductases are either classified based on their structure or based on their catalytic activity (Xu, 2005). Classification based on catalytic activity is valuable in the context of being applied in industry (Xu, 2005)., i.e. CV decolourisation in the present case. In this sense, however, CV decolourisation activity can also be used as a tool to find potential novel oxidoreductases and the organisms that produce them (Le Roes-Hill, et al., 2011). This approach has been used to search for potential lignin degrading enzymes such as laccase and peroxidases (including lignin peroxidases) (Le Roes-Hill, et al., 2011).

2.7 Summary and Approach to Research

2.7.1 Summary of literature review

The preceding sections have discussed various reaction pathways that have been identified in the de-colourisation of CV, the oxidoreductase enzymes that have been identified to catalyse these pathways as well as some of the organisms known to produce these enzymes with a view to developing a biocatalyst that could be used in the destruction of CV and other triphenylmethane dyes common in waste waters of textile manufacturing. Actinobacteria are of interest in this context, especially those originating from extreme environments such as the deep sea, due their adaptation to hostile environments and their potential use of oxidoreductase enzymes in the mineralisation of food sources.

CV-decolourising deep-sea actinobacteria could provide a valuable resource of interesting redox biocatalysts that can be manufactured in industry and applied in various applications. Potential applications could range from the clarification and decolourisation of dye containing wastewater, treatment and detoxification of contaminated sites containing aromatic compounds, modification of antimicrobial agents and various other synthesis and degradative applications.

The decolourisation of CV is a convenient test to determine the expression of oxidoreductase enzymes from any type of micro-organism, but in the present context it is at the same time a convenient method of identifying candidate organisms to act as whole-cell biocatalysts for the industrial destruction of recalcitrant substances such as CV. Hence the present study is focussed on the screening of a suitable actinobacterium from a selection of species sampled in the deep sea, methods to culture this bacterium, and characterisation of its CV breakdown pathway to inform its potential use as a waste water treatment biocatalyst.

2.7.2 Research Objectives

This summary then sets up the key objectives of the study as listed below:

- The first objective was to identify CV dye decolourising actinobacterial strains, screen for potential oxidative enzymes and select the best decolouring strain as a candidate for further study.
- The second objective was to determine the factors affecting the decolourisation ability of the selected candidate, such as growth pH, initial biomass, agitation, temperature, CV concentration and dye type as well as screen for the possible enzymes involved.
- The third objective was to identify the redox enzyme involved in the decolourisation of CV and then isolating and characterising the enzyme.
- The fourth objective was to identify the metabolites generated from CV after bacterial treatment with the candidate strain to elucidate the reaction pathway.

2.7.3 Research Approach

According to the four objectives set out above, four experimental programs were run and are presented in subsequent chapters as follows:

Chapter 3 focuses on screening of eleven deep-sea strains of actinobacteria that were previously selected for their ability to decolourise CV. The primary screen included the

decolourisation of CV in both solid and in liquid format. The tolerance of strains under various concentrations of CV was also determined. The mechanism of decolourisation was interpreted by using pH analysis, spectral scan analysis, as well as biodegradation and biosorption analysis. A secondary screen included the oxidative enzymes assays for laccase, peroxidase and polyphenol oxidase using 96-well liquid formats as well as cuvette formats using microtitre plate readers and spectrophotometer. In silico screening involved the evaluation and searching bioinformatics databases BRENDA and NCBI possible enzyme candidates and to confirm possible laccase, peroxidase and polyphenol oxidases present in closely related species.

Chapter 4 focuses on the selected candidate strain, *Gordonia sp*. JC 51. The growth kinetics of this strain were studied using various methods such as OD readings, wet weight, dry weight and protein determination. Various parameters affecting the strain's ability to decolourise dye such as growth pH, agitation, inoculum size, dye type, temperature and media components were elucidated. The *in vitro* decolourisation of CV by using cell free system was elucidated, and the location of the decolourising enzyme, whether it is intracellular, extracellular or membrane bound, was also determined.

Chapter 5 focuses on identifying the enzyme involved in the decolourisation of CV *by Gordonia sp*. JC 51. The first part of this chapter focuses on the partial purification of the enzyme using acetone precipitation, ammonium sulphate precipitation, PEG/dialysis and dialysis tools. These tools were used to concentrate and remove impurities from protein samples. The second part of the chapter focused on enzyme and protein analysis such as Bradford assay, various enzyme assays including triphenylmethane reductase, SDS-PAGE, Native PAGE and in-gel activity assays.

Chapter 6 focuses on the metabolites produced after decolourisation of CV. A spectral scan is used to monitor the appearance of products and disappearance of CV. TLC is used to follow the decolourisation process and monitor the appearance of new spots of the unknown products. LC/MS/MS analysis is employed to separate the complex reaction mixture after decolourisation, identify the peaks of different molecular weights and fractionate each peak to determine the structure of the compound. Databases such as MASSBANK and various other databases as well as a literature search were used to determine the structures of the compounds. The final degradative pathway of CV by *Gordonia* sp. JC 51 was thus determined.

3 Identifying deep-sea actinobacteria able to decolourise Crystal Violet and screening for oxidative enzyme activity

3.1 Introduction

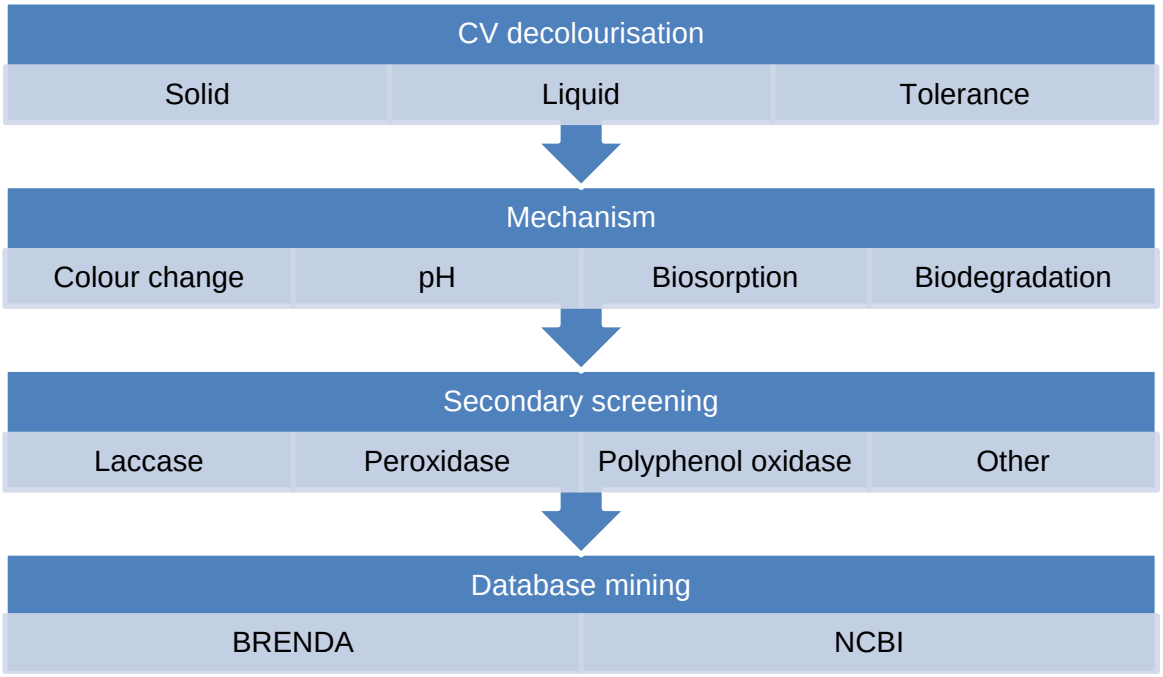


Figure 3.1 A segmented process to show the sequential steps used in identifying and characterising the CV decolourising deep-sea actinobacteria and finding the potential enzymes involved.

There are several strategies currently available to screen for novel oxidative enzymes. They could involve sequence-based screening or activity-based screening of environmental bacterial samples. Activity based screening approach involves the use of activity or function assays to monitor the rate of the reaction, monitoring the removal of substrate or the appearance of product. Visual observation serves as the simplest forms of monitoring changes occurring in a reaction. The simplest forms of observation are colour changes, precipitation forming or bubble formation. Assays could involve direct assays, coupling assays and stop/discontinue assays.

As discussed in Chapter 2, in the current study the main reaction of interest is the decolourisation of CV, which is monitoring the disappearance of a coloured substrate to a colourless compound through a biodegradation process involving active enzymes. This extends to the removal of the dye in solution as well as from cells. There is a hierarchical approach to confirming the decolourisation of CV. The first involves simple visualisation. On agar-plate based assays the inoculated microorganisms form decolourised halos around them without being stained by the dye. This confirms the biodegradation process. However, it is possible for the bacteria to grow without decolourising the dye but being stained themselves. These strains are biosorbers.

In liquid based assays the solution becomes colourless after turbidity is removed from solution. If the biomass pellet after centrifugation is stained the process is called biosorption. If the pellet remains unstained then the process is called biodegradation. However, the decolourisation process has been proven under various studies to be a more complex process and cannot be easily defined. A study by Pan et al. (2012) showed that it is possible for biosorption to occur and the stained bacteria, *Aeromonas hydrophila* DN322p undergo biotransformation. This bacterium eventually becomes unstained through decolourisation over time (Pan et al., 2012). It is possible that this process may just be a comparatively slower decolourisation process. In several other studies it has been shown that residual biosorption occurs in the cells of fungi and bacteria (Park et al., 2007; Bumpus and Brock, 1988).

As has been postulated in Chapter 2, culturable actinobacteria from the deep-sea could provide a potential source of interesting and unique oxidoreductase-based biocatalysts. The use of Triphenylmethane dye-based assays, including Crystal Violet (CV) has been used for screening lignin degrading oxidoreductases namely laccases, peroxidases and polyphenol oxidases and its application has increased over the years (Shedbalkar, et al., 2008; Kirby, 2006; Le Roes-Hill, et al., 2011). Other enzymes, such as triphenylmethane reductase has also been identified as an enzyme responsible for the decolourisation of triphenylmethane dyes (Jang, et al., 2005). The potential application of actinobacterial CV decolourising enzymes could range from enzymatic bleaching to biodegradation of closely related compounds.

Deep-sea actinobacteria isolated from sediments from the Izu-Bonin and Japan Trenches have the potential in providing valuable oxidoreductase-based biocatalysts for industry and the environment (Bull et al. 2005). A collection of 11 strains from the deep-sea collection

demonstrated the ability to partially or completely decolourise CV in a preliminary study. Strains identified were members of the genus *Gordonia, Rhodococcus, Williamsia* and *Pseudonocardia*. The ability of strains to grow and decolourise CV indicates their resistance to the antimicrobial effects of the dye. CV resistance may be caused by the possible aid of aromatic degrading oxidoreductases (including lignin degrading enzymes, laccases, peroxidases and polyphenol oxidases) and triphenylmethane specific decolourising enzymes (triphenylmethane reductases). Hence, in the present Chapter, the 11 candidate organisms were screened further to identify the best-performing strain for further study.

CV dye decolourisation tests in solid and liquid media were performed. The ability of strains to tolerate CV under various concentrations were also determined. Strains were further characterised by determining the mechanism of decolourisation. Strains able to biodegrade CV were selected and subsequently screened for laccase, peroxidase and polyphenol oxidase activity. Bioinformatics were used to determine and confirm the presence of potential enzyme candidates that were involved in the decolourisation process. The novel enzyme, triphenylmethane reductase was then further included in the screening.

3.2 Materials and Methods

3.2.1 Bacterial strains used

The deep-sea actinobacterial strains were provided by Prof. Alan T. Bull, University of Kent, UK. Strains used in this study was selected based on the ability to decolourise CV using the decolourisation assay in solid media as described by Le Roes-Hill et al., 2011 and in liquid format in microtitre plates. The selected strains used in the study are shown in **Table 3-1**. These strains were used in parallel activity, screening and tolerance tests unless otherwise stated.

Table 3-1 Deep-sea actinobacteria used in study

Species	Strain
Gordonia sp.	JC 18
Gordonia sp.	JC 25
Gordonia sp.	JC 50
Gordonia sp.	JC 51
Gordonia sp.	JC 70

Species	Strain
Rhodococcus sp.	JC 4
Rhodococcus sp.	JC 24
Rhodococcus sp.	JC 55
Rhodococcus sp.	JC 58
Pseudonocardia sp.	AB 630
Williamsia maris	DSM 44693

3.2.2 Maintenance of bacterial strains

Cultures were maintained both on YEME agar plates (Appendix G: G1) at 4°C and YEME broth supplemented with 20% (v/v) glycerol at -20°C. Gram staining was carried out using the standard Gram reaction to determine culture purity. For standard culture conditions a loopful of cells from YEME agar maintenance plates or cryostocks were inoculated into 10ml YEME broth in 50 ml Erlenmeyer flask. For larger flask volumes, a 1% (v/v) inoculum size was used (as specified) and the medium volume was at least 10% of the total volume capacity of the Erlenmeyer flask. Flasks were incubated at 30°C on a rotary shaker at 160 rpm for 3-7 days (or unless otherwise stated). When there was poor or no growth, incubation was extended for a further 1-2 days until active growth was observed.

3.2.3 Screening Strategy for detecting Crystal Violet decolourising bacteria and potential oxidative enzymes involved

The screening strategy used was based on screening programme developed by Dr Le Roes-Hill (2007, unpublished), which was further optimised and adjusted by the researcher. The tiered approach involved first the CV decolourisation in solid media (Section 3.2.3.1), followed by the CV decolourisation in liquid media (Section 3.2.3.2) and then a secondary screen involving oxidative enzyme assays for determining laccase (Section 3.2.3.3), peroxidase (Section 3.2.3.4) and polyphenol oxidase (Section 3.2.3.4) activities. Bioinformatic-based screening (Section 3.2.3.5) was used to evaluate if laccase, peroxidase and polyphenol oxidase were present in the genomes of species of the same genus of the CV biodegrading strains. The latter also used to screen for potential oxidoreductases that could be included in the study.

3.2.3.1 *Crystal Violet decolourisation assay in solid media*

The decolourisation ability of strains (**Table 3-1**), which is associated to the ability of strains to degrade lignin and produce oxidative enzymes, was assessed both in solid-phase and liquid

assays. For solid-phase dye decolourisation assays, bacteria were stab inoculated onto duplicate (unless otherwise stated) dye-containing agar plates with either ISP#2 (YEME) or ISP#9 media. CV was added to 30 mg/l or 60mg/l. Plates were incubated at 30°C and monitored at days 3, 5, 7, 10, 13 and 21 for colony growth and the development of decolourisation zones. The diameter of colony and clear halo (clearing) was measured in mm and used to determine the zone of clearing. The zone of clearing (in mm^2) was calculated according to the formula described by Le Roes-Hill et al. (2011) and as follows:

$$Zone\ of\ clearing\ = Area\ of\ clearing - Area\ of\ colony$$

The colour of the colonies was visually inspected. A negative result for enzyme activity was colonies with no clear halo after 21 days of active growth. Unstained colonies surrounded by a clear zone were positive for enzymatic decolourisation (biodegradation). However, stained colonies surrounded by a clear zone represents non-enzymatic decolourisation (biosorption) and, therefore, presented as a negative result for biocatalytic activity.

In order to determine if CV can be used as a carbon and energy source, all eleven strains were inoculated onto duplicate ISP9 medium containing 30 mg/l CV. This was incubated at 30°C for 3-5 days.

3.2.4 CV tolerance Test

In order to determine the tolerance of strains towards CV, tolerance test was conducted to determine the concentration of CV that was acceptable for growth of strains. The 11 test strains (**Table 3-1**) were each streaked onto separate YEME agar plates containing increasing concentrations, namely 0, 10, 20, 30, 40, 50 and 60 mg/l, of CV in duplicate and incubated at 30°C for 7 days (unless otherwise stated). Negative controls were included for all concentrations of CV. Tolerance was confirmed when colonies formed on the duplicate plates at equivalent CV concentrations. The morphology and decolourisation ability of strains were also observed. When strains could tolerate all concentrations tested the test was repeated with higher concentrations.

3.2.5 Crystal Violet decolourisation tests in liquid medium

For decolourisation of growing cultures CV was added to the YEME medium at the start of growth after autoclaving and strains were inoculated to this medium (i.e. the growth phase and reaction phase are the same). For decolourisation of mature cultures were first allowed to

grow for 3-7 days and then CV was added (i.e. there is first a growth phase followed by a reaction phase).

For growing cultures of deep-sea actinobacteria (n=11; Table 3-1) a loopful of tester strain was inoculated into 5 ml YEME broth supplemented with 30 mg/l CV in 50ml Erlenmeyer flask and incubated at 30°C with shaking at 160 rpm for 6 days. Triplicate flasks and negative controls were included. Flask 1, 2 and 3 were used to sample for days 1, 3 and 6 respectively. Duplicate samples (1 ml) were removed. The culture supernatant was used to determine CV decolourisation and used for partial UV/Vis spectral analysis.

For mature cultures of deep-sea actinobacteria (Table 3-1) a loopful of strains were inoculated into 10 ml YEME broth in 50ml Erlenmeyer flasks and incubated at 30°C with shaking at 160 rpm for 3 days. At the reaction phase the 3-day old culture was split into two. One 5 ml culture was used as before decolourisation and the other 5 ml culture was placed in 50 ml Erlenmeyer flask and CV was added to a final concentration of 30 mg/l using a 2%w/v CV stock. Reaction flasks were incubated at 30°C with shaking at 160 rpm for 1h. The culture supernatant was prepared as described in section and used to determine CV decolourisation and used to conduct a secondary screen screening for laccase, peroxidase and polyphenol oxidase. The cell pellet was used to extract residual dye using solvent as described in section and used to determine CV biosorption and CV biodegradation.

Recovery phase

Samples (1 ml) were removed at various time points after CV addition (0-6 days or 0-24 h) and the biomass harvested by centrifugation at 10,000 rpm for 5 min at room temperature. The cell-free culture supernatant was retained and used for the subsequent biochemical tests as described below. The remaining cell pellets were extracted with 1 ml ethanol (70% or absolute) (Deng, et al., 2008) and clarified by centrifugation at 10,000 rpm for 5 min at room temperature. The supernatant, called the cell pellet extract, was used for biochemical tests (biodegradation assay, biosorption assay).

3.2.6 Crystal Violet Spectrophotometric Assay

After decolourisation the culture supernatant (1 ml) and/or cell pellet extract (1 ml) was used to monitor the disappearance of CV at 590 nm (λ_{max}) using a UV-Vis spectrophotometer (Heλios α Unicam). Dye decolourisation and biodegradation of CV was determined by the modified method of Yatome, et al. (1993) and Deng, et al. (2008) respectively.

The blank (baseline) for measuring culture supernatant and cell pellet extract was YEME medium and 70% or absolute ethanol (depending on the solvent, the cell pellet extract was used), respectively. Where dye decolourisation was instantaneous, the time 0 samples were close to the baseline readings. For this reason, YEME containing 30 mg/l CV (the concentration used in treatment) was used as time 0. The 30 mg/l CV in ethanol represents the absorbance of sample if all CV was absorbed to cells and was extracted by ethanol. CV decolourisation percentage was determined by the formula described by Yatome, et al.(1993), which was calculated as follows:

$$Decolourisation\ (\%) = \frac{A_0 - A_1}{A_0} \times 100$$

Where A_0 is the initial absorbance (the absorbance taken at time 0) and A_1 is the final absorbance (this is also called observed absorbance).

Biodegradation percentage (complete removal of dye from cell pellet and media) was determined by the formula described by Deng, et al. (2008). Biodegradation was determined as follows:

$$Biodegradation\ (\%) = \frac{A - B - C}{A}$$

Where A is the initial absorbance, B is the final absorbance of culture supernatant and C is the final absorbance of cell pellet extract

For UV/Visible spectral scan studies, the culture supernatant (1 ml) was also used to scan the absorbance spectra of CV and metabolites between 200-800nm or 400-600nm to focus only on the disappearance of the peak at 590nm at various time points. Spectral scans were also used to monitor changes in peak absorbance and shift in wavelength. Shift in the peak from 590 nm to 540 nm is positive for *N*-demethylation. It was also used to confirm decolourisation of CV by strains. Peaks at the visible region indicate that solution has colour. Peaks absent at the visible region indicate that the solution is colourless.

3.2.7 Procedure used to screen for oxidative enzyme activity

Culture supernatants collected before and after decolourisation of CV was used to perform secondary screening and detect for the presence of laccase, peroxidase and polyphenol oxidase activity. The assays were performed in 96-well plate format using Standard 96-well microtitre plates (clear, flat bottom, LASEC). Each assay contained 150µl reaction mixture

(with substrate and buffer) and 50 µl enyme solution (culture supernatant). Blanks were also included which contained 150µl reaction mixture and 50 µl water. Positive controls were also included with a signal of at least thrice the absolute absorbance of blank. All assays were performed in triplicate at room temperature (22°C) and monitored at the maximum absorbance for each substrate (Sections 3.2.7.1 to 3.2.7.3) for 10 time points at 30 s intervals (5 min total) using an Anthos Zenyth 1100 multimode detector.

3.2.7.1 *Laccase assay*

The master mix for the laccase assay contained 1 mM 2,6 DMP (2,6-Dimethoxyphenol, Sigma) in 50 ml 100 mM Sodium phosphate buffer pH 6. An aliquot of 150 µl of the master mix was used per assay with 50 µl diluted sample/enzyme. The increase in absorbance was monitored at 468 nm (Solano, et al., 2001). For the positive control for laccase activity commercial laccase from *Trametes versicolor* (Fluka) was used.

3.2.7.2 *Polyphenol oxidase*

The master mix for the polyphenol oxidase assay contained 10 mM L-DOPA (L-3, 4-dihydroxyphenylalanine, Sigma) in 20 ml 50 mM Potassium Phosphate buffer pH 6. 150 µl was used per assay with 50 µl diluted sample/enzyme. The increase in absorbance was monitored at 475 nm (Lerch & Ettlinger, 1972). Commercial tyrosinase (*Agaricus bisporus,* Sigma) was used for the positive control.

3.2.7.3 *Peroxidase Assay*

Peroxidase activity was detected by using 3-Hydroxyanthranilic acid (3-HAA) as substrate based on the method described by Mester & Field (1998) with modifications. Briefly, a 0.05 M H_2O_2 stock was prepared by adding 75.5 µl 30% hydrogen peroxide (BDH) in deionised water to a total volume of 50 ml. The substrate solution of 1 mM 3-HAA (3-Hydroxyantranilic acid) was prepared in 100 mM Potassium phosphate buffer (pH 6). For the preparation of the master mix 40 µl of 0.05 M H_2O_2 was added to a 20 ml substrate solution. 150 µl was used per assay with 50 µl diluted sample/enzyme. The increase in absorbance was monitored for 5 min at 452 nm (Eggert, et al., 1995). Commercial peroxidase (Horseradish peroxidase, Sigma) was used as a positive control.

Commercial enzymes (Horseradish peroxidase, Sigma; laccase from *Trametes versicolor*, Fluka and *Agaricus bisporus,* Sigma) were used to test the respective enzyme assay prior to use in the microtitre plate screening assay. For all assays 1 U of enzyme activity was defined as the amount of enzyme required to produce an increase of 1 absorbance unit per minute at 22°C.

3.2.8 Bioinformatic-based screening of potential oxidoreductases that can decolourise Crystal Violet

Two main bioinformatic databases were used to search for the potential enzyme candidates involved in the decolourisation of CV. The first database used was BRENDA (2008 to 2018) using the website https://www.brenda-enzymes.org/. The search term used was “Crystal Violet” and the field selected was “substrate” and the search was then started. Only oxidoreductase-based enzymes were selected from the hits obtained. The second database used was NCBI (2008 to 2018) https://www.ncbi.nlm.nih.gov/. At the home page “all databases” was selected and the search word used was “triphenylmethane”, because CV was a triphenylmethane dye. This led to a page containing all the database links with the number of hits that are formed. The full-text articles were selected and the relevant articles for oxidoreductases was selected. The articles selected on the potential enzyme candidates was used as a tool for the detection of the specific oxidoreductases.

The NCBI database was used to determine if triphenylmethane reductase was found in representatives of *Gordonia*, *Rhodococcus*, *Pseudonocardia* and *Williamsia*. ” All databases” was used as an option for the database search, which obtains information from different sources and includes literature, genome, gene, protein and chemical databases. Hits obtained for genomes, genes and proteins were further investigated and the results obtained was used to generate a table.

3.3 Results and Discussion for Screening Actinobacteria

3.3.1 Description of the strains used for screening

Eleven deep-sea actinobacteria as per Table 3-1 were shown to decolourise CV in a preliminary study (personal communication, Le Roes-Hill, 2007 and 2014). Although all these strains were isolated from the deep-sea, they were able to grow under ambient temperatures, atmospheric pressure, neutral pH and aerobic conditions and did not have any special nutrient

requirements. The test strains were also previously identified to their genus level and were not pathogenic

All strains except *W. maris* DSM 44693 were filamentous actinobacteria and only *Pseudonocardia* sp. AB630 produced spores. All strains were Gram positive and showed positive catalase/peroxidase activity. On YEME agar plates *Gordonia* sp. JC 18 produced dull white/cream/light yellow colonies. *Gordonia* spp. JC 25, JC50, JC51 produced dull peach/orange colonies and JC 70 dull light/milky peach colonies, *Rhodococcus* spp. JC 4, JC24, JC 55 produced dull orange colonies and JC 58 dull yellow colonies. *W. maris* DSM44693 produced glossy bright orange colonies. *Pseudonocardia* sp. AB630 produced brown basal mycelium and white aerial mycelium.

The *Gordonia* spp. showed the highest growth (1.38-1.67 OD600 after 1 day growth) compared to the *Rhodococcus* spp (0.21-0.98 OD600 after 1 day growth), *W. maris* DSM44693 (0.15 OD600 after 1 day growth) and *Pseudonocardia* sp. AB630 (0.02OD600 after 1 day growth). The filamentous actinobacteria had variable growth morphology and produced clumps, therefore variations could occur when withdrawing culture samples for analysis. This was encountered by modified sampling as discussion in Section 3.2.2. Further description of strains is shown in Appendix A:Table A1.

3.3.2 Screening of isolates able to decolourise CV in solid media

As expected from the preliminary study all eleven actinobacterial isolates had the ability to decolourise CV, either partially or completely, in either solid or in liquid microtitre plate assays or both. Solid plate assays were repeated; the morphology, time decolourisation started, and decolourisation ability were also monitored.

Figure 3.2 shows the decolourisation results obtained from the 21-day decolourisation solid plate assay. All strains tested showed the ability to decolourise CV on solid plates. Decolourisation could be detected from 3 days, which was the minimum time needed to detect decolourisation activity. Decolourisation activity for strains ranged from 9-55 mm^2 after 3 days. Of the 10 strains assayed *Gordonia* sp. JC 25 (494 mm^2) and *Gordonia* sp. JC 18 (409 mm^2) showed the highest decolourisation activities after 21 days, whereas *W. maris* DSM44693 (33 mm^2) showed the lowest decolourisation activity.

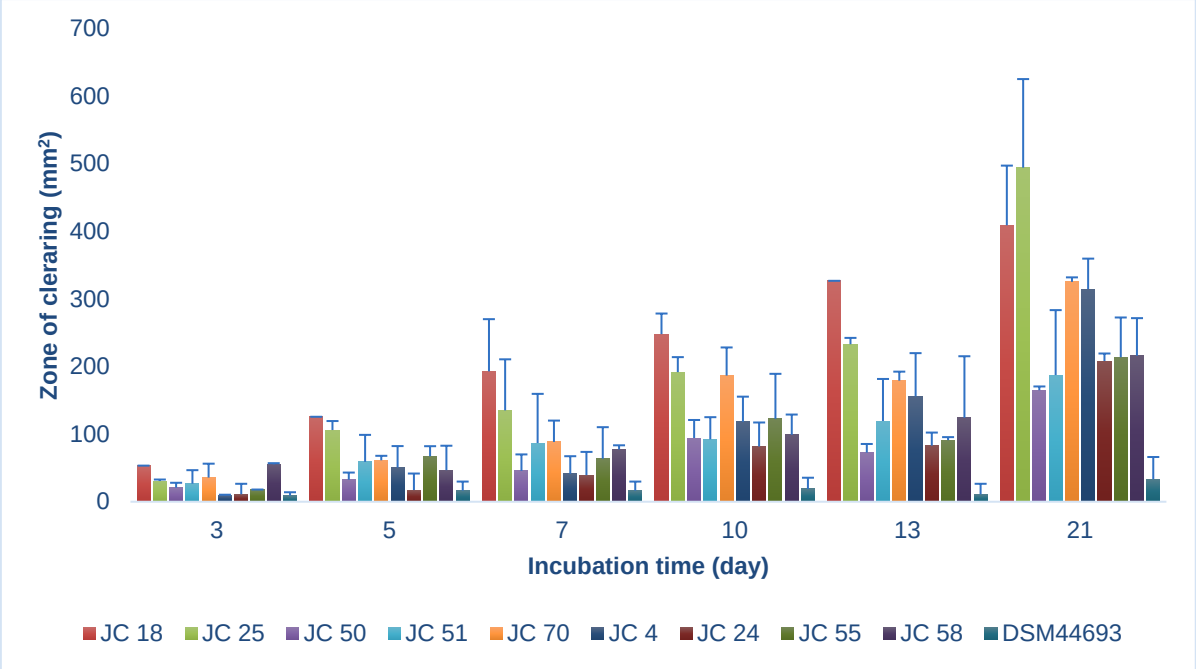


Figure 3.2 The zone of clearing of ten deep-sea actinobacterial strains grown on YEME agar plates containing 30 mg/l CV for 21 days. Results are presented as mean and error bars correspond to the standard deviation from duplicates. Controls were also included and produced no colonies or decolourised halos.

Pseudonocardia sp. AB630 was omitted from the assay because it grew irregularly and even detached from the surface of the agar medium. The colonies of *Pseudonocardia* sp. AB630 were elevated as they grew vertically instead of outwards from point of inoculation. However, decolourised zones were also observed at the point of attachment to the agar medium. These results confirm that the eleven strains tested decolourise CV and could potentially have oxidative enzyme activity.

The CV tolerance was used in this study to characterise and compare the ability of strains to grow in the presence of CV. This test was performed to ensure that only the most tolerant strains were carried forward into the rest of this study, as well as to determine the concentration at which CV became inhibitory to growth. Strains were considered tolerant to the CV concentration if colony formation occurs.

For CV tolerance test all deep-sea actinobacteria were able to grow on CV concentrations of between 10 and 60 mg/l, except *Williamsia maris* DSM44693 which could tolerate only up to 30 mg/l. Low concentrations of CV (10 mg/l) greatly reduced the growth of all eleven strains and caused morphological changes of the bacterial cell colonies of those that grew. Variable

colony sizes were observed for the colonies that grew on CV-containing plates. Figure 3.3 shows examples of three strains (AB630, JC 4 and JC 51) that illustrate the effects CV has on growth and cell morphology. Higher concentrations of CV were also monitored up to 408 mg/l, which only *Gordonia* sp. JC 51 could simultaneously grow on and decolourise. *Gordonia* sp. JC 51 also showed reproducible and high tolerance towards CV.

CV was also tested as a sole carbon source. All eleven strains showed growth, but only JC 51, JC 25, JC50, JC 55 and DSM44693 showed positive decolourisation activity. This may suggest that these strains can metabolize CV and do not need added glucose for decolourisation. AB630 and JC 58 showed no decolourisation, whereas the rest showed variable results.

To summarise all eleven strains could decolourise 30 mg/l CV on solid medium. However, AB630 had irregular growth which hampered the ability to measure the zone of clearing. Ten out of the 11 strains could tolerate 60 mg/l CV when tested. Only one strain (JC 51) could grow and decolourise CV at 1 mM. All eleven strains could grow on ISP#9 medium with 30 mg/l CV as carbon-source, but only 5 out of the 11 strains could decolourise CV under these conditions. This may suggest that CV decolourisation is growth phase dependent as strains require an appropriate amount of growth before decolourisation occurred. This could mean that they need to express genes encoding CV decolourising enzymes.

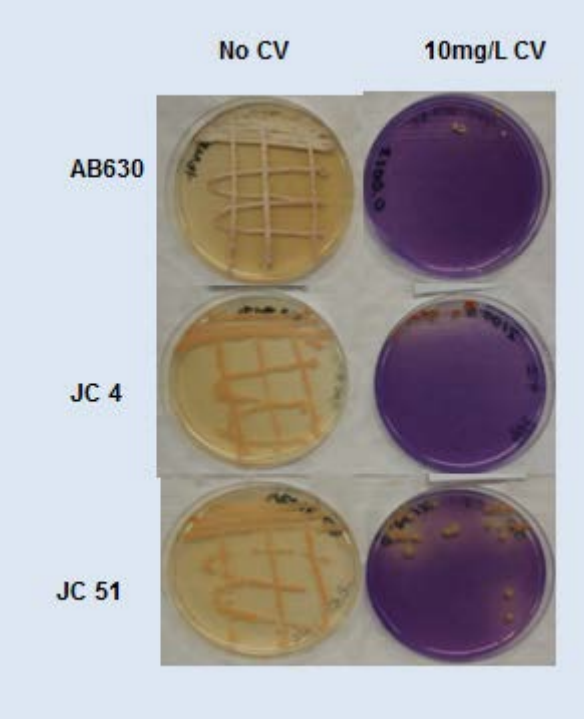


Figure 3.3 Illustration of the effects of 10 mg/l CV concentration has on strains *Pseudonocardia* sp. AB630, *Rhodococcus* sp. JC4 and *Gordonia* sp. JC51. CV effects the morphology of the cells of *Pseudonocardia* sp. AB630, caused hyperpigmentation of cells of *Rhodococcus* sp. JC 4 and reduces the growth of *Gordonia* sp. JC 51 and all strains.

3.3.3 Screening of isolates able to decolourise CV in liquid media

CV decolourisation of deep-sea actinobacteria was tested in liquid format as discussed in Section 3.2.5. Figure 3.4 shows the results from the screening of the eleven deep-sea actinobacterial strains (Table 3-1). Only two (*Gordonia* spp. JC50 and JC51) of the eleven strains could decolourise 30 mg/l CV above 88% within 1 day, above 92% within 3 days and above 97% within 6 days. The strain *Gordonia* sp. JC 51 was the best of all the strains tested.

The criterion for true decolourisation was limited to the ability of strains to decolourise CV above 80%. In the second phase the ability of strains to decolourise CV in liquid were studied. Firstly, the ability of strains to decolourise 30 mg/l CV when introduced at initial point of growth and monitoring the decolourisation extent over a period of 6 days were tested. Figure 3.4 shows the decolourisation ability of strains for these conditions. Only 2 (JC 51 and JC 50) of the 11 strains could decolourise CV above 88% within 1 day of growth. After 3 days these two strains could decolourise CV above 92% and above 97% after 6 days. At day 6 JC 70 could

decolourise CV at 82%. The environment (Negative Control) only contributed to 11% decolourisation over a period of 6 days. Spectral scan studies confirm the ability of JC 50 and JC 51 to remove CV because the major peak at 590 nm is reduced (Figure 3.5).

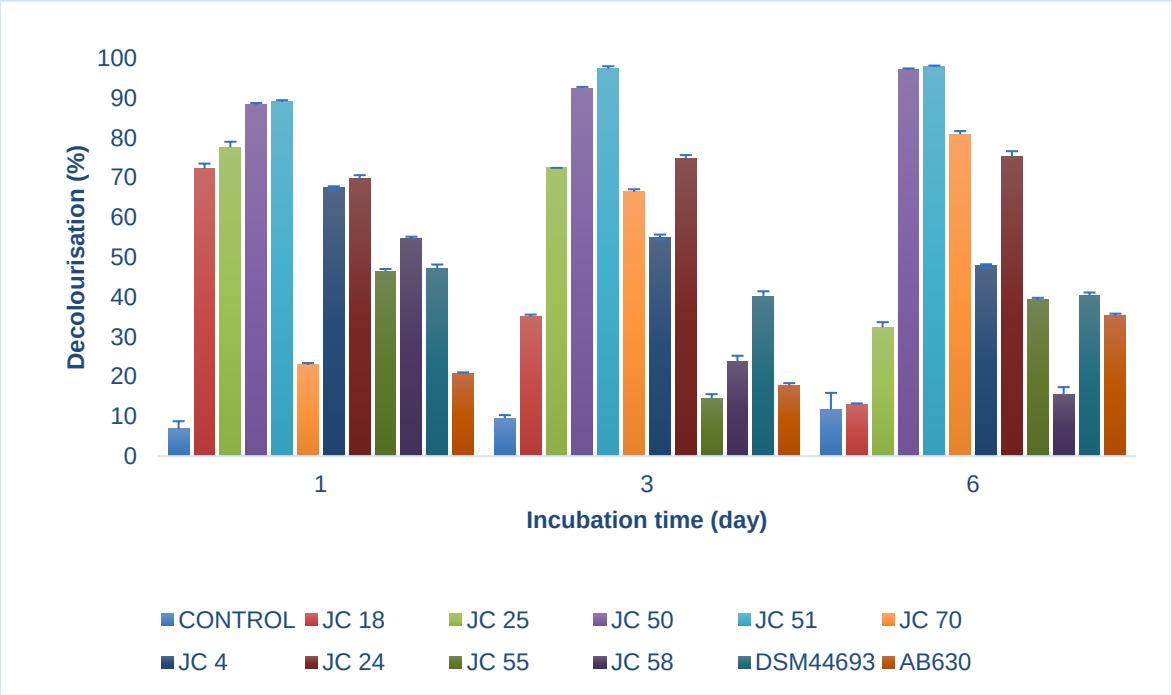
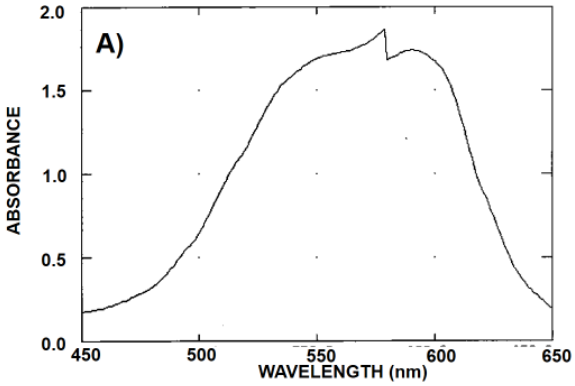


Figure 3.4 Decolourisation ability of deep-sea actinobacteria. Values represent the mean and error bars represent standard deviation of duplicate samples.


A)
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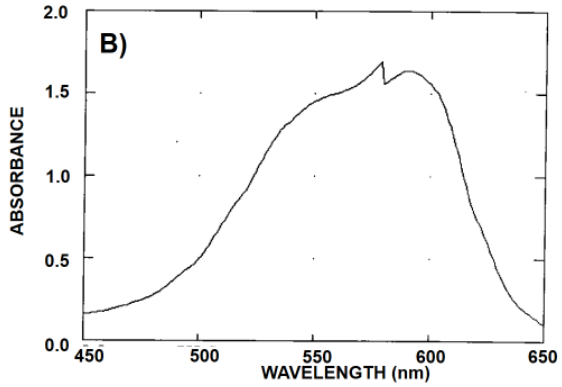
B)
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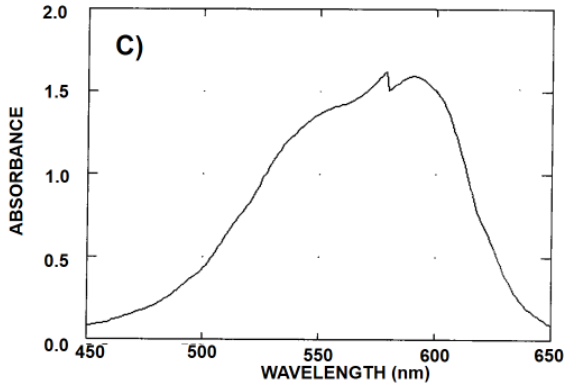
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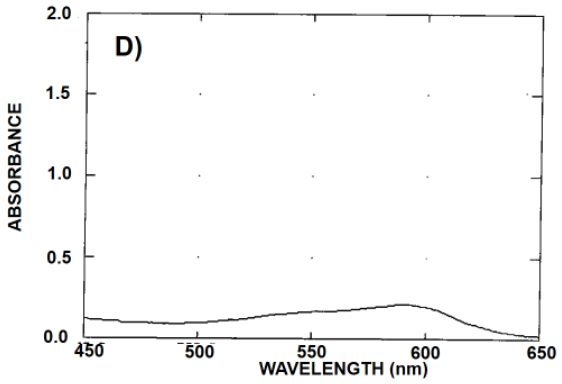
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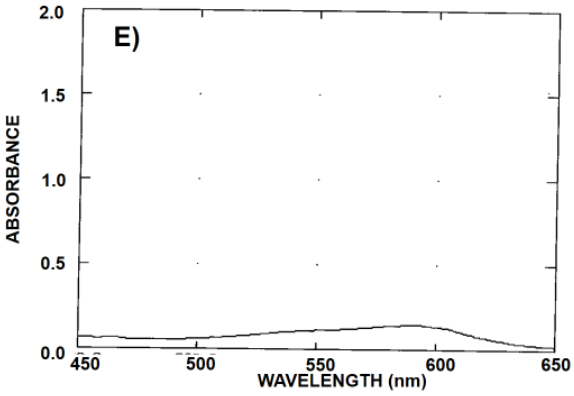
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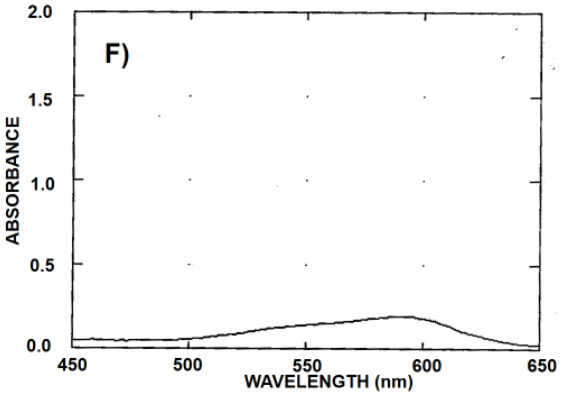
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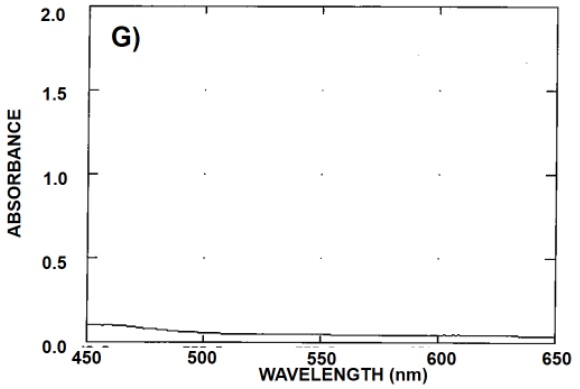
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WAVELENGTH (nm)

Figure 3.5 Comparison between the spectral scan of culture supernatant of *Gordonia* spp. JC 50 and JC51 with Negative Control at 450-650nm. **A)** Reference Control (Time 0) with 30 mg/l CV in YEME. **B)** Negative Control (30 mg/l CV in YEME uninoculated control incubated for 1 day) **C)** Negative Control (30 mg/l CV in YEME uninoculated control incubated for 3 days) **D)** JC 50 (culture supernatant from JC 50 after growing in YEME with 30 mg/l CV for 1 day) **E)** JC 50 (culture supernatant from JC 50 after growing in YEME with 30 mg/l CV for 3days) **F)** JC 51(culture supernatant from JC 51 after growing in YEME with 30 mg/l CV for 1 day) **G)** JC 51 (culture supernatant from JC 51 after growing in YEME with 3 mg/l CV for 3days)

Figure 3.5 shows the spectral scans (450-650nm) of CV after 1 and 3 days of incubation with *Gordonia* sp. JC 50 and JC 51 and including the untreated controls at 0, 1 and 3 days. These spectra demonstrate complete removal of the major visible light absorbance peak at 590nm for 30 mg/l CV incubated in the presence of *Gordonia* sp. JC 51 after 3 days of incubation. For both *Gordonia* spp. JC 50 and 51 the decolourisation process was not caused by the shift in the peak from 590nm to 540nm, which means the *N*-demethylated products were not found in the medium, as compared to the study by Chen et al. (2007a) using *Pseudomonas putida* which found that the N-demethylated products of CV resulted in hypsochromical shift of the maximum absorption peaks in the UV/Vis spectrum. In the study with deep-sea actinobacteria this shift was not observed.

3.3.4 Determining the mechanism of decolourisation

As previously discussed in the literature review there are two mechanisms of decolourisation, biodegradation and biosorption. These mechanisms of decolourisation were observed for the actinobacterial isolates tested within the course of this investigation (Figure 3.6). All ten strains tested (JC 50 was omitted) could decolourise CV at greater than 90% in liquid media. Five of these (*Gordonia* spp. JC 18, JC 25, JC 51, JC 70 and Williamsia maris DSM44693) showed that biodegradation was the main mechanism for decolourisation. Four (*Rhodococcus* spp. JC 24, JC55, JC 58 and *Pseudonocardia* sp. AB630) out of the ten strains showed that decolourisation was solely caused by biosorption. *Rhodococcus* sp. JC 4 was the only strain from the *Rhodococcus* strains used that partially biodegraded CV. These results indicate that decolourisation by the *Gordonia* spp. and *Williamsia maris* DSM44693 as well as *Rhodococcus* sp. JC 4 involves complex mechanisms. The decolourisation involves a combination of biodegradation by enzymes and biosorption of the dye to the cells.

Biodegradation is regarded as an enzymatic process and therefore laccase, peroxidase and polyphenol oxidase were screened for. Secreted (extracellular) ligninolytic enzymes bind non-specifically to the substrate, which means they can degrade a wide variety of recalcitrant

compounds and complex mixtures of pollutants including dyes (Kaushik and Malik, 2008). Secreted enzymes are sought after as they are not limited by substrate uptake into the bacterial cell and are easily assessable with regards to purification, which make them economical as a biocatalyst.

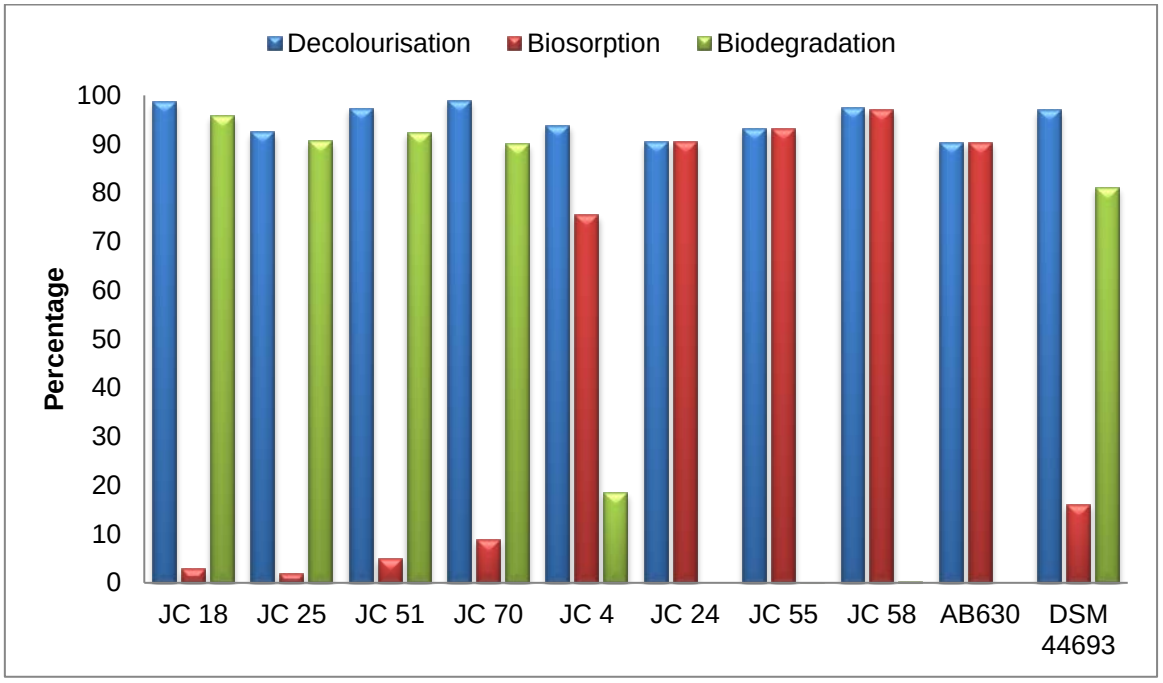


Figure 3.6 The decolourisation mechanisms with ten deep-sea actinobacteria in YEME broth containing 30 mg/l CV. Decolourisation represents the removal of dye from the medium. Biosorption (dye bound to cells) and biodegradation (dye consumed by cells) both represent the removal of dye from the medium.

The mechanism of decolourisation by extracellular oxidative enzymes was determined for all eleven strains, but only the biodegraders *Gordonia* spp. JC 18, JC 25, JC 51, JC 70 and *Williamsia maris* DSM44693 will be discussed. The enzyme activities were determined before and after 1h treatment with 30 mg/l CV and results are shown in Table 3.2. The activities were very low, because the highest activity was 0.004U/ml for extracellular polyphenol oxidase from *Williamsia maris* DSM44693 before treatment with CV. The low levels detected for extracellular laccase, peroxidase and polyphenol oxidase for *Gordonia* spp. JC 18, JC 25, JC 51, JC 70 and *Williamsia maris* DSM44693 make them unsuitable for industrial biocatalyst. One of the criteria for an ideal industrial biocatalyst is that the desired enzyme (such as

laccase, peroxidase and polyphenol oxidase) be secreted in the media at high levels (above 0.05U/ml). Extracellular enzymes require no extensive purification procedure, which reduces costs. The low activities detected may suggest that the enzymes involved in the decolourisation are either intracellular or not the ones tested for.

Table 3-2 Extracellular enzyme activity profile of strains able to decolourise CV via biodegradation

Strain	Laccase Activity (U/ml) [a]		Polyphenol oxidase Activity (U/ml) [b]		Peroxidase Activity (U/ml) [c]	
	Before Induction	After 1 h	Before Induction	After 1 h	Before Induction	After 1 h
JC 18	<0.001	<0.001	0.001±0.0008	0.002±0.0009	0.001±0.0001	0.001±0.0001
JC 25	<0.001	0	0.001±0.0002	0	<0.001	0.001±0.0001
JC 51	0.001 ±0.0012	0	0.002±0.0007	0	0.001±0.0002	<0.001
JC 70	0	0	0.003±0.0007	0.002±0.0004	0.001±0.0001	0.001±0
DSM 44693	<0.001	0	0.004±0.0008	0.002±0.0018	<0.001	<0.001

Values represent the mean of triplicate samples with SD (± standard deviation of the mean).
Substrates were a) 2,6 DMP, b) L-DOPA and c)3-HAA.

The strategy used in this study makes use of testing both the before- and after-1h decolourisation extracellular samples. Initial laccase assays involved the use of ABTS and guaiacol. The activities were not always found after decolourisation. Most samples taken after decolourisation showed a decreasing instead of an increasing slope. This could be attributed to either of the following:

- the ABTS is converted instantaneously and the reverse reaction becomes favourable,
- after the ABTS radical is instantaneously produced it is subsequently broken down, or
- there is an interfering substance present in the sample.

Peroxidase assays also showed problems such as bubble formation. The appearance of bubbles could be the conversion of hydrogen peroxide to oxygen gas, which is released and possibly catalysed by the presence of catalase. The presence of catalase would greatly interfere with the enzyme activity for peroxidases as both enzymes would compete for hydrogen peroxide. The fact that no substrate is converted suggest that this is not a peroxidase, or possibly the sample contains interfering substrates that may compete with

substrate and cause side reactions that are not detected. For example, for *Gordonia* sp. JC 51 the peroxidase activity was found only after extended reaction time. The fact that extended time is required to observe a colour change suggest that the enzyme activities were extremely low. An extracellular actinobacterial peroxidase screening study by Mercer et al. (1996) had to concentrate culture supernatants in order to detect peroxidase activity. However, low oxidative enzyme activities are not uncommon and have been reported.

Le Roes-Hill et al. (2011) used dye decolourisation as a crude indication of oxidative enzyme production. In the study they found 5 out of 39 actinobacteria that were able to partially decolourise CV. Four out of the five CV decolourisers had both laccase and peroxidase activities. Strangely, the strain that produced the highest decolourisation of CV did not produce any laccase, peroxidase or tyrosinase activity. This suggests that there could be another enzyme involved that has not been included in the screening programme. Hence also in the current study it is possible that another enzyme might be involved in the decolourisation of CV.

There are a few studies that point to this possibility. Yan, et al. (2009) studied the biodegradation of CV by the white rot fungus *Pleurotus ostreatus* BP and showed that pure laccase with the specific activity of 500 U/mg could only decolourise 2.8% of 20 mg/l CV within 24 h. However, the low molecular mass fraction (LMMF) of less than <5 kDa could decolourise CV at 25%. Higher decolourisation rates could be obtained with the combination of LMMF and laccase. Native and synthetic mediators of low molecular mass can increase the rate of dye decolourisation and synthetic mediators can improve dye degradation with laccase (Yan, et al., 2009). (Fan, et al., 2011) have shown that the decolourisation of CV by free laccase was slow without the presence of a mediator, purified recombinant laccase at 0.1 U/ml reaction could decolourise 68.3% of 20 mg/l CV after 12h without mediator and 82.45% with mediator. Bumpus and Brock (1988) purified lignin peroxidase from *Phanerochaete chrysosporium,* which biodegraded CV via a sequential N-demethylation process. They found that the fungus could also decolourise CV in non-ligninolytic (nitrogen-sufficient) cultures, which suggest that another mechanism also exists for this fungus.

3.3.5 Bioinformatics based screening

Given the possibility of the presence of a novel oxidative enzyme responsible for the decolourisation of CV in actinobacteria, bioinformatics-based screening was used, which is an *in-silico* approach to evaluate the potential enzymes involved in decolourisation of CV.

The bioinformatic approach used the mining of databases such as bioinformatics tools such as BRENDA (Braunschweig Enzyme Database; http://www.brenda-enzymes.org) and NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/). NCBI contains various bioinformatic information ranging from DNA (genes, genomes), Protein (structure, sequences) and Chemical/ metabolite information. BRENDA has all enzymes that have been described and have an EC number.

The BRENDA database was searched with the search term “Crystal Violet” and the field selected was “substrate”. There was only one positive hit which was for the enzyme peroxidase (EC1.11.1.7). No hits for “triphenylmethane”. (2008 and 16 January 2018 output was the same).

The NCBI database was searched using all associated databases, and the search word used was “triphenylmethane”. Only full text journals were selected the search leads to a page containing 17 databases containing the word including databases leading to literature databases, health, genomes, genes, proteins and chemicals. In 2008 there was limited data about triphenylmethane, and the articles provided were of triphenylmethane reductase from *Citrobacter*. Triphenylmethane reductase was not found in the BRENDA database.

“Triphenylmethane Reductase from *Citrobacter* sp. Strain KCTC 18061P: Purification, Characterization, Gene Cloning, and Overexpression of a Functional Protein in *Escherichia coli” by Jang et al. 2005* was obtained and used as a guide to identify and characterise the enzyme triphenylmethane reductase from deep-sea actinobacteria.

In the present study it was found that *Gordonia spp.* JC 18, JC 25, JC 51, JC 70 and *Williamsia maris* DSM44693 could decolourise 30 mg/l CV via biodegradation at 96, 96, 92, 90 and 81%, respectively, within 1 h. This suggests that the enzyme activities are very high for these strains, but measured activities were low for extracellular laccase, peroxidase and polyphenol oxidase. In order to understand, confirm results and search for potential enzymes involved in CV decolourisation, laccase, peroxidase, polyphenol oxidase and tyrosinase was searched for in the genomes of the genera *Gordonia* and *Williamsia* using NCBI and BRENDA and the results are shown in **Table 3-3**. No tyrosinase was found in genomes of *Williamsia* and *Gordonia*. Possible laccase, peroxidase and polyphenol oxidase enzymes were found in genes and/or protein sequences in these genomes. When searching for enzymes involved in decolourisation of triphenylmethane dyes using NCBI an enzyme called triphenylmethane reductase was found. Triphenylmethane reductase from *Citrobacter* sp. strain KCTC 18061P

was first discovered by Jang, et al. (2005). This enzyme could decolourise CV to Leucocrystal Violet (LCV) with NADH as a cofactor. This enzyme was also searched for in genomes of *Williamsia* and *Gordonia* and 2 and 25 protein sequences were found respectively. Further investigation revealed that these enzymes were not called triphenylmethane reductase, but NAD(P)H quinone oxidoreductases. This means that triphenylmethane reductase has not been identified for the genome of *Gordonia.* However, NAD(P)H quinone oxidoreductases are considered part of the "Triphenylmethane-like" proteins.

It was also found that the enzyme triphenylmethane oxygenase exist and has been found in the bacterium *Aeromonas*.

Table 3-3 Screening results of bioinformatics databases BRENDA and NCBI for oxidative enzymes involved in CV decolourisation via biodegradation

Enzyme	***Gordonia***	***Williamsia***
Laccase	BRENDA=0 NCBI: genes=3 Protein sequence=51	BRENDA=0 NCBI: genes=0 Protein sequence=2
Peroxidase	BRENDA=0 NCBI: genes=30 Protein sequence=532	BRENDA=0 NCBI: genes=0 Protein sequence=15
Polyphenol oxidase	BRENDA=0 NCBI: genes=3 Protein sequence=51	BRENDA=0 NCBI: genes=0 Protein sequence=2
Tyrosinase	BRENDA=0 NCBI: genes=0 Protein sequence=0	BRENDA=0 NCBI: genes=0 Protein sequence=0
Triphenylmethane reductase	BRENDA=0 NCBI: genes=1 Protein sequence=25	BRENDA=0 NCBI: genes=0 Protein sequence=2

3.4 Conclusion

It can be concluded that all eleven deep-sea actinobacteria tested could decolourise CV, but only 5 strains could decolourise CV via biodegradation. Low levels of extracellular laccase, peroxidase and polyphenol oxidase activities produced by biodegraders suggested that another enzyme may be involved. Database mining tools indicate that Triphenylmethane reductase may be a potential candidate oxidoreductase responsible for the decolourisation of CV and should be screened for.

Gordonia sp. JC 51 was selected as a candidate for further investigation within the scope of this study, because it was the only strain that had the ability to consistently decolourise CV at high activities. It could also tolerate and decolourise CV at 1 mM on YEME agar plates. The next phase of the study was to determine if *Gordonia* sp. JC 51 has triphenylmethane reductase activity and to assess the ability of this strain to decolourise CV under various conditions.

4 Decolourisation of Crystal Violet by *Gordonia* sp. JC 51

4.1 Introduction

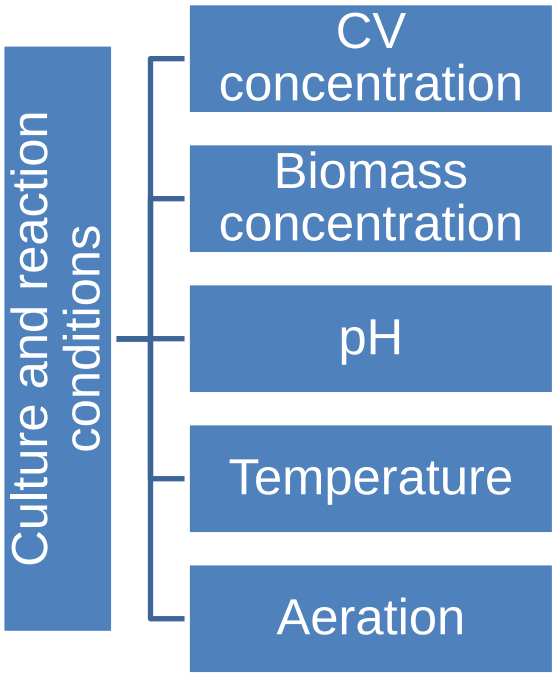


Figure 4.1 A Horizontal Multilevel hierarchy used to represent the culture and reaction conditions tested for CV decolourisation by *Gordonia* sp. JC 51. The CV concentration, biomass concentration, pH, temperature and aeration were the growth and reaction conditions tested.

The use of bacterial consortia in the treatment of complex industrial wastewaters have proven to be efficient. It is therefore important to understand and characterise single microorganisms that have potential use in these bacterial consortia and how to develop them into efficient whole-cell biocatalysts for environmental application. The first step would be to obtain a highly efficient dye decolourising strain that maintains efficiency under a range of operating conditions. As discussed in the literature review, most microorganisms are unable to decolourise CV. The previous chapter has identified the actinobacterium *Gordonia* sp JC 51 as a potential candidate for efficient CV decolourisation using certain oxidoreductases.

In this section of the present study *Gordonia* sp. JC 51 will be focused on for the ability to decolourise CV under various culturing conditions including variation in parameters such as aeration, pH, temperature, CV concentration, inoculum concentration and time of induction.

4.2 Materials and Methods

4.2.1 Dyes and Chemicals

CV was purchased by Merck. All chemical reagents used were analytical grade. The concentrations used were given in the tables or figures unless otherwise stated.

4.2.2 Microorganism

The CV dye decolourising actinobacterial strain *Gordonia* sp. JC 51 was initially isolated from Izu-Bonin trench at great depth and provided by Professor Alan Bull (University of Kent, UK). *Gordonia* sp. JC 51 was characterised by the modification of several techniques described by Shedbalkar et al. (2008), Kaushik & Malik (2009) and An et al. (2002). Based on the screening study described in Chapter 3, *Gordonia* sp. JC 51 was selected for further study as it was identified as the most efficient in CV biodegradation.

4.2.3 Decolourisation of CV by living *Gordonia* sp. JC 51 cultures

The CV decolourisation medium was YEME broth containing 4 g/l yeast extract, 10 g/l malt extract and 4g/l glucose with different concentrations of CV dye. *Gordonia* sp. JC 51 was precultured in YEME broth at 30°C with shaking at 160 rpm for 3 days (unless otherwise stated) and used as an inoculum to inoculate the decolourising medium with an initial cell concentration above 10 mg/l wcw (wet cell weight).

After decolourisation culture samples (1 ml) were removed at various time points, and centrifuged at 10,000 rpm for 5 min. The supernatant (Culture supernatant) was used to measure absorbance of CV dye in solution (A_s). The cell pellets were extracted with 1 ml 70% ethanol (or absolute ethanol) and centrifuged at 10,000 rpm for 5 min. The supernatant (Cell pellet extract) was used the measure the absorbance CV dye extracted from cell pellet (A_p).

4.2.4 Bacterial cell fraction preparation and activity test

100 ml culture was centrifuged at 10,000 rpm for 30 min. The supernatant was used as culture supernatant. The cell pellet was washed twice with buffer 50mM sodium phosphate buffer (pH 7.0) and the resuspended cells in 100ml buffer were sonicated on ice (30 s, 11, 4 cycles for 4

min) by using VirSonic Ultrasonic cell disruptor 100. Cell debris were removed by centrifugation at 10,000 rpm for 30min. The supernatant was used as cell lysate. The protein concentration was determined by the Bradford method (Bradford, 1976) using BSA (bovine serum albumin) as standard. Culture supernatant and cell lysate was used to determine enzyme activities, Laccase, peroxidase, polyphenol oxidase and triphenylmethane reductase.

4.2.5 Growth studies of *Gordonia* sp. JC 51

For growth curve studies a loopful of cells of *Gordonia* sp. JC 51 from streak plates was inoculated into 10 ml YEME broth (pH 7) in 50 ml Erlenmeyer flasks (32 flasks were used). Negative controls were also included which contained uninoculated medium used to confirm that growth was not due to contamination of medium. Flasks were incubated at 30°C, shaking at 160 rpm, for 12 days. The biomass (wet and dry), pH and protein concentration (intracellular, extracellular and total) were determined as per the following sections.

4.2.6 Preparation of extracellular, intracellular and total soluble protein fractions

Total soluble protein fractions were prepared by placing 1 ml samples of culture in 1.5 ml microcentrifuge tubes in duplicate. Cells were disrupted by sonicating (VirSonic Ultrasonic cell disruptor 100) for 10 s pulsation and 10 s rest on ice, repeated for a total of 4 min. The disrupted cells and total soluble protein were separated by centrifugation at 10,000 rpm for 5 min. The supernatant was transferred to 1.5 ml microcentrifuge tubes and kept on ice until use.

The culture supernatant and cell lysate were prepared by placing 1 ml samples of culture in 1.5 ml microcentrifuge tubes in duplicate and centrifuging at 10,000 rpm for 5 min. The supernatant was transferred to another 1.5 ml microcentrifuge tube. This was the culture supernatant and kept on ice until use. The remaining cell pellet was washed (with buffer), centrifuged at 10,000 rpm for 5 min, the supernatant removed and resuspended in 1 ml 50 mM sodium phosphate buffer (pH 6). The cell suspension was disrupted by sonicating (VirSonic Ultrasonic cell disruptor 100) for 10 s pulsation and 10 s rest on ice, repeated for a total of 4 min. The crude cell was centrifuged at 10,000 rpm for 5 min. The supernatant was the cell lysate was transferred to 1.5 ml microcentrifuge tubes and kept on ice until use.

4.2.7 Analysis methods

4.2.7.1 *Decolourisation*

Decolourising activity was modified using the method previously described (Yatome, et al., 1993). Decolourisation % = $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the initial absorbance of the control, A_1 is the absorbance of the sample solution after decolourisation. Biodegradation activity was modified using the method previously described (Deng, et al., 2008). Biodegradation %= $[(A_0 - A_s - A_p)/A_0] \times 100$, where A_0 is the initial absorbance of the control, A_s is the absorbance of culture supernatant after decolourisation and A_p is the absorbance of the cell pellet extract after decolourisation. The maximum wavelength of CV was 590 nm and the spectrophotometer was used. Microtitre plate assays were performed in triplicate and spectrophotometric assays in duplicate unless otherwise stated.

4.2.7.2 *Determining wet and dry biomass*

After growth, duplicate 1 ml samples of culture were transferred into 1.5 ml microcentrifuge tubes which had been oven-dried and then pre-weighed. The samples were centrifuged at 10,000 rpm for 5 min. Supernatant was removed and the remaining cell pellet was washed and re-centrifuged. All liquid was removed, and the weight determined. Dry weight of cells was determined by placing the microcentrifuge tube containing the cell pellet in an 80°C oven for 24 h. The weight was then determined. The weight determined was then subtracted from the predetermined weight of the microcentrifuge.

4.2.7.3 *Determining the pH of media*

The pH of samples collected at various time points was determined by using a Cyberscan 1000 pH meter.

4.2.7.4 *Bradford assay*

The Bradford Assay (Bradford, 1976), was performed to determine the protein concentration of samples (such as culture supernatant, cell lysate and other enzyme solutions) using bovine serum albumin (BSA) as a standard. A standard of BSA was created through serially diluting a 1mg/l BSA stock (Appendix G; G28) in the concentration range of 0 to100μg/ml. A 1.1ml final volume Bradford assay, which contained 100 μl protein sample and 1ml Bradford Reagent (Sigma), was placed in a 2ml plastic cuvette and allowed to incubate for 5min at room temperature (22°C) before the absorbance was measured at 590nm (Appendix J: J1 and Table J1). Blanks were included, which contained 100 μl water instead of protein sample.

The unknown protein concentration of the samples was determined by comparing the absorbance readings with the absorbance of standards of known concentrations (Appendix H: Figure H1)

4.2.8 Procedures used to determine the effect of various variables on the ability of strain to decolourise CV

The standard culturing of *Gordonia* sp. JC 51 was done in YEME at 30°C, shaking at 160rpm for 1-7 days. Temperature tests strains were grown for 1 day, treated with 30 mg/l CV and incubated for 1h at different temperatures (4,22,30,37 and 55°C). Aeration tests strains were grown for 6 days (200 ml in 1L Erlenmeyer flask) either shaking at 160rpm or static, treated with 100 mg/l CV and samples removed at various time intervals (0,1,2,4,5,6 and 24 h). For culture pH test, cultures were grown under different initial pH (5,7 and 9) for 3 days (50ml in 500ml Erlenmeyer flask), incubated for 18 h. For initial CV concentration tests strain was grown for 7 days (100 ml) and to a series of ten 100 ml Erlenmeyer flasks 10ml culture was added to each flask. To each flask different concentrations of CV was added ranging from 0-2000 μM, incubated for 30min and 1 h.

For initial biomass tests a 6-day-old culture was used to prepare 100 mg/l wet weight of cells. This stock was used to make 5, 10, 20, 50, 60, 70, 80, 90,100 mg/l cell biomass samples in 1ml in 1.5ml microcentrifuge tubes containing 100 mg/l CV in water and incubated for 18 h.

For all tests, samples were collected (1-2 ml), centrifuged at 10000 rpm for 5 min and 1ml culture supernatant used for analysis of CV decolourisation.

4.2.9 Testing decolourisation under various concentrations of CV

Tester strains, *Gordonia* sp. JC 51 and *Rhodococcus* sp. JC 4, were cultured in YEME broth for 7 days at 30°C, shaking at 160 rpm. On the seventh day of growth CV was added at various concentrations (0, 10, 30, 60 and 100 mg/l). For each concentration duplicates were performed. Controls were also included which contained all medium components but no culture. Flasks were incubated at 30°C, shaking at 160 rpm, for 18 h. Duplicate samples were collected into 1.5 ml centrifuge tubes and centrifuged at 10,000 rpm for 5 min. One millilitre culture supernatant was used to determine the decolourisation as described previously (Section 2.2.2.3). Culture samples were used to determine viability by testing growth on YEME agar plates. A positive test result was the appearance of colonies of test strain. Indicating that cells are viable (still alive) after exposure and decolourisation of CV. A negative test result was

when there was no growth (no appearance of colonies of Test strain). Negative result would indicate that cells die after being exposed to CV at the test concentrations.

4.2.10 Procedure used to determine the location and catalytic ability of strains by studying the decolourisation by whole cells, lysed cells and lysed heated cells

Cells were cultured at 30°C, shaking at 160 rpm for 3 days in 100 ml YEME (pH7). Duplicates were made. Cultures were then split up into three fractions. One culture batch was placed aside and was designated culture untreated (UC), the other two batches were sonicated by pulsing at level 10 for 10 s and resting for 10 s for 12 cycles (approximately 4 min in total). The one batch of sonicated culture (CS) sample was stored on ice. The other batch of sonicated culture was heated at 80°C for 1 h and called culture sonicated and heated (CSH). UC, CS and CSH were then placed in Erlenmeyer flasks, treated with 30 mg/l CV and incubated at 30°C, shaking at 160 rpm for 1 h. Two 1 ml samples were removed from each flask, placed in 1.5 ml microcentrifuge tubes and centrifuged at 10,000 rpm for 5 min. The supernatant and pellet were used to determine the adsorption and biodegradation.

4.2.11 Enzyme Assays

4.2.11.1 *Procedures used to identify the main enzymes responsible for CV decolourisation.*

Laccase (Section 3.2.8.1), peroxidase (Section 3.2.8.2) and polyphenol oxidase (Section 3.2.8.3) were assayed for.

4.2.11.2 *Procedure used to determine triphenylmethane reductase*

To determine triphenylmethane reductase (TMR) the method as described by Jang, et al. (2005) using CV as a substrate. A 1 ml reaction mixture contained 20 μM CV, 20 mM Sodium phosphate buffer pH 7, 0.1 mM NADH (prepared fresh) and 100μl enzyme solution. The decrease in absorbance was monitored at 590nm for 2 min (ε= 110916$M^{-1}cm^{-1}$).

4.2.11.3 *Procedure used to determine the need for NADH in decolourisation*

To determine if NADH was required the TMR assay was performed with and without the addition of NADH.

4.3 Results and Discussion

4.3.1 Growth curve analysis of *Gordonia* sp. JC 51

Preliminary studies that involved the growth analysis of all eleven strains revealed that *Gordonia* and *Rhodococcus* spp. formed pellets when grown. This interfered greatly with growth curve analysis. There are different methods for determining growth such as wet biomass, dry biomass, optical density reading and protein concentration. It was not possible to correlate cell weight with cell adsorption, because the strain went through various morphological and biochemical changes. Also, it was noted that various metabolic and secretory products were produced by the strain. This could contribute to weight and interfere with absorption readings. The colour of strains was observed to greatly increase in intensity over time. This should therefore be taken into consideration.

The growth curve for *Gordonia* sp. JC 51 was determined and is presented in Figure 4.2. The best method for determining the stages of growth was the use of wet biomass but using the OD reading at 600 nm for initial stage of growth could be valuable as it is more sensitive. Both the wet biomass and absorbance reading for a period of 4 days correspond to each other and showing that highest point of growth for *Gordonia* sp. JC 51 is on day 4.

Figure 4.2 shows the growth curve of *Gordonia* sp. JC 51 for the period of 12 days. The dry weight does not show much change over the course of the 12 days. However, the wet weight shows that on Day 3-4 there is a peak in the growth of cells and that there is a stationary phase between 4-12 days. Between 4 and 12 days the total soluble protein increases from 182 to 314 μg/ml.

The effect CV has on the growth of *Gordonia* sp. JC 51 was also investigated. The initial biomass was 57 mg/ml±6wet weight and 4 mg/ml±1 dry weight. When CV was added to growth media at 0 mg/l, 30 mg/l and 100 mg/l the average wet biomass was 51 mg/ml (±6), 43 mg/ml (±6) and 15 mg/ml (±2) respectively after 3 days growth. This demonstrates that an increase in CV concentration results in a decrease in biomass i.e. reduced growth.

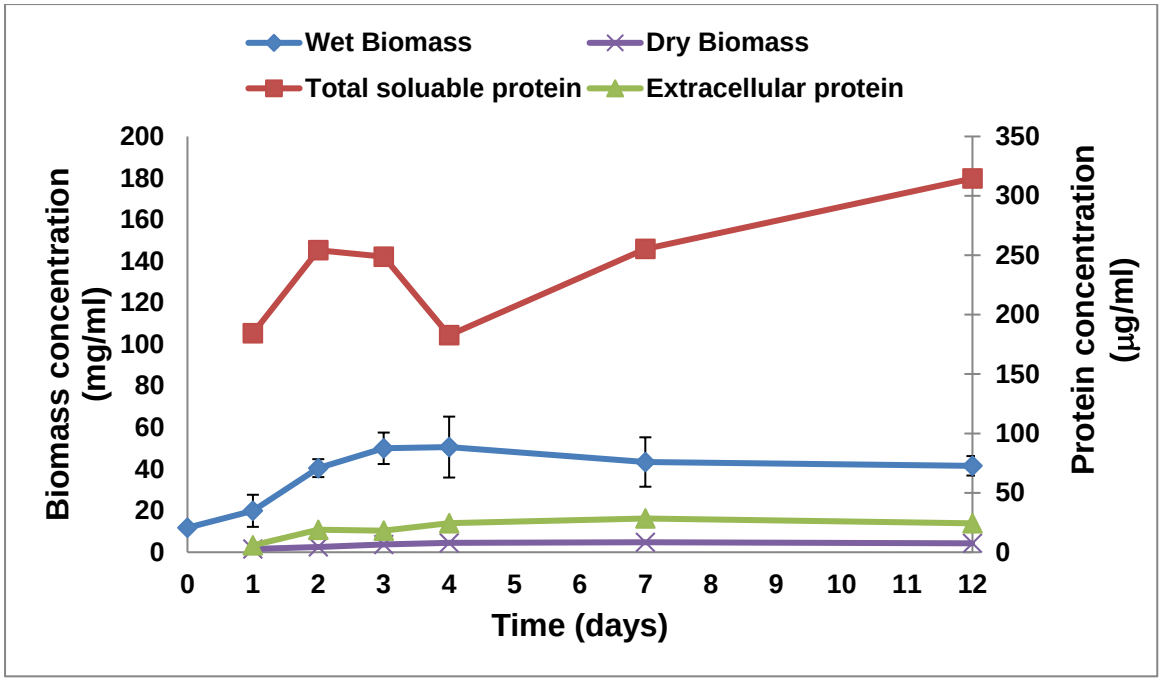


Figure 4.2 Growth curve of *Gordonia* sp. JC51. Datapoints represent the mean of at least duplicate samples and error bars represent standard deviation.

4.3.2 The effect of different variables on decolourisation ability of the strain *Gordonia* sp. JC 51

4.3.2.1 *Initial biomass concentration*

Figure 4.3 shows the relationship of between the wet cell biomass and decolourisation. After 18 h of incubation 10-100 mg/ml JC 51 cells caused decolourisation of more than 90% for 100 mg/ml CV. At the concentration of 5 mg/ml initial biomass of *Gordonia* sp. JC 51 only an average of 7.55% CV was decolourised. At cell concentrations from 0-20 mg/ml an increase in cell concentration increases decolourisation activity. This means that the more biologically active cells available that the higher the capacity to express decolourising enzymes. After 20 mg/ml the strain reaches the highest decolourisation threshold and an increase in biomass does not affect decolourisation activity. The initial growth biomass of the growth curves of *Gordonia* sp. JC 51 (Figure 4.2) was between 11.8 and 13.1 mg/l (biomass above 10 mg/l obtained more than 91% in this study) , which explains why decolourisation was high for this strain after 18-24 h in previous tests (Section 3.2.5)

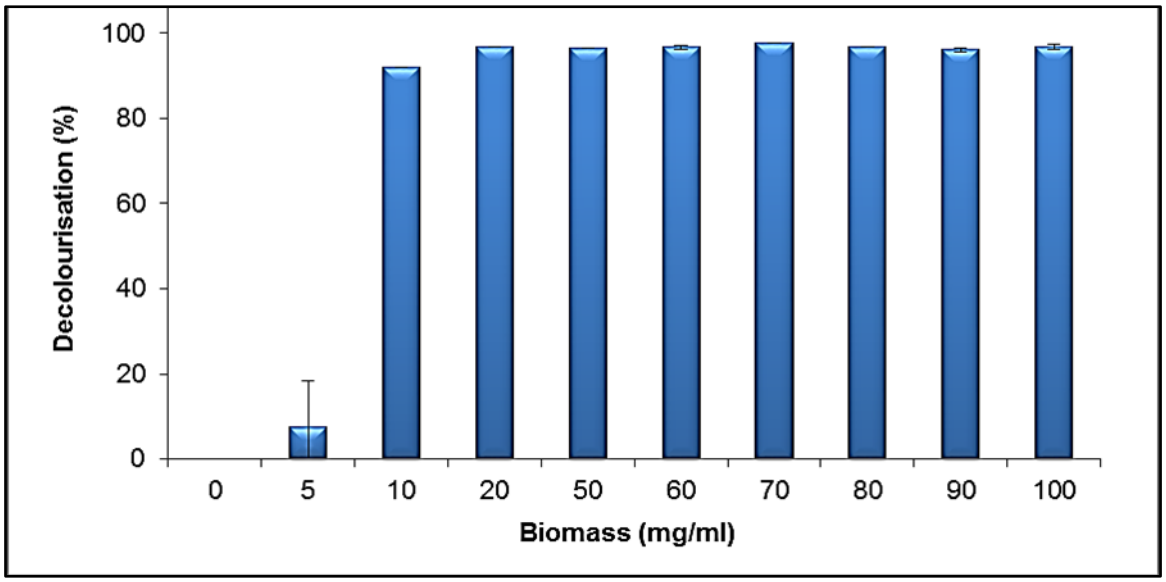


Figure 4.3 The correlation between biomass and decolourisation percentage of *Gordonia* sp. JC51 at 18 h after the initial CV concentration of 100 mg/l was added. Results represent the average of duplicates and the error bars represent the standard deviation.

4.3.2.2 *Aeration*

Figure 4.4 shows that the decolourisation of CV occurred more rapidly under agitation (aerated conditions) than under static conditions. The decolourisation of 100 mg/l CV for aerated and static conditions was above 90% within 1 h and 4 h respectively. The end point is not affected by the agitation, but only the initial rate. JC 51 grew better in agitation than static condition as biomass was 30 mg/l and 19 mg/l after 6 days of incubation. The higher rate of decolourisation for aeration could be the result of higher biomass concentration. The increased rate could be due to improved mass transfer or aeration. At an industrial perspective the ability of *Gordonia* sp. JC 51 to decolourise CV for both agitation and static conditions could be beneficial.

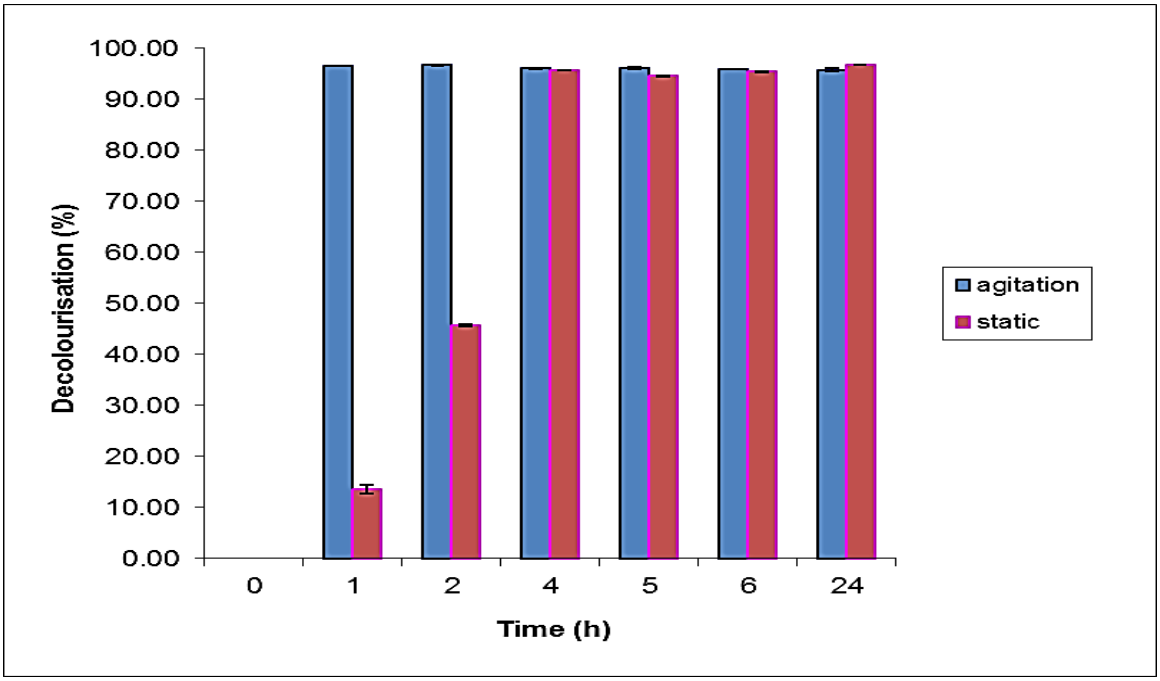


Figure 4.4 The differences between agitation and static conditions have on decolourisation for *Gordonia* sp. JC 51. The results represent the average of duplicates and the error bars represent the standard deviation.

4.3.2.3 *Temperature*

In order to determine the effects temperature had on the ability of *Gordonia* sp. JC 51 to decolourise CV the temperatures 4-55°C was tested. It was found that the temperature range tested had little or no effect on the ability of *Gordonia* sp. JC 51 to decolourise CV. All temperature conditions showed a decolourisation percentage of above 92% (Figure 4.5). The temperature of the reaction shows no significance between 4 and 55°C based on the removal of dye (decolourisation). However, observing the pellet it is clear that at 22 and 30°C biodegradation occurs as the cell pellets are unstained at the end of the reaction. At 4 and 55°C the pellets are stained and indicates that the decolourisation mechanism is biosorption. For *Gordonia* sp. JC 51 the optimum temperature for biodegradation is at 22 and 30°C. However, for the removal of dye from the solution the temperatures between 4-55°C would be effiecient.

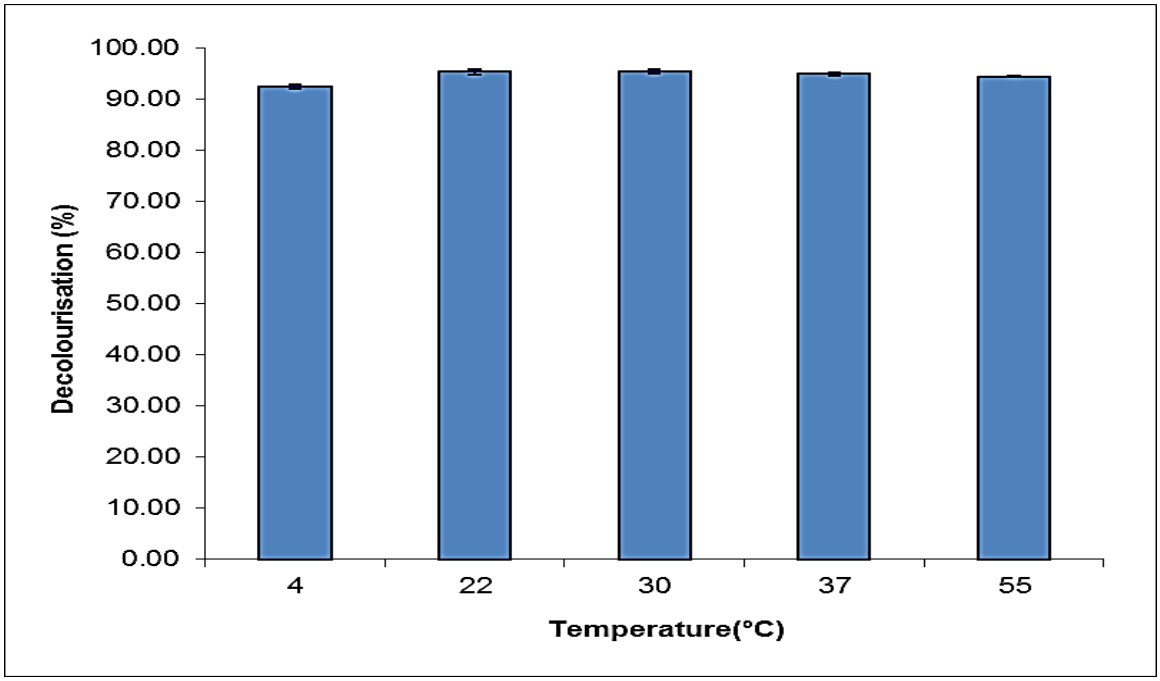


Figure 4.5 The effect of temperature on decolourisation of JC51 after 1 h. The results represent the average of duplicates and the error bars represent the standard deviation.

4.3.2.4 *pH*

The effect pH had on growth and CV decolourisation ability of *Gordonia* sp. JC 51 was determined using pH 5 for acidic conditions, pH 7 for neutral conditions and pH 9 for alkaline conditions.

Strains were able to grow at all three conditions tested. Figure 4.6 shows that decolourisation for pH 5, 7 and 9 were high and above 82%. There is no significant difference between pH 5 and 7. At pH 9 the decolourisation, while still high, is significantly lower than the other two conditions. This indicates that the neutral or slightly acid decolourisation conditions are optimal. However, a wider range of pH would need to be tested if the target wastewater demanded it. The pH was also followed during decolourisation of CV and it was found that pH did not contribute in the decolourisation of CV.

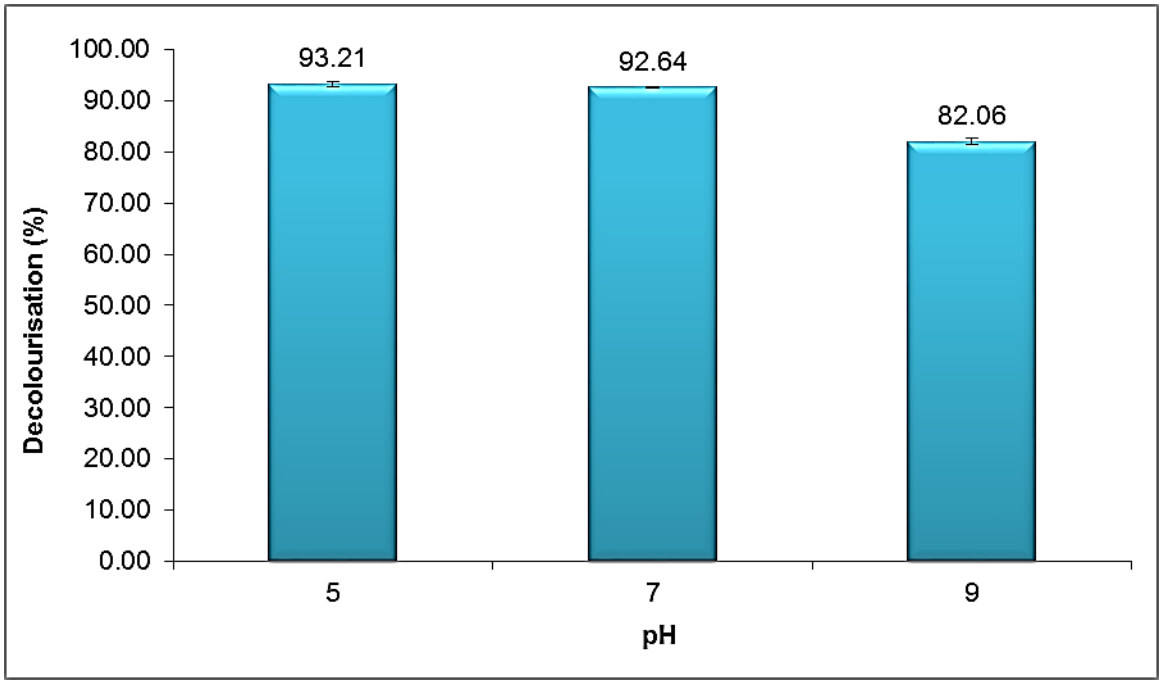


Figure 4.6 The decolourisation ability of *Gordonia* sp. JC 51 at pH 5, 7 and 9. Error bars represent standard deviations of duplicates.

4.3.2.5 *Dye concentration*

In order to characterise *Gordonia* sp. JC 51 the ability of strain to decolourise CV at different concentrations of the dye (30-2000μM) were tested.

In Chapter 2 (Section 3.2.4) the tolerance of stains (*Gordonia* spp. JC18, JC25, JC50, JC51, JC70; *Rhodococcus* spp. JC4, JC24, JC55, JC58; *Williamsia maris* DSM44693 and *Pseudonocardia* sp. AB630) for CV was determined in solid medium using various concentrations of CV ranging from 10-60 mg/l. The comparison of the decolourisation ability of *Gordonia* sp. JC 51 and *Rhodococcus* sp. JC 4 at various concentrations of CV after 18 h of incubation is shown in Figure 4.7.

In this section of the study the ability of *Gordonia* JC 51 and *Rhodococcus* sp. JC 4 to decolourise CV under various concentrations was compared, but in liquid format and extending the concentrations tested to 100 mg/l (Section6.2.1). Both JC 51 and JC 4 could decolourise CV at rates above 89% for concentrations 10-60 mg/l after 18h, but only JC51 could decolourise CV (91%) at 100 mg/l. Culture samples from JC51 after treatment with 100 mg/l CV were tested for viability by streaking on YEME plates. High growth was found on

plates, which proved that JC 51 cells were still viable at 100 mg/l. This means that JC 51 can both decolourise and tolerate CV at 100 mg/l.

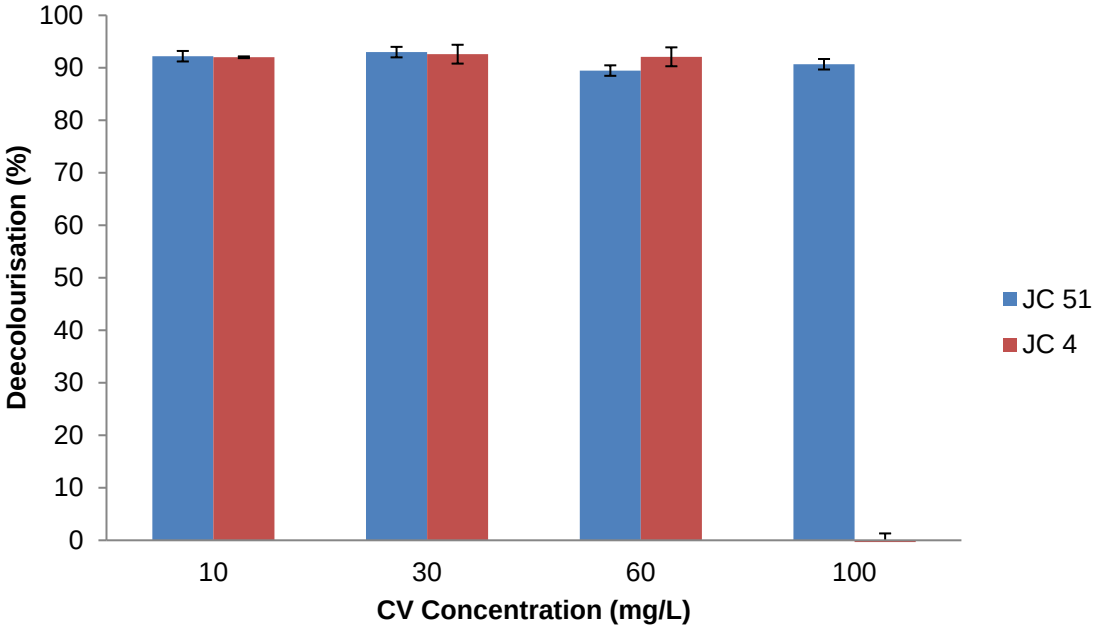


Figure 4.7 The comparison of the decolourisation ability of *Gordonia* sp. JC 51 and *Rhodococcus* sp. JC 4 at various concentrations of CV after 18 h of incubation at 30°C shaking at 160 rpm. Data represent the average of two replicates and error bars represent the standard deviations.

The ability of *Gordonia* sp. JC 51 to decolourise CV at various concentrations ranging from 30-2000 μM were tested, and the results are shown in Figure 4.8 Between 30-500 μM more than 95% CV was decolourised within 1h (60 min). At 1000 and 2000 μM *Gordonia* sp. JC 51 could decolourise CV at 72 and 16 % respectively after 1 h.

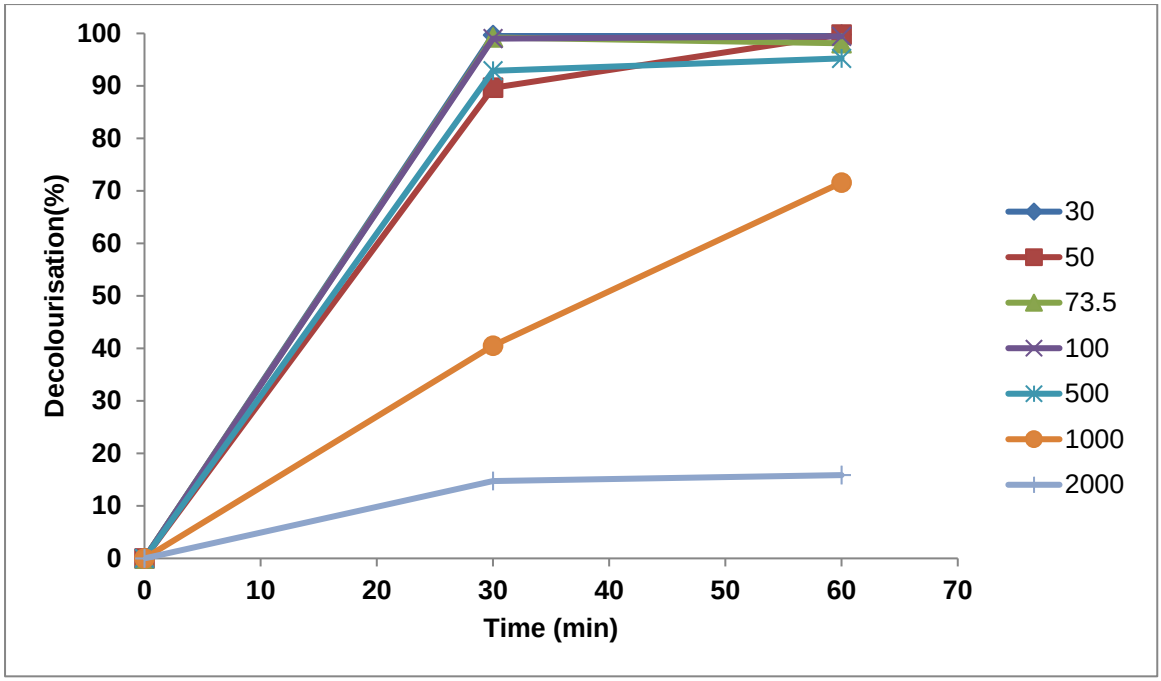


Figure 4.8 The effect of dye concentration on the ability of JC 51 to decolourise CV. The concentrations assessed were: 30 μM (12.24 mg/l), 50 μM (20.4 mg/l), 73.5 μM (30 mg/l),100 μM (40.8 mg/l), 500 μM (204 mg/l),1000 μM (407.98 mg/l) and 2000 μM (815.96 mg/l), as indicated.

4.3.3 Confirming if decolourisation is an enzymatic process

Cultures were subject to two treatments, the first was sonication and the second was sonicated and heated. These two treatments represent cell-free samples. Enzymes are responsible for the biodegradation of CV. The heat-inactivation of enzymes prevents decolourisation via biodegradation. CV can be decolourised by attachment to cell components such as proteins, lipids, DNA. Decolourisation could be due to binding to cell components and therefore suggest CV-adduct formation.

Decolourisation was determined under three culture conditions of *Gordonia* sp. JC 51, which were: 1) culture untreated (UC), 2) culture sonicated (CS) and 3) culture sonicated and heated (CSH). The different treatments were prepared as described in section 4.2.10.

Figure 4.9 shows the results for the UC, CS and CSH samples of *Gordonia* sp. JC 51. All three treatment conditions (UC, CS, CSH) could decolourise CV highly efficiently, because it could reach decolourisation levels above 88%. UC and CS have similar decolourising capabilities. Biodegradation was attributed to active enzymes being present in the UC and CS samples.

No biodegradation was found in the CSH sample, because enzymes were heat-inactivated. Decolourisation for CSH was mainly due to CV binding to proteins, lipids and other components released from the cell. This experiment demonstrates that:

1. enzymes are responsible for the biodegradation of CV
2. decolourisation can occur by enzymatic (biodegradation) and non-enzymatic (biosorption) mechanisms
3. decolourisation can occur in living (whole cells) and non-living (cell-free) conditions

The limitations of this experiment is that it only confirms if enzymes are responsible for the biodegradation of CV. Since the enzymes are mixed in solution and not separated the assay does not differentiate the original location of the enzymes (i.e. if the enzymes that cause decolourisation are found mainly in the intracellular cytosol, bound within the membrane of the bacterial cell or secreted extracellular enzymes found in the culture medium).

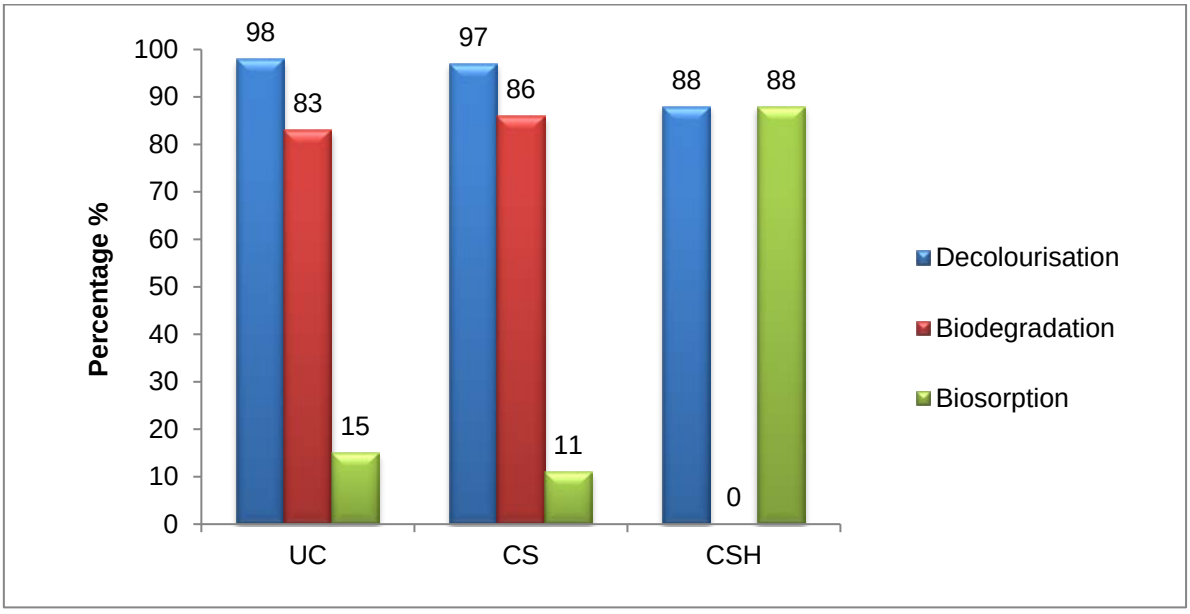


Figure 4.9 Representative samples of UC (Untreated culture), CS (Culture sonicated) and CSH (Culture sonicated and heat treated) of *Gordonia* sp. JC 51 and decolourisation, biodegradation and biosorption of CV (attachment of the dye to disrupted cell constituents).

Previous enzymatic screening involved laccase, peroxidase and polyphenol oxidase (PPO) and focused on the culture supernatant, but here both culture supernatant and cell lysate were analysed. Triphenylmethane reductase (TMR) was also included, because a study (Jang, et

al., 2005) has demonstrated that it can be involved in the decolourisation of CV as was discussed in Chapter 2.

Figure 4.10 illustrates the enzyme activity profile of both culture supernatant and cell lysate protein samples of *Gordonia* sp. JC 51.

The activities were noted to vary according to the different batches. Batch to batch variations were common. There was no activity detected for laccase. Peroxidase has insignificant specific activity for both intracellular and extracellular fractions. PPO has low, but significant extracellular and insignificant intracellular specific activities. TMR has high extracellular activity, but insignificant intracellular activity. It is possible that other unknown enzymes are involved in the decolourisation of CV. It was found that at various times after decolourisation that the enzyme profiles change.

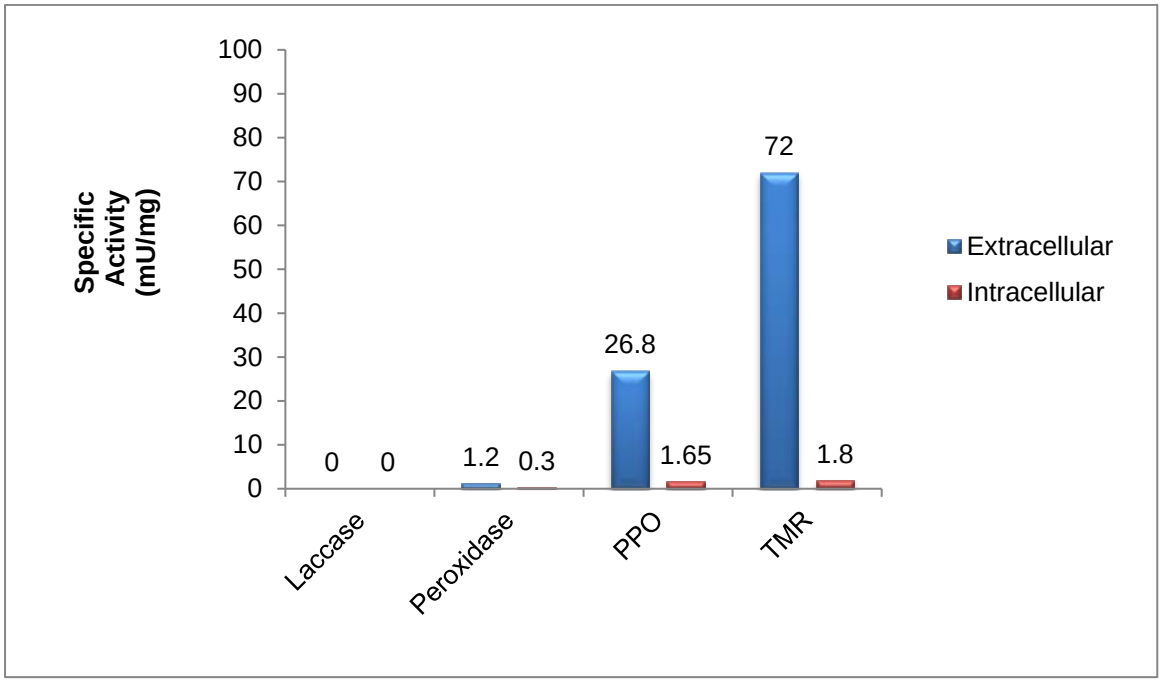


Figure 4.10 Identification of the known dye decolourising enzymes produced by *Gordonia* sp. JC 51

4.3.3.1 *The effect NADH has on decolourisation activity of TMR*

Before triphenylmethane reductase could be confirmed as the main enzyme responsible for the decolourisation of CV by *Gordonia* sp. JC 51, the dependence of NADH in the assay was determined. Triphenylmethane reductase requires NADH to convert CV to LCV (Reaction 1).

$$CV+NADH \rightarrow LCV + NAD \qquad \text{(Reaction 1)}$$

Culture supernatant and cell lysates from *Gordonia* sp. JC 51 were tested using the triphenylmethane reductase assay performed as previously described (Section 4.2.11), but also without NADH (Section 4.2.11.3).

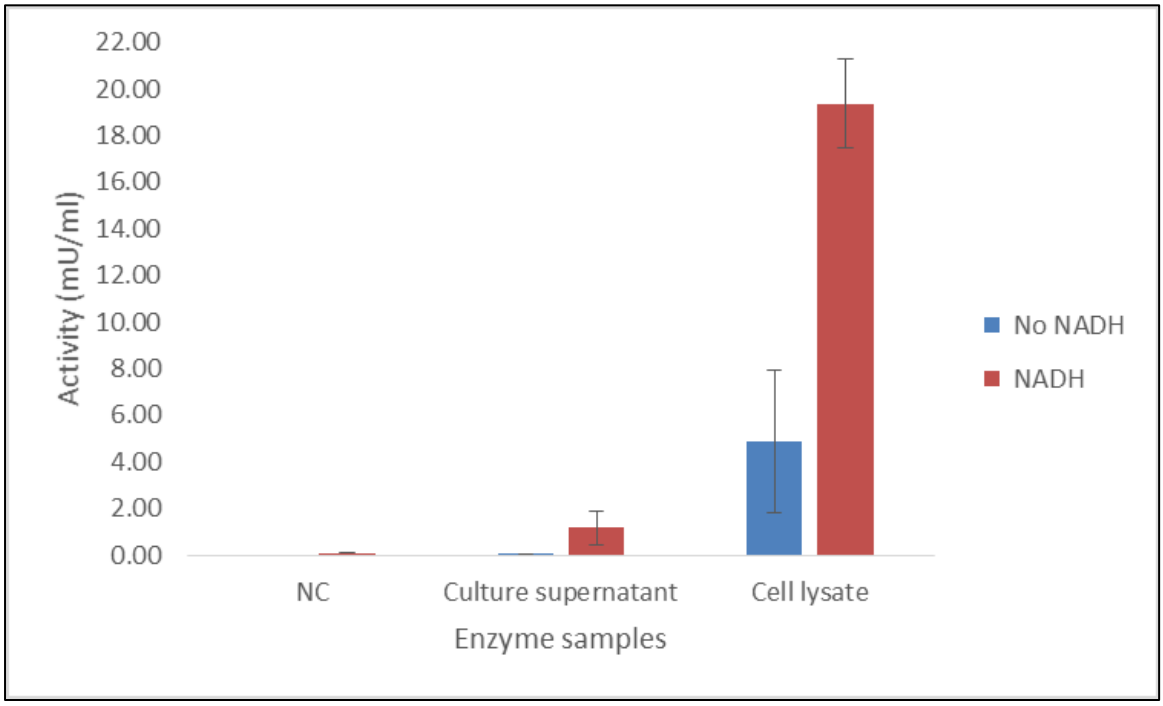


Figure 4.11 The effect NADH has on CV decolourisation activity of enzyme samples from *Gordonia* sp. JC 51. NC: Negative control. Data represented by the average of three independent samples (n=3) and error bars represent standard deviations.

As shown in Figure 4.11 the culture supernatant and cell lysate performed better in the presence of NADH than without. The fact that there is activity without NADH suggest that there are NADH independent enzymes that may also be involved in the decolourisation process in conjunction with triphenylmethane reductase. These enzymes could be peroxidases,

polyphenol oxidases and laccases (Sections 3.3.4 and 0). In this study NADH clearly enhances the enzymatic decolourisation of CV, which shifted the focus of the study to the enzyme Triphenylmethane reductase.

4.4 Discussion

Microbial decolourisation could be either via biodegradation or biosorption (Deng, et al., 2008). In this study, *Gordonia* sp. JC 51 mainly decolourised CV by biodegradation, meaning that most of the dye was removed from both the culture supernatant and the cell pellet. To measure the dye found in the cell pellet the solvent ethanol was used. Ethanol was effective and removed all dye in the cell pellet as no visible dye remained.

Triphenylmethane dyes could be degraded under aerobic or anaerobic conditions. *Gordonia* sp. JC 51 demonstrated the ability to decolourise CV under static and shaking conditions. However, shaking conditions the decolourisation was instantaneous. Suggesting that the enzymes involved were already present.

During the decolourisation process of *Gordonia* sp. JC 51, the violet CV dye decreased in colour and eventually turned colourless in water or back to the original colour of the media (such as yellow for YEME). Decolourisation could occur instantaneously when enzymes were already present.

There are two main mechanism for the biodegradation of triphenylmethane dyes, which involve the attack of the chromophore structure or the attack on the auxochrome structure of the dye. Triphenylmethane reductase have been reported to attack the chromophore structure of the CV dye using NADH as cofactor (Jang, et al., 2005). The lignin peroxidase has been reported to biodegrade CV by the sequential N-demethylation of CV, which eventually leads to decolourised products.

In conclusion, *Gordonia* sp. JC 51 have different mechanisms and pathways to attack CV and degrade it. *Gordonia* sp. JC 51 demonstrated high decolourisation efficiency at pH 5-9, 4-55°C and static and shaking conditions. This deep-sea actinobacterial strain showed potential for applications in the treatment of industrial wastewater and bioremediation of triphenylmethane dye from contaminated environments.

5 Isolation, purification and characterisation of Triphenylmethane reductase from *Gordonia* sp. JC 51

5.1 Introduction

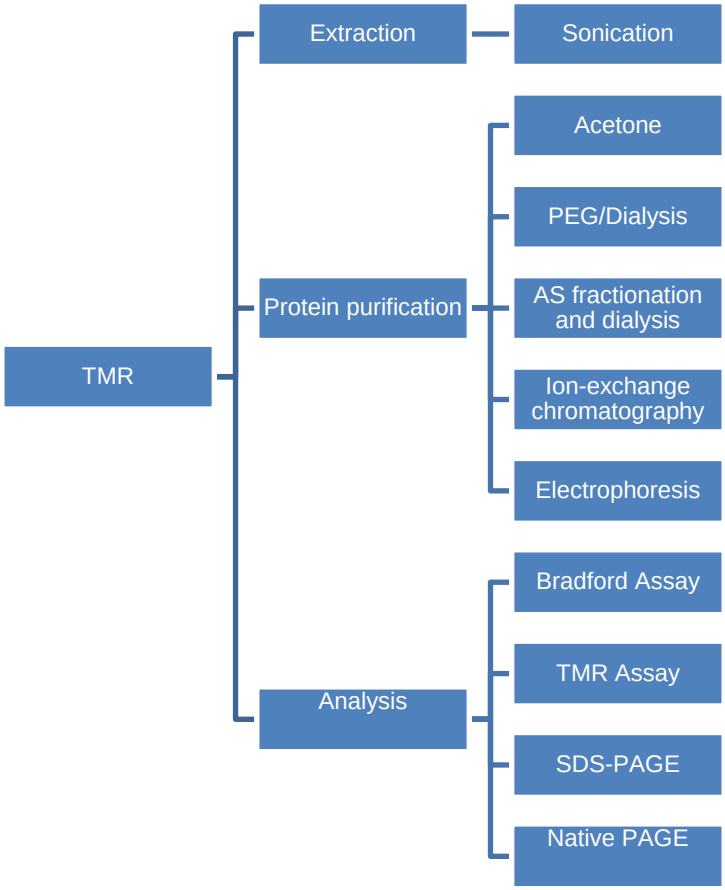


Figure 5.1 The horizontal organisation chart showing the methods used to extract, purify and analyse TMR. TMR was extracted by sonication. The enzyme was purified by acetone, PEG/dialysis, AS fractionation and dialysis, ion-exchange chromatography and electrophoresis. The enzyme was analysed by using Bradford Assay for determining protein concentration and TMR assay for measuring TMR activity. SDS-PAGE and Native PAGE was used to separate protein and enzymes respectively.

Triphenylmethane reductase (TMR) has not been isolated from actinobacteria. As shown in Chapter 4, bioinformatic databases containing genomic and proteomic sequence and structure data reveal that the enzyme may be more common to the CV decolourising actinobacteria. A *Rhodococcus* sp has been studied by (Li et al., 2014) for the ability to decolourise and degrade CV and Methyl Violet. The products found included leuco-dyes. Although lignin peroxidase was suggested to be the main enzyme involved in the process, through bioinformatic screen it was found that triphenylmethane reductase was found in a *Rhodococcus*. In Chapter 3 CV has been used to identify *Gordonia* strains as some strains show resistance towards the dye and has further shown that TMR could potentially play a role. This was further corroborated in Chapter 4 indicating that TMR was indeed preferentially expressed. However, TMP has never been isolated from actinobacteria and this is therefore the focus of the present chapter.

5.2 Different recovery methods for obtaining proteins

There are different recovery methods for obtaining pure proteins. The standard processes used to obtain extracellular proteins and enzymes are as follows: After fermentation cells are separated using centrifugation or filtration. Alternatively, sedimentation or flotation methods could also be used. The clarified medium is separated from the cells and used as a primary source of extracellular enzymes. This is called either extracellular fluid, culture supernatant or culture filtrate depending on the mechanism of separation. The culture supernatant is then concentrated using either precipitation, chromatography or filtration techniques. This is then subject to further purification using high resolution techniques such as chromatography, electrophoresis and dialysis. The final step would either be crystallisation, filtration, gel chromatography and/or drying such as lyophilisation.

For intracellular enzymes, after cell separation, cells are collected and undergo cell disruption through either mechanical or non-mechanical methods. Mechanical methods could be homogenization or sonication. Non-mechanical could be osmotic shock, autolysis, the use of enzymes and detergents. After cell disruption the resultant mixture is then clarified by centrifugation or filtration. Subsequently the clarified solution may undergo a concentration step using precipitation and later high-resolution techniques as mentioned for extracellular enzymes.

Based on the protein purification handbook (Amersham) the three-phase strategy is described. These three phases involved a capture phase, which main aim is to isolate,

concentrate and stabilise the protein. The second phase is the intermediate purification phase and the key aim is to remove most of the impurities. The third and final phase, which is called the polishing phase has the objective is to obtain high purity of the enzyme by eliminating trace amount of impurities or closely related enzymes.

There are no reports on the isolation of triphenylmethane reductase from actinobacteria. Based on the study by Jang et al. (2005), the strategy for obtaining triphenylmethane reductase involved extracting intracellular enzyme. The cells were first grown in medium containing antibiotics and cells were harvested by centrifugation. Cells were washed with sodium phosphate buffer at pH 7 and the cells frozen at -70°C. The frozen cells were resuspended in buffer and the cell was disrupted using sonication and clarified by centrifugation. The supernatant was used as crude enzyme and starting material for purification. The purification involved Ion-exchange chromatography (IEX) followed by dialysis of active fractions then hydrophobic interaction chromatography (HIC) followed by the dialysis of the active fractions. This was then subject to a second IEX step followed by concentration with ultrafiltration and stored at -20°C.

5.3 Materials and Methods

5.3.1 Growth phase

Gordonia sp. JC 51 was inoculated into 50ml Erlenmeyer flask containing 10 ml YEME broth (pH 7) and incubated at 30°C for 3 days.

5.3.2 Reaction phase

To 3-day-old cultures CV was added to make a final concentration of 30 mg/l. The reaction was incubated at 30°C for 1 h and shaking at 160 rpm using a rotary shaker.

5.3.3 Recovery of crude extracellular enzymes

After the decolourisation reaction samples were collected and placed on ice, followed by centrifugation at 10,000 rpm for 5 min at room temperature (22°C). The supernatant was transferred to fresh tube and used as a source of extracellular enzymes. This was kept on ice or 4°C prior to use.

5.3.4 Recovery of crude cell lysate for intracellular and membrane enzymes

After decolourisation the reaction samples were collected and placed on ice. These were subsequently centrifuged at 10,000 rpm for 5 min at room temperature. The supernatant was removed, and the remaining cell pellet was washed twice with 20mM sodium phosphate pH 7 and resuspended in the same buffer. The resuspension was then sonicated for 4-5 min on ice and subsequently centrifuged at 10, 000 rpm for 5 min at room temperature. The supernatant was transfered to a sterile microtube and called the cell lysate. The cell lysate contained both intracellular and membrane enzymes. The pellet was also collected and called the insoluable fraction.

5.3.5 Recovery of crude total soluble enzymes

After the decolourisation reaction samples were collected and placed on ice. The culture was sonicated for 4-5 min on ice and then centrifuged at 10, 000 rpm for 5 min at room temperature. The supernatant was transfered to a sterile microtube and called the crude total soluable enzymes. The crude total soluble enzymes contained extracellular, intracellular and membrane enzymes in solution.

5.3.6 Protein purification by acetone precipitation

All TMR purification methods was performed at 4°C. For acetone precipitation 1-part enzyme solution was added to 4 parts ice cold acetone, which was subsequently kept at -20°C for 90min or overnight.

Protein samples were precipitated using 80% v/v ice cold acetone. The solution was kept overnight at -20°C. The protein pellet was collected by centrifugation at 10,000 rpm for 5 min and the supernatant discarded. Each microcentrifuge tube was inverted on paper towel to remove remaining liquid and the pellet left to air dry.

The pellet was re-suspended in 20 μl sodium phosphate buffer for test tube 1 (Storage sample), 20 μl 1 X non-denaturing sample buffer for test tube 2 (Native-PAGE) and 20 μl 1 X denaturing sample buffer for test tube 3 (SDS-PAGE). For cell lysate 60 μl buffer volume was used instead. The samples were stored at 4°C until used.

5.3.7 Bradford Assay

The Bradford Assay (Bradford, 1976), was performed to determine the protein concentration of enzyme samples using bovine serum albumin (BSA) as a standard.

5.3.8 Triphenylmethane reductase Assay

Triphenylmethane reductase activity was determined by using CV as substrate and NADH as co-factor as described in the method by Jang et al. (2005), but with modification in temperature from 40°C to room temperature (22°C). A 1 ml reaction mixture contained 20 μM CV, 20 mM Sodium phosphate buffer pH 7, 0.1 mM NADH (prepared fresh) and 100 μl enzyme solution. The decrease in absorbance was monitored at 590nm for 2 min (ε= 110916$M^{-1}cm^{-1}$). 1U of enzyme activity was considered as the amount that catalysed the reduction of 1μmol CV/min.

5.3.9 Protein electrophoresis

There were two methods used, which were denaturing SDS-PAGE and non-denaturing SDS-PAGE. Both methods are identical except that for non-denaturing SDS-PAGE the samples were not denatured by reducing agents and heat-inactivated. Non-denaturing conditions leave the enzymes biologically active.

SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was performed using a 5% stacking and 12% separating gel, according to the method of Laemmli (1970), using Bio-Rad Mini-Protean Tetracell System. Enzymes samples and controls were loaded on gels at 20 μl per well. PageRuler™ Prestained Protein Ladder (Fermentas; 5 μl) was included in each gel.

Native PAGE was performed using the same method as for SDS-PAGE, but without SDS and samples were kept biologically active (non-denaturing conditions).

After the separation of denatured enzyme samples using SDS-PAGE the gels were stained with Coomassie Blue to detect proteins in the samples. This was used to determine the homogeneity if the samples used as well as the protein profile. For the detection of proteins in the polyacrylamide gel the gel was removed and washed several times with deionised water. The water was removed and replaced with fixing solution, which just covered the gel. This was left for 30 min with gentle agitation. The fixing solution was removed, replaced with Coomassie stain solution (Coomassie Brilliant Blue R-250) and left agitating overnight. The stain was removed, washed with deionised water before destaining solution was added. This was left agitating. Destaining solution was replaced several times until protein bands were visible and the blue background cleared. The destaining solution was then removed and the gel image captured by scanning or photograph.

Native PAGE is a technique used to study the composition and structure of Native proteins leaving both the confirmation (structure) of the protein and bioactivity (such as enzyme activity) intact. The objective of using Native PAGE is to maintain the enzyme in its Native state. Since Native PAGE retains Native (non-denaturing) enzymes/proteins the activity can therefore be verified using in-gel assays

After the separation of non-denatured enzyme samples (native/active enzymes) on Native PAGE gels the triphenylmethane reductase activity was performed as described by Jang et al. (2005) using a CV solution containing NADH. After Native PAGE, the gel was placed in a substrate solution containing 73.5μM CV and 0.1 mM NADH, and this mixture was incubated at 22°C until a clear band developed in the background of the dye-stained gel. When no clear band developed within 10 min the gel was incubated for a longer period (maximum overnight for 16 hrs). Nondenaturing SDS-PAGE was used as an alternative to Native PAGE for monitoring activity.

For the double staining method, the Native PAGE was first treated with the CV and NADH activity stain, washed several times with sterile deionized water followed by Coomassie Blue staining.

For preparative PAGE large gels were used the maximum sample capacity was added per well throughout all wells. Samples were non-denaturing and did not include Bromophenol blue tracking dye as this dye was thought to interfere with the detection. Only samples at the first and last wells contained tracking dye, Bromophenol blue. After electrophoresis the gel was washed with Sodium phosphate buffer pH 7 and stained with CV and NADH. When a clear band appeared the band was excised, transferred to 50ml centrifuge tube, further cut into smaller pieces and soaked in Sodium phosphate buffer overnight. The solution (5ml) was transferred to 5ml tube. The purified enzyme was tested for triphenylmethane reductase activity.

5.4 Results and Discussion for characterising the unknown CV decolourising enzyme

5.4.1 Protein separation of Triphenylmethane Reductase

When *Gordonia* sp. JC 51 was grown for 3 days in 10 ml YEME in 50ml Erlenmeyer flask in triplicate and treated with 30 mg/l CV it could decolourise CV at 99% within 1 h (Table 5-1). This represents high enzyme activity. When intracellular, extracellular and total soluble protein samples were prepared and tested for triphenylmethane reductase activity the measured activities were fairly low <5 mU/ml (Table 5-1).

The protein samples were further concentrated with 80% (v/v) acetone and prepared as non-denaturing (Native) and denaturing protein samples, which were run on Native PAGE and SDS-PAGE respectively. SDS-PAGE was stained with Coomassie to visualise protein bands, which appeared blue. The protein profiles for intracellular, culture supernatant, total soluble protein and cell debris are shown in Figure 5.2-A. The culture supernatant and total soluble protein profiles could not be visible under the conditions tested, possibly because of the protein concentrations being low (<30 μg protein per well).

Table 5-1 Decolourisation extent of *Gordonia* sp. JC 51 treated with 30 mg/l CV and protein concentration, Triphenylmethane reductase activity and specific activity of intracellular, extracellular and total soluble protein of strain

	DC (%)	**Protein Concentration (μg/ml)**			**TMR activity (mU/ml)**			**TMR Specific Activity (mU/mg)**		
Flask	**After 1 h**	**In**	**Ex**	**TSP**	**In**	**Ex**	**TSP**	**In**	**Ex**	**TSP**
1	99	639	79.8	148.6	5	1	0	2.35	1.25	0
2	99	652.2	139.4	137.8	5	5	-	2.3	3.5	-
3	99	246.6	133.4	170.2	1	4	3	1.22	2.96	2.63
Average	99	512.6	117.53	152.2	3.67	3.33	1.5	1.96	2.57	1.32
SD	0.000	230.46	32.82	16.50	2.31	2.08	2.12	0.64	1.18	1.86

DC = Decolourisation extent, In= Intracellular protein, Ex= Extracellular protein, culture supernatant, TSP= Total soluble protein, TMR= Triphenylmethane reductase, SD=Standard Deviation. Large variations were caused by samples taken from 3 different batches and not samples taken from the same batch. Cells were not homogeneous as they form clumps.

Native PAGE was initially stained with CV and NADH. No decolourised bands appeared within the first hour of the staining procedure and therefore it was incubated overnight. Only laccase and the intracellular protein fraction showed activity (Figure 5.2-B1). Although laccase did not produce a distinct clear band, it did however produce a pink streak. The intracellular protein sample of *Gordonia* sp. JC 51 produced a purple streak, which was more intense than the background. It also produced 3 distinct bands, called A, B and C. Band A produced a pink band, band B produced a colourless band and band C an intense purple band. When the CV activity stain was removed from the Native PAGE it was subsequently stained with Coomassie to detect the protein. The results are shown in Figure 5.2-B2. There were 2 blue bands present, which correlated with bands B and C of Figure 5.2-B1. Band C was not detected in denaturing SDS-PAGE and seemed to have a molecular weight higher than 130 kDa. Band B was detected in all gels and was approximately 69kDa. Band A was not detected in the Native PAGE Coomassie stain (Figure 5.2-B2), but was detected in the SDS-PAGE Coomassie stain (Figure 5.2-A) and was 26.7kDa.

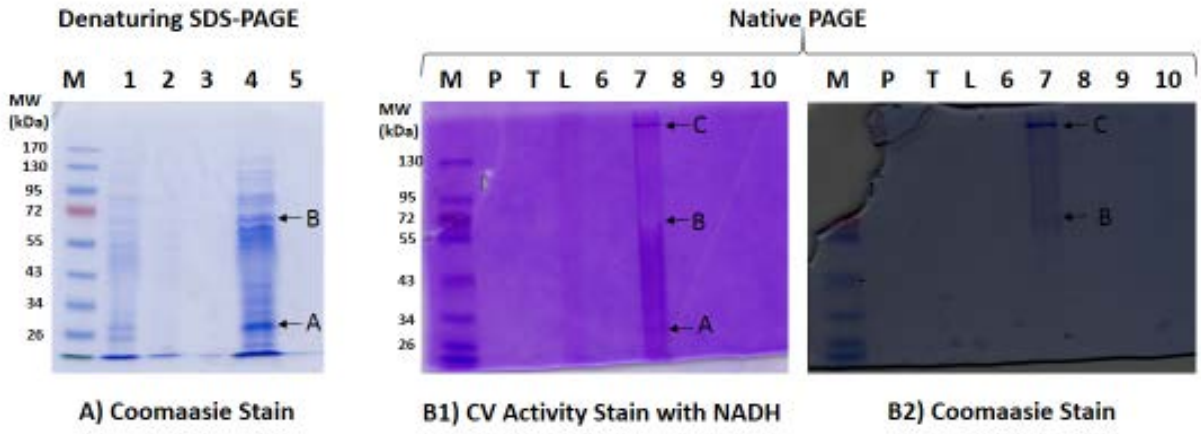


Figure 5.2 Protein electrophoresis analysis of acetone extracts from *Gordonia* sp. JC 51 after 1 h treatment with 30 mg/l CV. A) Denaturing SDS-PAGE stained with Coomassie; B1) Native PAGE stained with CV and NADH; and B2) Native PAGE stained with Coomassie. Lane M: PageRuler™ Prestained Protein Ladder; Lane P: Peroxidase; Lane T: Tyrosinase; Lane L: Laccase; Lanes 1 and 10: Cell debris fraction, Lanes 2 and 9:Total soluble protein fraction (28.64 μg ± 1.53), Lanes 3 and 8: Culture supernatant protein fraction (27.5 μg ± 0.71), Lane 4 and 7: Intracellular protein fraction(43.04 μg ± 0.62), Lane 5 and 6: Negative Control. Band A: 26.7 kDa, pink when stained with CV and NADH. Band B: 69 kDa, colourless when stained with CV and NADH. Band C: Unknown weight above 130 kDa, dark intense purple band when stained with CV and NADH.

The protein profiles (culture supernatant, intracellular and total protein) from *Gordonia* sp. JC 51 grown for 3 days in 10 ml YEME untreated and treated with 30 mg/l CV for 1 hr are shown in Figure 5.3-A and B respectively. The culture supernatant protein was not detected in Figure 5.3-A under the conditions tested. Although the culture supernatant and total soluble protein were not detected for denatured SDS-PAGE stained with Coomassie for samples taken after 1h treatment with 30 mg/l CV, the gel was allowed to dry for 2-3 days and shrink in size. It was found under these conditions that the bands would become visible. The protein profiles could be used as a guide to determine what proteins are expressed by the strain and determine what the contaminating proteins are. Even under concentrated conditions and under high protein concentrations it was found that detecting culture supernatant protein was difficult.

5.4.2 Isolation of Triphenylmethane Reductase

Various attempts were made to isolate the triphenylmethane reductase enzyme, which included ion-exchange chromatography, acetone precipitation, ethanol precipitation, ammonium sulphate precipitation/fractionation and PEG/dialysis.

Gordonia sp. JC 51 was grown for 4 days in 100 ml YEME to 42.5 mg/ml wet biomass. One was left untreated and the other treated with 30 mg/l CV for 1h, which could decolourise CV to 94.76%±0.08. In order to prepare enzymes for purification, both untreated and treated total protein samples were left “unconcentrated”. The untreated total protein was 93.2 μg/ml and treated was 127 μg/ml. A large gel was used with one half untreated total protein and the other half containing treated total protein. No bromophenol blue was added to samples except the first and last lanes, because it was a dye that could potentially interfere with the activity. When CV solution was added initially, no activity was detected. Only when NADH was included did a clear band appear across the gel near the dye front, which suggest that the enzyme has low molecular weight. This was initially thought to be an artefact, but by the use of confirmation tests revealed that it was caused by enzymes (data not shown) and that activity was also found in the stacking gel. Triphenylmethane reductase activity was found in both the untreated and treated total protein sample (Figure 5.3-C and -D) suggesting that the enzyme might be constitutive and not induced. When the reaction solution was removed the colourless band turned purple, which was reversible and revert back to colourless when reaction solution was reintroduced (Figure 5.4).

After the colourless band was cut and dissolved in buffer and the 5ml extract (purified enzyme) was tested for TMR activity, 0.005 Abs/min was produced (0.05 Ab/min/ml). Converted to U/ml

(Unit defined as 1μmol converted per min) the activity was less than <0.001 U/ml(<1mU/ml) triphenylmethane reductase activity. Activity was not linear as the enzyme activity increased and then decreased, which demonstrates and confirms the reversibility of the reaction.

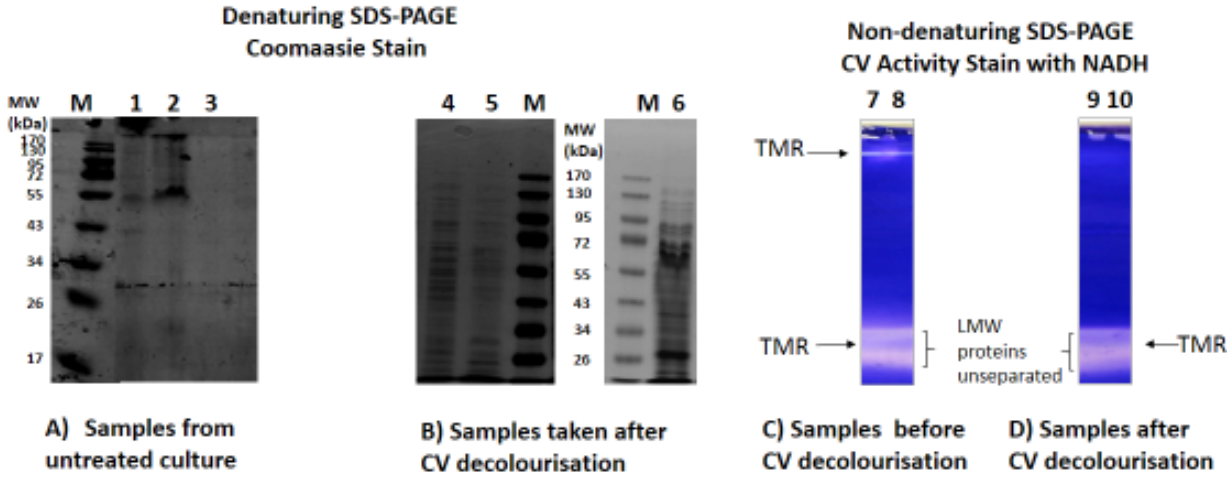


Figure 5.3SDS-PAGE analysis of protein. Denaturing SDS-PAGE stained with Coomassie of protein samples taken from untreated (A) and treated (B) with 30 mg/l CV after 1 h cultures of *Gordonia* sp. JC 51. Non-denatured SDS-PAGE stained with CV and NADH of protein samples taken from untreated (C) and treated (D) with 30 mg/l CV after 1 h cultures of *Gordonia* sp. JC 51. Lane M: PageRuler™ Prestained Protein Ladder, Lane 1, 4 and 7-10: Total soluble protein, Lane 2 and 6: intracellular protein, Lane 3 and 5: Culture supernatant protein. LMW= Low molecular weight proteins. TMR= Triphenylmethane reductase

Based on the results obtained it confirms that triphenylmethane reductase activity is present in *Gordonia* sp. JC 51. The initial decolourisation is reversible and can either result in LCV converting to CV or similar N-demethylated products. Acetone may be useful for concentrating protein for SDS-PAGE, but not suitable for maintaining enzyme activity and may denature the protein irreversibly (Jansen and Ryden, 1998; Scopes, 1994 in Protein Purification Handbook, Amersham Biosciences 2001 -Code 18-1132-29). This could mean that the pink band A (Figure 5.2) observed may in fact be a partially weak enzyme activity as the band is not fully decolourised. It could also mean that the enzyme involved that changes the CV to a pink product may be another reaction involved. The degradative studies in Chapter 6 may further explain and confirm the enzymatic degradation of CV by *Gordonia* sp. JC 51.

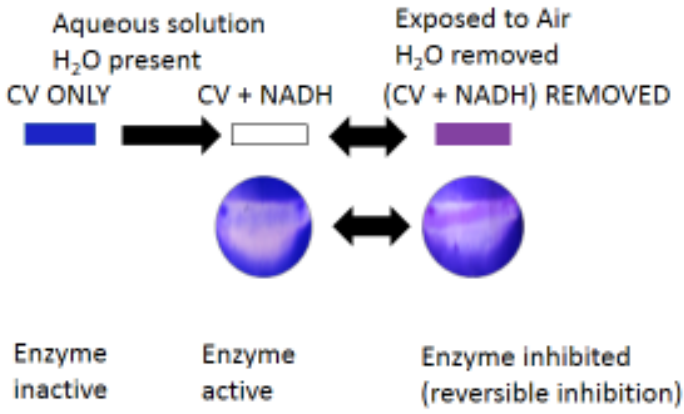


Figure 5.4 Characteristics of triphenylmethane reductase of *Gordonia* sp. JC 51. The enzyme TMR of *Gordonia* sp. JC 51 is inactive without the presence of NADH and does not decolourise CV. When CV and NADH are present the enzyme decolourises CV and produces a decolourised band. When the solution is removed containing both CV and NADH the enzyme is inhibited as it is exposed to air and the colourless LCV is converted to a purple compound, which may either be CV or N-demethylated intermediate of CV that has similar colour.

The fact that decolourisation occurs via biodegradation indicates that the process is enzymatic. This is further confirmed when heated unclarified cell-free lysates were treated with CV and could not biodegrade CV. To determine if NADH was required for decolourisation the fractions were tested with and without this cofactor. It was found that there are multiple enzyme mechanism responsible for decolourisation. In spectrophotometry it was found that increased intensity followed by a decrease in absorbance. This could be attributed to adduct formation with proteins or the initial binding step involved. Acetone extracts showed that there are in fact dark bands for some proteins. However, one band was decolourised, another was dark and another was pink. The pink band could be due to N-demethylation. When samples were stored with glycerol and PMSF and the samples were prepared, they had a transparent, colourless band close to the dye front. This indicates that the enzymes have low molecular weight. Coomassie in Native PAGE was not an effective method. It revealed that there was a major band. The rest of the protein could not be viewed. The band was excised and the activity determined. Isolation of the enzyme for identification and kinetic analysis was not easy. Alternative methods for the isolation of TMR should be further established, but the data generated here can serve as a guide.

5.5 Conclusion

In summary, it was found that *Gordonia* sp. JC 51 exhibits triphenylmethane reductase activity. Although various attempts were made to purify triphenylmethane reductase from *Gordonia* sp. JC 51, the enzyme could not be isolated or characterised in its purified form. This therefore prevented the determination of the amino acid sequences and therefore the biophysical parameters of this enzyme remain unknown for *Gordonia* sp. JC 51.

Activities were better visualised with SDS-PAGE under non-denaturing conditions than with Native PAGE. Since the conversion of CV to LCV can result in the appearance of a purple coloured band when the CV and NADH solution is removed it could result in false negative results for triphenylmethane reductase. Therefore the degradation products should be identified to establish the reaction pathway, which can serve as a pointer to the biocatalysts involved.

The next chapter will look at the disappearance of CV and the appearance of the products formed when CV is degraded.

6 Identifying the different metabolites produced during the biodegradation of Crystal Violet by *Gordonia* sp. JC 51

6.1 Introduction

Currently there are several methods available that can be used to identify and characterise the reaction products of Crystal Violet decolourisation and degradation. These methods include UV/Visible spectrophotometry (UV/Vis Spec), Thin layer chromatography (TLC), Tandem mass spectroscopy (LC/MS/MS) and various other methods (Jang et al., 2005; Pan et al., 2012; Chen et al., 2007a; Stolze et al., 2012; Fan et al., 2009; Chen et al., 2008).

In previous chapters, it was determined that the enzyme responsible for initial decolourisation of Crystal Violet (CV) was likely the enzyme triphenylmethane reductase (TMR). In order to confirm that TMR is indeed the enzyme responsible for decolourisation of CV, the product Leucocrystal Violet (LCV) needs to be detected. Since the initial decolourisation is reversible and LCV converts to a purple product when exposed to air, it is assumed that the decolourisation process requires the further degradation of the dye to more “stable” decolourised products. In order to understand the mechanism of decolourisation and degradation of CV the use of various analytical tools need to be used such as Spectral scan studies, thin layer chromatography and LC/MS/MS.

Several studies have demonstrated the use of spectral scan studies using UV/Vis spectrophotometry to detect the disappearance of the substrate CV at 590nm and the appearance of new products. For example LCV in dichloromethane has a peak at 260 nm and an increase in absorbance at this wavelength suggest the possible formation of this compound (Pan, et al., 2012; Jang, et al., 2005). The sequential *N*-demethylation of CV can also be detected by spectral scan analysis, whereby there is a shift hyposochromically from 590 nm (589.4) to 542.9nm (Chen, et al., 2007a).

In order to understand the mechanism of decolourisation and biodegradation of CV and the potential enzymes that may be involved in the process, the use of the combination of spectral scan studies, thin layer chromatography and LC/MS/MS may be beneficial. Spectral scan studies can reveal if the products produced are colourless (UV region <400nm) or have colour

(visible light region >400nm). Thin layer chromatography can demonstrate the disappearance of the substrate and the appearance of new products. The dye visible spots under visible light may gradually disappear with different time points and eventually new spots will appear either visible as well or only visible under UV light or upon exposure to iodine vapours. LC/MS/MS has become a method of choice for studying metabolism and degradation studies. The process makes use of the separation of LC, which can separate the mixture to different peaks, which are further separated by the first MS into different molecular weight and the different peaks can be further subject to a second MS for fragmentation to identify the compounds. The fragmentation patterns can be compared to MassBank and other sources in the literature.

Gordonia sp. JC 51 was treated with CV and the reaction product was collected and analysed by spectral scan studies, TLC and LC/MS/MS. In this section of the thesis the mechanism of decolourisation and biodegradation of CV by *Gordonia* sp. JC 51 will be reported.

6.2 Materials and Methods used for identifying the degradative products

6.2.1 Spectral Scan studies used to monitor the biotransformation of CV in nutrient limited conditions

Gordonia sp. JC 51 (1ml culture stock was centrifuged and the cell pellet harvested, washed with water and resuspended in 1ml water) was added to 4ml water (5ml total volume in 50ml Erlenmeyer flask) and treated with 10 mg/l CV and incubated at 30°C, shaking at 160 rpm for 24 hours. After incubation 1ml sample was removed, centrifuged at 10,000 rpm for 5min and the supernatant was used to measure the UV/Vis spectrum from 200-800nm. The disappearance of the peak at 590nm and appearance of new peaks were monitored. The spectrum was compared to control 10 mg/l CV in water, which represented time 0.

6.2.2 TLC analysis procedure of the biotransformation of CV to colourless products by *Gordonia* sp. JC 51

A reaction mixture (50ml in 500ml Erlenmeyer flask) containing *Gordonia* sp. JC 51 culture and 1.25 ml 2% (w/v) CV (final concentration 0.5g/l) was incubated with shaking (160 rpm) at 30°C for 24 h. 2 ml samples were collected at various time points (0,1, 2, 3, 20, 24 h) and centrifuged at 10,000 rpm for 5 min. The cell pellet colour was monitored and the supernatant used for TLC analysis and CV decolourisation assay. For TLC analysis, 5 μl supernatant was applied onto 0.25 mm thick silica gel GF_{254} plates (Merck) and developed with propanol: water:

glacial acetic acid (90:9:1 v/v/v) as mobile phase. Developed TLC plates were visualized by using visible, 256nm UV light and iodine vapour. R_f values for CV and *N, N, N', N', N''*-pentamethylpararosaniline were 0.23 and 0.33 respectively. The standards CV (0.23), LCV (0.75-0.76), MK (0.78-0.79), DB (0.76-0.79) and HB (0.83-0.84) were also tested and R_f values determined. YEME broth, untreated culture and 0.5g/l CV in YEME served as Abiotic control, Biotic control and standard respectively.

6.2.3 Determining the degradation products of Crystal Violet to colourless compounds by *Gordonia* sp. JC 51 using LC/MS/MS

A reaction mixture (10 ml in 50ml Erlenmeyer flask) containing washed cell suspension of *Gordonia* sp. JC 51 in 10 ml water and 30 mg/l CV solution was incubated with shaking (160 rpm) at 30°C for 1 h. From centrifugation at 10,000rpm for 5min the culture supernatant (1ml) and cell pellet was prepared. The cell pellet was re-suspended in 1 ml absolute ethanol to extract the adsorbed CV and CV products. This was centrifuged for 10,000 rpm for 5 min. The supernatant, which is the cell pellet extract was used to analyse CV metabolites bound to the membrane. The culture supernatant and cell pellet extract were centrifuged at 10,000 rpm for 10 min for preparation for LC/MS/MS analysis. The samples were transferred to HPLC vials, which were kept at 4°C until analysed.

LC/MS/MS was performed on a Dionex HPLC system (Dionex Softron, Germering, Germany) equipped with a binary solvent manager and autosampler coupled to a Brucker ESI Q-TOF mass spectrometer (Bruker Daltonik GmbH, Germany). The products were separated by reversed phase chromatography on a Waters Sunfire C18 column 5 µm; 4.6 × 150 mm (Dublin, Ireland) using gradient elution at a flow rate of 1.0 ml min^{-1}, an injection volume of 10 µl and an oven temperature of 30°C. The gradient was set up as follows: 98% A to 90% A (5 min); from 90% A to 25% A in 15 min; from 25% A to 10% A in 1 min; held at 10% for 14 min before increasing back to 98% and equilibrating for 10 min. Solvent A - 0.1% formic acid, solvent B - acetonitrile. MS spectra were acquired in positive mode using the full scan and auto MS/MS (collision energy 25 eV) scan modes with dual spray for reference mass solution. Electrospray voltage was set to -3500 V. Dry gas flow was set to 9 l min^{-1} with a temperature of 300 °C and nebulizer gas pressure was set to 35 psi. HPLC data was analysed using the Chromeleon software while mass spectrometry data was analysed using Compass DataAnalysis.

6.3 Results and Discussion for the identification of the degradative products of CV by *Gordonia* sp. JC 51

6.3.1 The use of spectral scan analysis to monitor the biotransformation of CV by *Gordonia* sp. JC 51 in nutrient deprived conditions

When *Gordonia* sp. JC 51 cells were placed in nutrient limited conditions with only 10 mg/l CV in water for 24 hours, it decolourised CV. UV/Visible spectral analysis showed a 99% decrease in absorbance at 590 nm (**Figure 6.1**) and no peaks were detected at the visible light spectrum (400-700nm).This indicates that the reaction products were colourless. The reaction products also had a peak at 256nm (**Figure 6.1**), which is part of the UV region of the spectrum. This indicates that the compound has UV-absorbing properties. Previous spectral scan analysis was hampered by YEME medium having a strong absorbance at the UV region, which prevented the detection of UV-absorbing compounds. Therefore the dye decolourisation reactions were tested in water instead of YEME media. The strain could also grow and decolourise CV on ISP#9 medium with only 30 mg/l CV as a sole carbon source (Data not shown).

The ability of *Gordonia* sp. JC 51 to decolourise CV without the need for additional nutrients makes it potentially valuable in terms of applying it as a whole cell biocatalyst to decolourise dyes. The products produced by *Gordonia* sp. JC 51 remains unidentified, however spectral scan studies have provided some clues as to the properties of the products such as the maximum absorption peak. The fact that the compound has an adsorption peak at UV region suggest that the strain disrupted the chromophore structure of the CV dye during the biotransformation process, which involves attacking the central carbon in CV. Spectral scan studies of 10 mg/l CV in water at pH13 and pH 13.8 (adjusted with 1M NaOH) a peak at 255 and 254 appeared respectively with the disappearance of the peak at 590nm (Data not shown).

UV/Vis Spectral Scan studies have shown the disappearance of CV and the appearance of new peaks such as either LCV at 260nm (Jang et al, 2005) or Michler's ketone at 348nm (Yatome, et al. (1993). The *N*-demethylated process of CV can also be detected by the hypsochromic shift of the absorbance maximum from 591 nm to 544 nm (Bumpus and Brock, 1988).

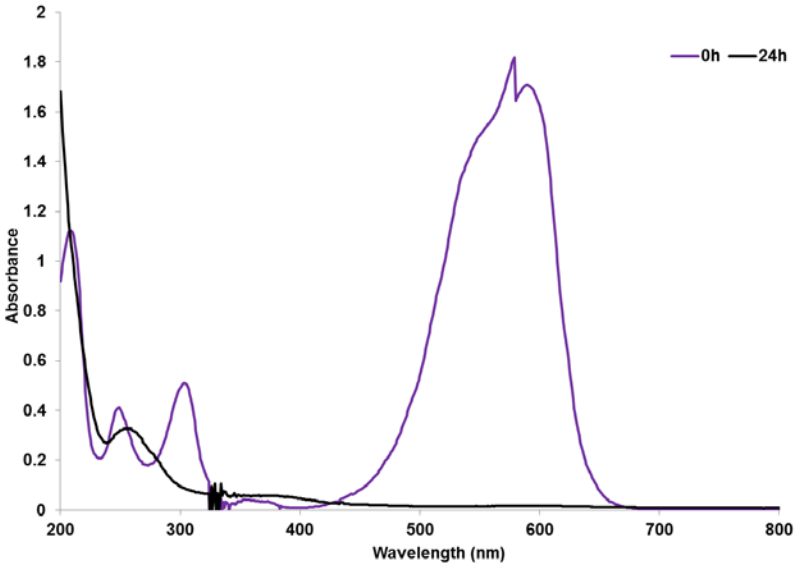


Figure 6.1 UV/Vis Spectra of the supernatant reaction samples of the biotransformation of 10 mg/l Crystal Violet in water with *Gordonia* sp. JC 51 after 24 hours.

6.3.2 Thin layer chromatography and decolourisation tests to monitor the biodegradation of high concentration of CV by *Gordonia* sp. JC 51

In order to follow the initial steps leading to the decolourisation of CV by *Gordonia* sp. JC 51 high concentrations of CV was used. Preliminary studies showed that the organism could not only decolourise high concentrations of CV (100 mg/l), but that the cultures were also still viable after decolourisation. Based on these studies 0.5g/l CV (1.226 mM) concentration was selected for the TLC analysis. In conjunction with TLC tests both decolourisation and pellet colour was observed.

TLC analysis showed that at the initial stages before decolourisation that CV and *N,N,N',N',N"*-pentamethylpararosaniline (also known as *N, N*-Dimethyl-*N',N'*-dimethyl-*N"*-methylpararosaniline; DDMPR and Methyl Violet) were present together. DMMPR was removed before CV. As time progressed CV disappeared and a new product formed. This product was not detected in visible light, but was detected when it was exposed to UV light and iodine vapour. This indicated that the compound was colourless, UV-absorbing and an organic compound with a carbon-carbon double bond system (C=C). The unknown compound had an R_f value of 0.61, which was different to Crystal Violet (R_f value = 0.23). Initially it was thought that this compound was Leucocrystal Violet, but it did not correlate with authentic LCV (R_f = 0.75-0.76) or other tested standards such as MK (R_f =0.78-0.79), DB (R_f =0.76-0.79)

and HB (R_f = 0.83-0.84). It is therefore possible that other colourless compounds may also be produced (these forms were later found to be Leuco-*N*-demethylated derivatives of Crystal Violet, which will be discussed later). At 20h the decolourisation was 92% complete, but based on CV concentration the conversion was 99.77% as only 2.8 μM CV remained in solution.

The cell pellet of JC 51 was observed and it was found that the cells took on the intense colour of the dye (CV) initially through biosorption which eventually faded as the time progressed (see Figure 6.2 and Table 6-1). At 20 and 24h the pellets had a very faint violet colour, which confirms that biodegradation was taking place within the cells subsequent to biosorption. Since both biosorption and biodegradation mechanisms are used by *Gordonia* sp. JC 51 it would be interesting to determine the unknown compounds produced in the cell pellet extract and culture supernatant. Thus far it was determined that the compound/s produced is visible under UV light.

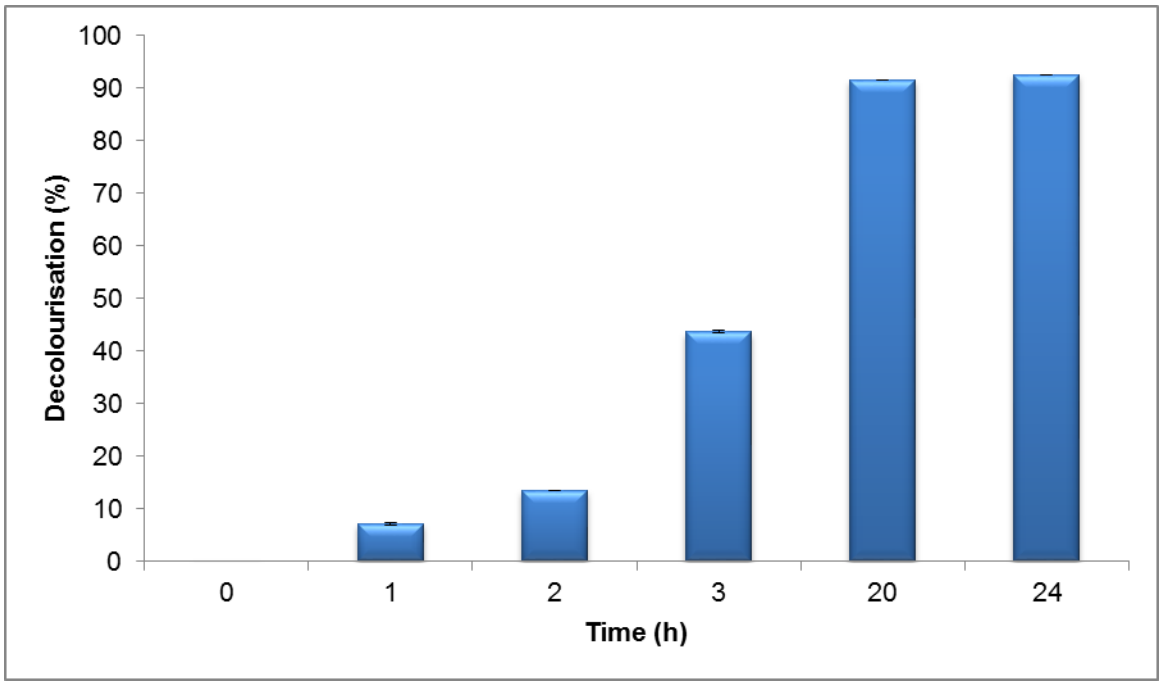


Figure 6.2 The decolourisation of 0.5g/L CV by *Gordonia* JC 51 from 0 to 24 hrs.
Each data point represents the average and error bars represent the standard deviation of duplicates

Table 6-1: Properties of samples taken from *Gordonia* sp. JC 51 at different time points based on decolourisation, kinetic rate, intermediates formed and the colour of cell pellet.

Time (h)	**Decolourisation (%)**	**Kinetic rate (Ab/h)[a]**	**CV, intermediates and other products detected by TLC**	**Pellet colour**
0	0	0	CV, MV	Dark purple
1	7	-0.14 (0-1h)	CV, MV	Purple
2	14	-0.14 (1-2h)	CV, MV	Purple
3	44	-0.59 (2-3h)	CV	Purple
20	92	-0.06 (3-20h)	Unknown UV colourless absorbing compound (R_f 6.1)	Very faint purple
24	93	-0.01 (20-24h)	Unknown, colourless UV absorbing compound (R_f. Value 6.1)	Very faint purple

Initial conditions: 0.5g/l CV in YEME, 50ml in 500ml Erlenmeyer flask, 30°C, 160rpm shaking with rotary shaker, whole cultures of *Gordonia* JC 51.

a: The absorbance reaction rate is a negative slope

MV disappeared first before CV. Once CV disappeared and an unknown colourless, UV-absorbing compound appeared. Initially it was thought that this compound was LCV, but it did not correlate to LCV, MK, p-Dimethylaminobenzaldyde or 4-hydroxybenzaldehyde. The cells

take up the dye and become intensely coloured. Over time the dye in the cells gradually fades until only residual dye remains. The fact that *Gordonia* sp. JC 51 was able to decolourise 93% CV when 0.5g/L dye was added to the culture within 24 h demonstrates the strong degradative ability of the strain. The best rate was -0.59 Ab/h between 2-3h, which is 30% decolourisation/h.

Thin layer Chromatography has been used as a method to detect the appearance of LCV (Jang, et al., 2005), *N*-demethylated products of CV (Bumpus & Brock, 1988) and Michler's Ketone (Yatome, et al., 1993) after CV decolourisation or degradation. Michler's ketone can be detected using TLC plates Kieselgel $60F_{254}$ and DC-Fertigplatten RP-$8F_{254S}$ with the mobile phases n-hexane-ethylacetate (4:6v/v) and methanol-water (9:1, v/v) respectively (Yatome, et al., 1993) under UV light at 254 nm. The R_f value was 0.67 and 0.61 respectively (Yatome, et al., 1993). Bumpus and Brock (1988) used methyl violet as a standard for detecting CV (R_f=0.26,blue), *N,N,N',N',N*"-pentamethylpararosaniline(R_f=0.35, bluish purple), *N,N,N',N*"-tetramethylpararosaniline(R_f=0.43, purple), and *N,N',N*"-trimethylpararosaniline (R_f= 0.61, pink) using the Silica Gel 60 G F-254 plates and the mobile phase propanol-H_2O-glacial acetic acid (90:9:1,v/v/v). LCV can be detected using n-hexane-ethylacetate (4:6v/v) and propanol-H_2O-glacial acetic acid (90:9:1,v/v/v) as mobile phase and Kieselgel 60 F254 TLC plate as stationary phase (Jang, et al., 2005).

6.3.3 Separation and Identification of the degradation of CV products by *Gordonia* sp. JC 51 cells

Previous tests based on UV/Vis spectra and TLC showed (Figure 6.3) that colourless compounds were formed when CV undergoes biocatalytic decolourisation using *Gordonia* sp. JC 51 as whole cell biocatalyst. However, these colourless compounds and their structures were unknown. The use of LC/MS/MS provides information about the number of compounds that are present in the reaction sample, their retention times under specific conditions as well as the molecular weights of each component in a mixture and also their fragmentation patterns and the molecular weight of all fragments formed. Based on this information it is possible to determine the structure of the unknown compound. Once the structure is determined it is then possible to determine the reactions involved and the elucidate the pathway of degradation.

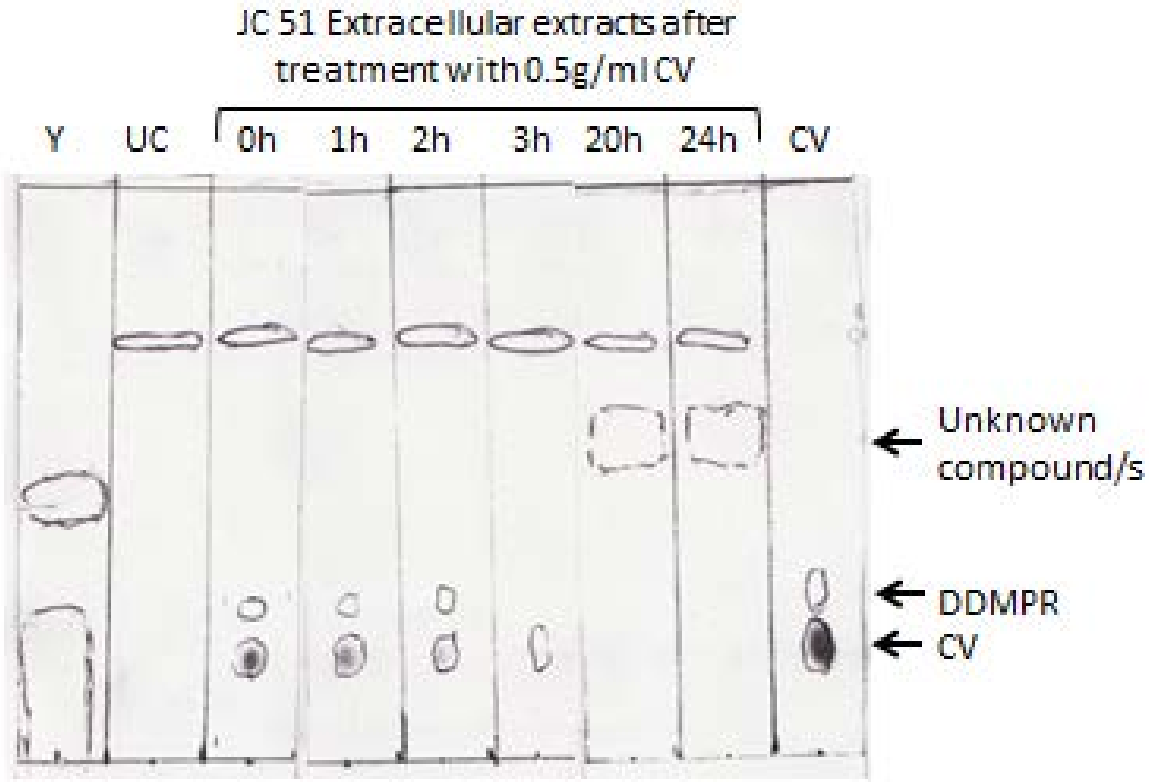


Figure 6.3. TLC analysis of JC 51 culture supernatant after treatment with 0.5g/l CV from 0-24h Lanes Y=YEME (medium control, no inoculum), UC= Untreated Control (JC 51 culture supernatant sample before CV added), CV= Crystal Violet Solution. Spots represented in diagram were marked after viewing under UV light. Dark spots represent compounds that were observed under visible light.

Gordonia sp. JC 51 permanent stocks were selected based on the ability to obtain sufficient biomass after 3 days of growth in 10ml YEME and ability to retain bioactivity, which is the ability of strains to decolourise at least 80% of 30mg/l CV within 1h at 30°C while shaking at 160rpm. The stocks used could obtain 97% decolourisation of 30mg/l CV within 1h. Untreated 3 day cultures prepared under standard conditions were used to prepare culture supernatant and cells by centrifugation based on the method described earlier. Cells were washed with water and resuspended with water. The resuspension was transferred to a sterile 50ml Erlenmeyer flask and a 10ml aqueous solution containing cells was treated with CV to a final concentration of 30 mg/l. To a 5ml culture supernatant CV was added to a final concentration of 30mg/l. Both were incubated at 30°C for 1h. No decolourisation was observed for the culture supernatant sample and therefore it was not used to determine LC/MS/MS analysis. Aqueous

solutions of CV containing washed cells in one batch showed partial and in the second batch showed almost complete decolourisation of CV within 1h, without added nutrients. No colour changes were observed once decolourisation occurred, which suggests that decolourisation was stable (Figure 6.4).

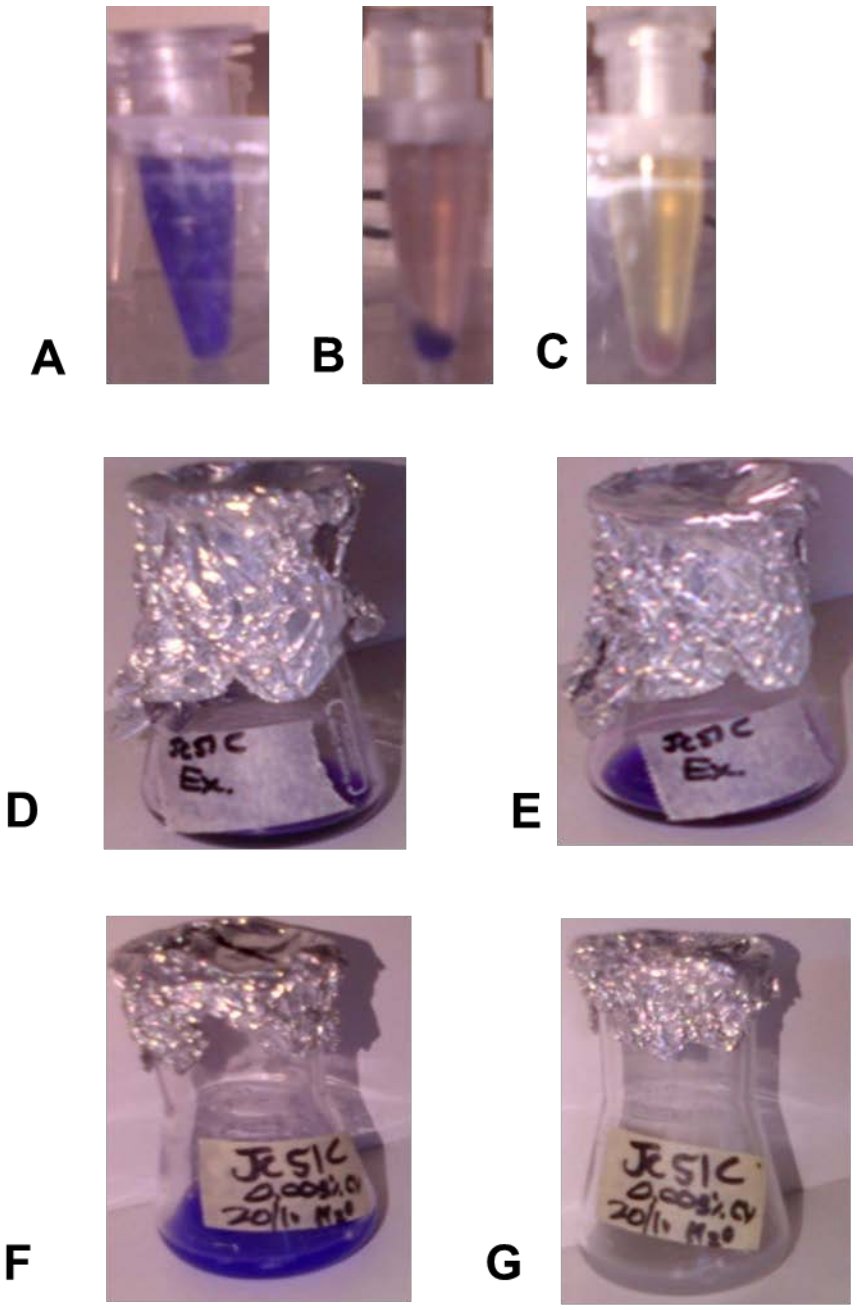


Figure 6.4 The decolourisation ability of the total culture, the culture supernatant and cells of *Gordonia* sp. JC 51 when treated with 30mg/l CV after 1h. **A** represents control without whole cell biocatalyst in YEME media containing 30mg/l CV. **B** and **C** show samples of JC51C total

cultures taken at time 0 and 1h when treated with 30mg/l CV in YEME respectively. **D** and **E** show the reaction of the culture supernatant of JC51 with 30mg/L CV at time 0 and 1h respectively. **F** and **G** show the reaction of JC51 cells in water as whole cell biocatalyst with 30 mg/L CV at time 0 and 1h respectively. No decolourisation activity was found for extracellular cell-free culture supernatant. Decolourisation was found for total culture in media (with nutrients) and cells in water (without added nutrients).

The CV degradation metabolites produced after 1h treatment of 30 mg/l CV in water with *Gordonia* sp. JC 51 (Figure 6.5) was analyzed using LC/MS/MS. The culture supernatants and cell pellet extracts of *Gordonia* sp. JC 51 after 1h showed reduced CV peak in HPLC chromatograms, when compared to Negative control (time=0) and CV standard (Figure 6.6 A and B).

For quality control purposes only samples from batches that could decolourise CV above 97% within 1 hour were used for LC/MS/MS analysis (which means only 2 out of 5 batches met the criteria and were used). The whole cell biocatalyst was prepared from untreated 3 day old cultures. The 10ml reaction mixture contained whole cell biocatalyst, *Gordonia* sp. JC 51, and 30 mg/l CV in water. Decolourisation of 30 mg/l CV occurred after 1h as shown in Figure 5.5. *Gordonia* sp. JC 51 cells could successfully decolourise CV in water only, without additional nutrient requirements, buffers, metal ions and cofactors. The samples used in LC/MS/MS analysis were the Negative Control, CV standard, Culture supernatant and cell pellet extract of *Gordonia* sp. JC 51 after 1h.

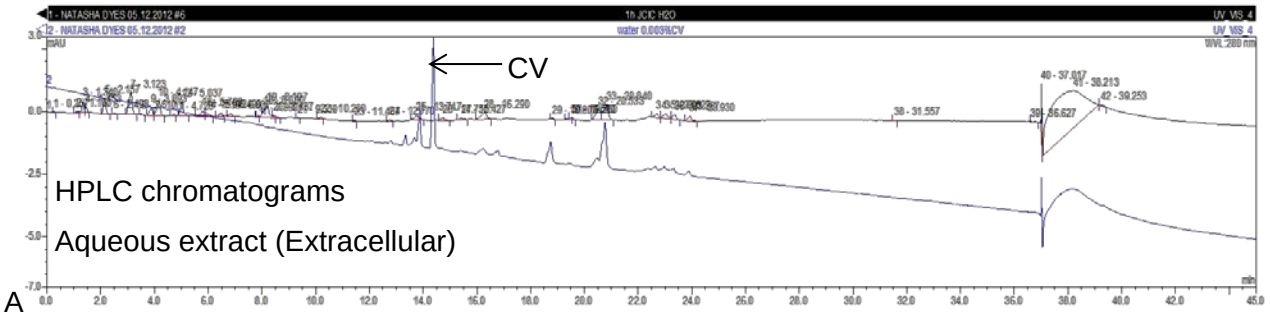
Cells could successfully decolourise CV in water only. The liquid and solid fractions were separated by centrifugation. Any remaining metabolites of CV were removed from cells using ethanol as a de-adsorbent. Both the liquid fraction and the cell-bound extracted metabolites were analysed by LC/MS/MS to determine the metabolites produced. The structures of CV degradation were determined by use of MassBank, database mining and literature survey. In the liquid fraction (

Figure **6.5**-A) there is a clear reduction of the dominant peak of CV when compared to the control (30mg/l CV in water, Time 0). Likewise, in the cell-pellet extract (

Figure **6.5**-B) there is a reduction in the dominant peak of CV when compared to the reference control (30mg/l CV in ethanol).

The peak heights of CV for the reference control, time 0 control, culture supernatant and cell pellet extract were 21.527, 4.414, 0.103 and 0.253 mAU respectively. A new peak appeared in the HPLC chromatogram of cell pellet extract obtained from *Gordonia* sp. JC 51 cells after 1 hour treatment with 30 mg/l CV (**Figure 6.6**). This peak was identified as LCV according to the LC/MS/MS analysis.

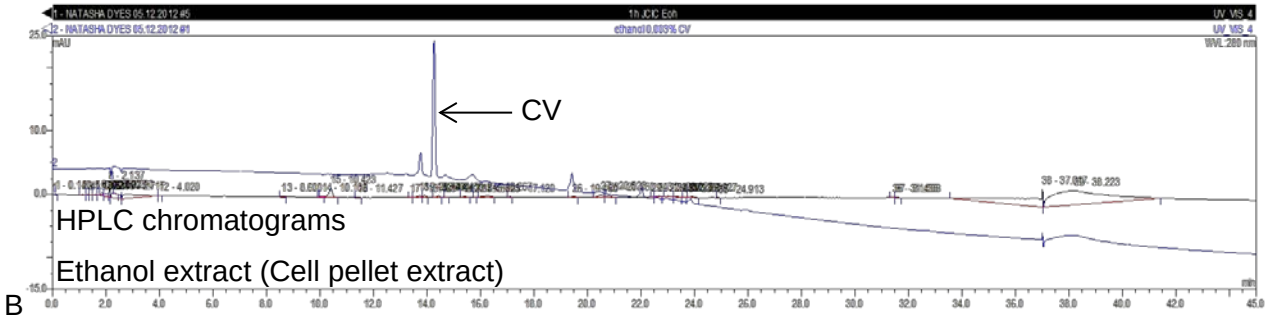
Since the degradation of CV was of interest compounds greater than 407.99 m/z, which is the actual molecular weight of CV will not be discussed. *N*-Methyl-*N*'-methyl-*N*"-hydroxymethylpararosaniline (MMHPR, 346.0453m/z) and MK (269.1582m/z) was found in the extracellular aqueous fraction as well as several other compounds ranging from 99.5095m/z to polymeric compounds at 1150.0747m/z. In the cell pellet extracts of *Gordonia* sp. JC51 CV (372.252m/z), LCV (374.2510m/z), MMPR (316.1564m/z), DMHPR (360.2372m/z), DDMPR (358.14m/z), DMMPR (344.2048m/z), MK (269.1599m/z) as well as several other compounds ranging from 86.0935m/z to polymeric compounds 1177.5357m/z were found. Degradation of CV took place in the extracellular environment as well as near the cell membrane of the *Gordonia* sp. JC 51 cell.



A

Sample: Aqueous culture supernatant from JC 51 after 1 h treatment with 30 mg/L CV

Control: 30 mg/L CV in water (Time 0)



B

Sample: Cell pellet extract from JC 51 after 1 h treatment with 30 mg/l CV

Control: 30 mg/L CV in ethanol

Figure 6.5: LC/MS/MS results of *Gordonia* sp. JC 51 metabolites after 1 h treatment extracted from culture supernatant and cell pellet extract. A)HPLC Chromatogram of *Gordonia* sp. JC 51 metabolites of Crystal Violet in culture supernatant after 1 h treatment with 30 mg/l CV in water (black line) compared to 30 mg/l CV in water before the addition of whole cells (blue line, Time 0).B) HPLC Chromatogram of *Gordonia* sp. JC 51 metabolites of CV in cell pellet extract after 1 h treatment with 30 mg/l CV in water (black line) compared to 30 mg/l CV in ethanol (blue line, control).

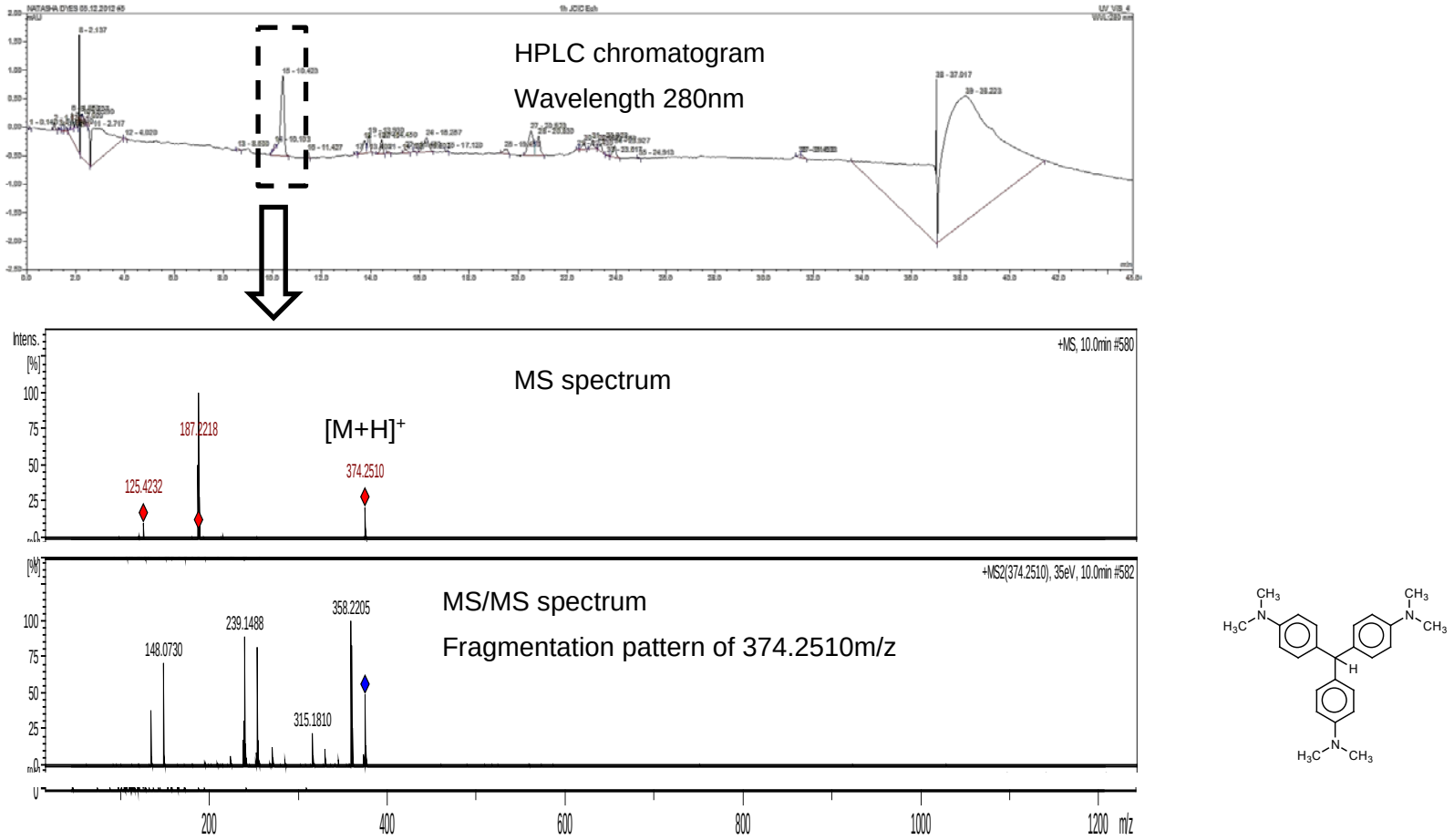


Figure 6.6 LC/MS/MS analysis of cell pellet extract obtained from *Gordonia* sp. JC 51 cells after 1 hour treatment with 30 mg/l CV

6.3.4 Structural characterisation of the biocatalysis products of CV decolourisation and degradation by *Gordonia* sp. JC 51 using LC/MS/MS analysis

LC/MS/MS analysis was used to identify the reaction products of CV decolourisation and degradation by *Gordonia* sp. JC 51 in water without the addition of nutrients.

6.3.4.1 *LC/MS/MS analysis*

LC/MS/MS analysis confirmed that LCV was one of the initial compounds formed after decolourisation. This confirmed the involvement of triphenylmethane reductase. Washed *Gordonia* sp. JC 51 was able to convert CV to colourless or degraded products through several pathways involving the reactions 1) reduction, 2) N-demethylation and oxidative cleavage. JC 51 converted CV to LCV, N-demethylated dyes of CV, N-demethylated leuco-dyes of CV, aminobenzophenones, aminophenols, aminobenzaldehydes and mineralized products. The conversion of CV to various intermediates suggest that other enzymes are also involved. Previous enzyme screening showed positive activities for polyphenol oxidase, triphenylmethane reductase and peroxidase.

6.3.4.2 *The intermediates detected*

Crystal Violet reference standard in ethanol (RC) at 280nm showed a total of 33 peaks of which the main peak was 14.277 min with a peak height of 21.527 mAU and Area of 2.2523 Mau*min. This peak at 14.3 min was 372.2438 m/z, which is the cation of Crystal Violet. Fragment pattern determined by a second ms at 35 eV only fragmented to one other compound. MassBank was used to confirm results.

The uncatalysed control containing CV in water only (UC) showed a total of 35 peaks at 280 nm (summarised in Table 6-2) The main peak was at 14.383 min with a peak height of 4.414 mAU and Area of 0.4253 Mu*min. The peak contained a major peak at 372.2387 m/z, which is CV. It also contained a number of 3 contaminating peaks, which was 186.6210,1027.7253 and 1049.449 m/z.

The samples JC 51C.H_20 and JC51D.H_20 represent samples of the reaction solution after JC 51 was treated with CV for 1 hour. JC 51C.EOH and JC51D.EOH represent the ethanolic extracts obtained from the cell pellets of JC 51, which also represent the insoluble or precipitated compounds that were soluble in ethanol. These compounds were eluted or

desorbed from cells with ethanol. JC 51C decolourised CV. JC51D partially decolourised CV. This can be seen in Figure 6.4 containing the flasks. JC 51C.H_20 and JC51D.H_20 represent the compounds found free in solution and JC 51C.EOH and JC51D.EOH represent the bound dye and intermediates that were attached to the cells and insoluble compounds. JC 51C.H_20, JC51D.H_20, JC 51C.EOH and JC51D.EOH showed 42, 41, 39 and 32 peaks respectively in HPLC chromatogram. Most of the peaks were less than 0.8 Mau.

Table 6-2 Crystal Violet detection in controls and samples of *Gordonia* sp. JC 51 after decolourisation by LC/MS/MS.

Sample	**RT**	**Area (Mu*min)**	**Height (mAU)**	**MS1**	**MS2**
RC	14.277	2.2523	21.527	372.2438	372.2466
	14.707	0.1007	0.47	ND	ND
UC	14.383	0.4253	4.414	372.2357	372.2370
	14.687	0.0095	0.074	ND	ND
S1.E (JC 51C.H_20)	14.5	ND	ND	372.2381	372.2369
	14.75?	0.0157	0.103	ND	ND
S2.E (JC51D.H_20)	ND	-	-	-	-
	14.72	0.0087	0.079	ND	ND
S1.P(JC 51C.EOH)	14.4	ND	ND	372.2352	372.2361
	14.45	0.0287	0.253	ND	ND
S2.P (JC51D.EOH)	14.733	0.033	0.037	ND	ND
	14.4	0.2299	2.244	372.2337	372.2347

6.3.5 The degradation pathways of CV by *Gordonia* sp. JC 51

The proposed pathway of CV degradation is shown in Figure 6.7. It is proposed that CV is reduced by the addition of hydrogen to form LCV, due to the activity of the postulated presence of the enzyme triphenylmethane reductase. Since LCV is found in the cell pellet extract it is suggested that triphenylmethane reductase might be found in the cell membrane region.

In the literature it was found that LCV could easily revert back to CV when exposed to halogens such as chlorine ions and iodine as well as oxygen. Peroxidases are found to convert LCV to CV. Catalase prevents the conversion of LCV back to CV as it competes for the hydrogen peroxide. The TLC analysis (Figure 6.3) has demonstrated that the compound converts to a purple/pinkish colour when exposed to iodine vapours and this confirms that LCV is formed. Also in previous Native PAGE results (Figures 5.1 through 5.3) a clear colourless band is formed when exposed to NADH with CV which means LCV is formed. Previous Spectral scan results have also shown that colourless compounds in the UV region are produced after exposure to CV with *Gordonia* sp. JC 51. Since various other compounds are simultaneously formed and different pathways are used by this bacterium it therefore makes the detection of triphenylmethane reductase difficult because the reaction is reversible. Slight changes in the decrease of CV could be due to *N*-demethylation. Extracting compounds from the cells is important as it helps in the identification of the intermediates. In this study it was found that the reactions for CV decolourisation occur extracellular as well as near the cell membrane, and therefore the enzymes involved may be obtained in this region and not the extracellular environment. The sensitivity of LC/MS/MS has allowed for the detection of very minute concentrations of products.

In the literature there are several proposed mechanisms of *N*'demethylation. Figure 6.4 shows that there is a significant decrease in colour after 1 h, but the *N*-demethylated products found are coloured compounds. These coloured compounds may be residual compounds that are detected by LC/MS/MS at low levels.

Spectral scan studies of shake flask reactions involving 10 mg/l CV in water treated with cells for 24 hours revealed that *Gordonia* sp. JC 51 could decolourise CV under starvation conditions. There was a disappearance of the peak at 590nm and a new peak appeared at the UV region. The shift from light spectrum to UV spectrum indicates decolourisation has occurred as well as a new compound has formed.

Thin layer chromatography studies involving shake flask reactions involving 6-day old cultures grown in YEME and subsequently treated with 0.5 g/l CV for 24 hours and monitored at various time intervals. It was found that after 20 hours decolourisation occurred. At the point of decolourisation a decolourised product was formed, which could not be seen under visible light, only under UV and exposure to iodine vapors. Under the exposure of iodine vapors a pale pink/light purple spot appeared, which was suggested to be either LCV or p-aminobenzaldehyde by monitoring standard reactions.

6.3.6 *Gordonia* sp. JC 51 metabolised CV via three pathways

Based on the LC/MS/MS data three pathways in CV degradation were indicated: reduction, N-demethylation and oxidative cleavage (see Figure 6.7 through 6.10). For the reduction pathway CV was converted to LCV. In the literature CV is either converted to LCV via the addition of an electron or via hydrogenation. In this study N-demethylated products of CV were also converted to their reduced leuco-dyes. Since NADH was required for the decolourisation of CV when enzyme solutions were used it is very likely that triphenylmethane reductase is the enzyme involved. For the N-demethylation pathway the methyl groups of the dye were sequentially removed and replaced with hydrogen. Lignin peroxidase have been shown to attack CV via N-demethylation, but required H_2O_2 as cofactor. For the oxidative cleavage pathway CV was cleaved at the central C-phenyl bond using oxidative enzymes that use oxygen and produce benzophenones and phenols. This may possibly lead to ring opening of the aromatic compounds and eventually mineralisation.

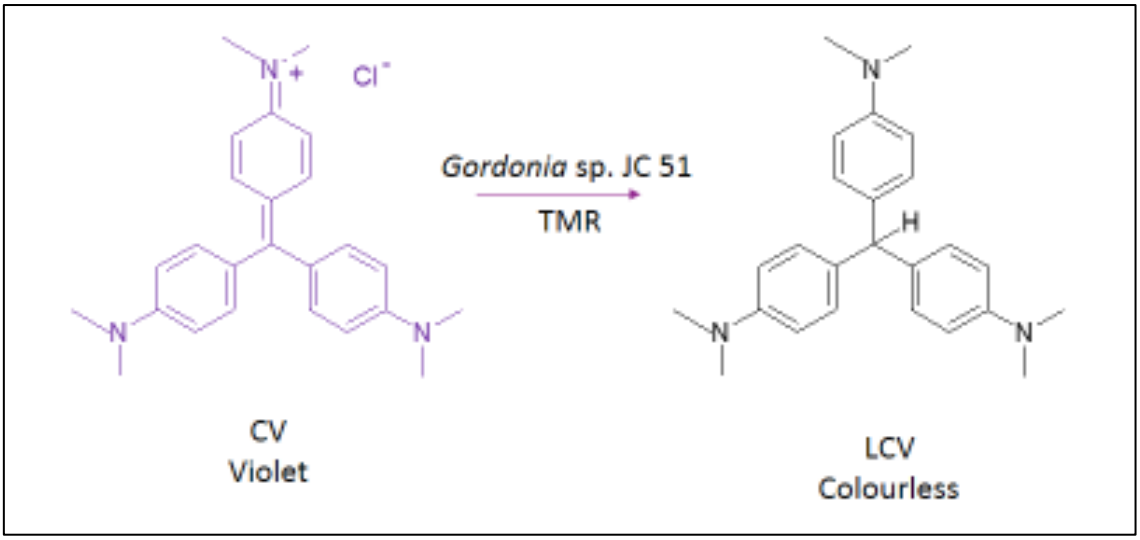


Figure 6.7 Reduction of CV to LCV by TMR in whole cells of *Gordonia* sp. JC 51.

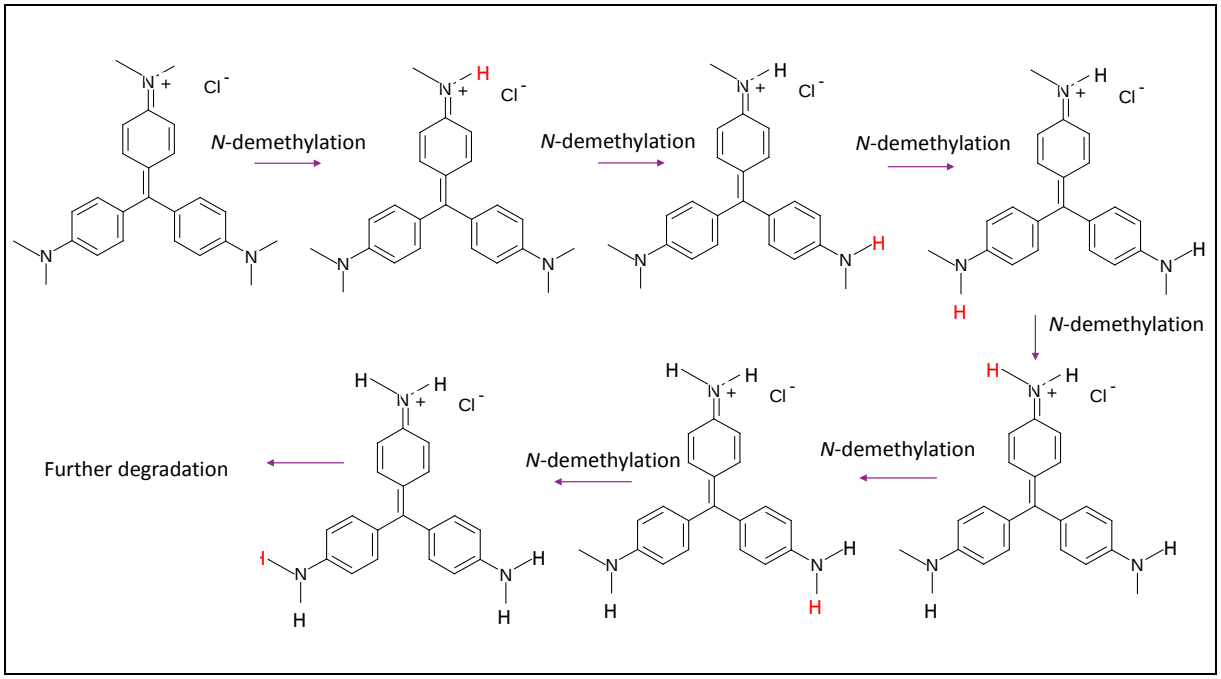


Figure 6.8 Proposed pathway 1: Sequential *N*-demethylation of CV to various colour intermediates.

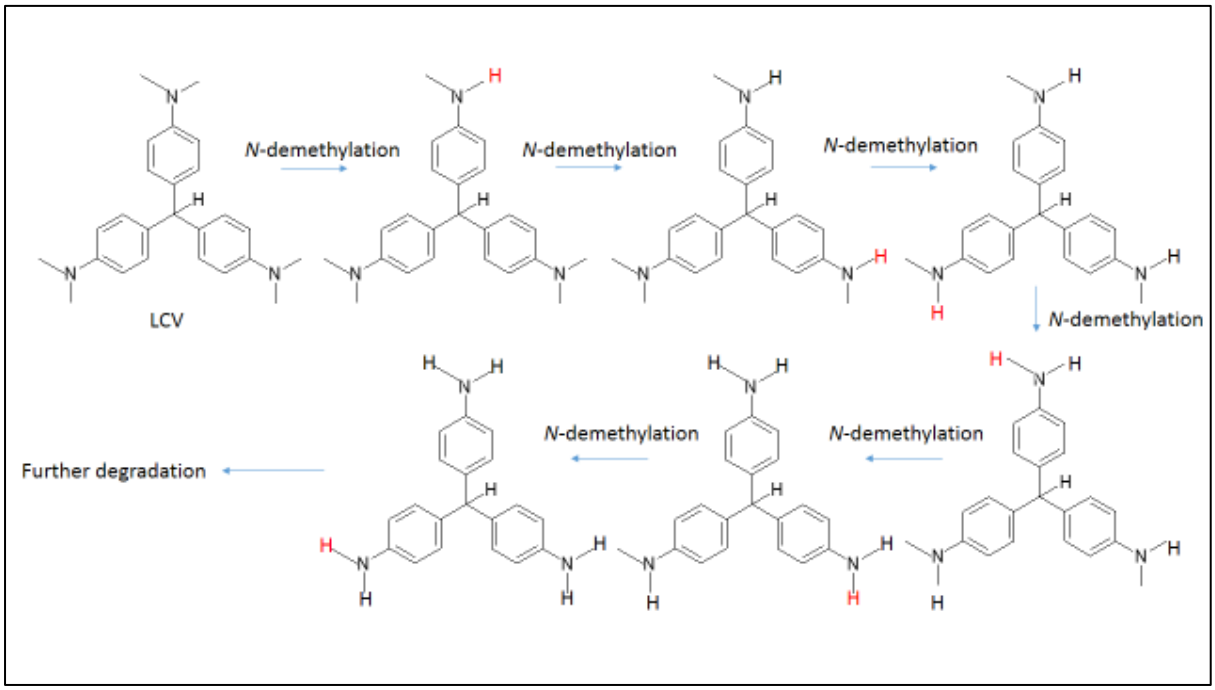


Figure 6.9 Proposed pathway 2: Sequential *N*-demethylation of LCV to various colourless derivatives

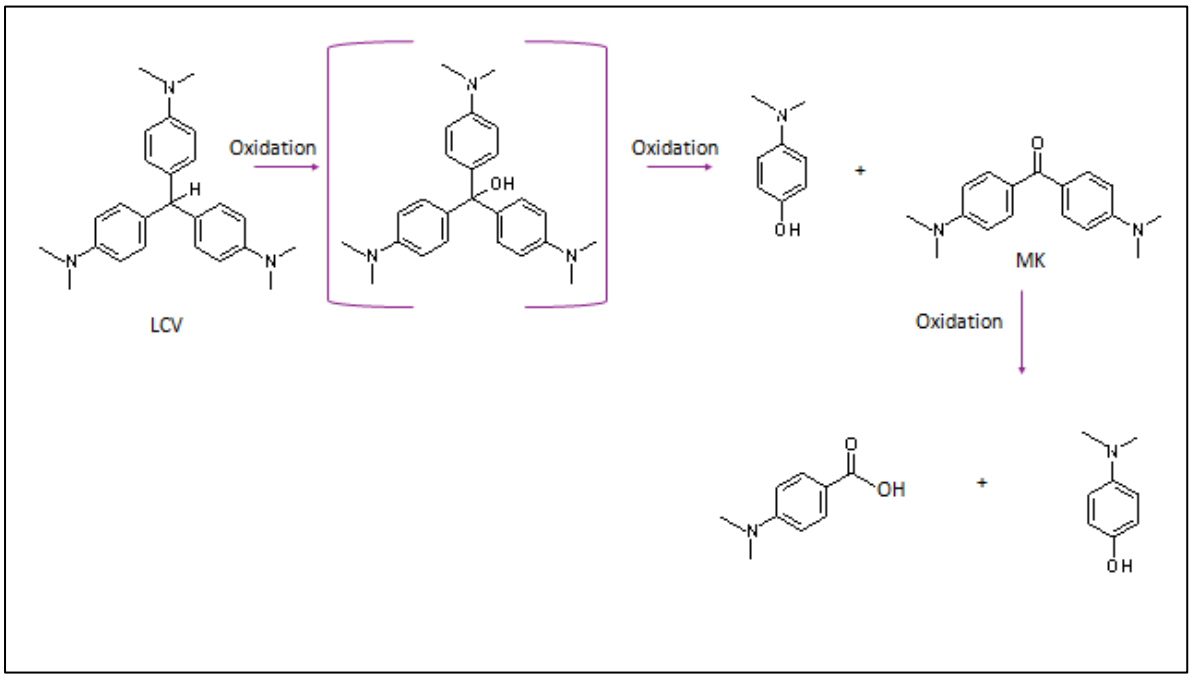


Figure 6.10 Proposed pathway 3: Overoxidation of LCV via stepwise cleavage resulting in depolymerisation. Trimers broken down to dimers and monomers.

6.4 Conclusion

This study revealed that *Gordonia* sp. JC 51 could be used as a whole cell biocatalyst to degrade and decolourise CV at high concentrations and also in nutrient limited conditions. Spectral scan studies showed that the products produced were colourless and had absorbance peaks in the UV region. High concentrations of 0.5 g/l CV could be removed and produced colourless compounds that had similar properties to LCV. LC/MS/MS analysis confirmed that LCV is a produced after CV decolourisation. However, it was not the only compound produced. The early stages of degradation and decolourisation of CV was elucidated. Thus far, it seems that the decolourisation of CV and its degradation pathway progresses via multiple pathways. Crystal Violet degradation involves reduction, oxidative cleavage, *N*-demethylation and *N*-demethoxylation reactions. The fact that LCV was found confirms that triphenylmethane reductase was likely to be the enzyme involved in the decolourisation of CV.

7 Conclusion and Recommendations

The evidence presented has shown that several deep-sea actinobacteria could decolourise and tolerate CV. The main mechanisms of decolourisation by the deep-sea actinobacteria studied were biosorption and biodegradation. Members of *Gordonia* and *Williamsia maris* DSM 4493 mainly removed CV via biodegradation in liquid culture, whereas *Rhodococcus* and *Pseudonocardia* sp. AB630 mainly employed biosorption. Secondary screening using laccase, peroxidase and polyphenol oxidase enzyme assays showed low or no activity. This evidence highlighted that another enzyme was involved in the initial decolourisation of CV. Database mining provided information on a suitable enzyme candidate that could be involved in the decolourisation of CV by these strains, which was triphenylmethane reductase (TMR).

Gordonia sp. JC 51 was selected as a candidate to further characterise the decolourisation of CV and determine the optimal growth and reaction conditions. High decolourisation efficiency (>80%) was obtained for *Gordonia* sp. JC 51 when the inoculum wet biomass concentration was between 10-100 mg/ml, reaction temperatures between 4-55°C, growth pH between 5-9 and CV concentrations between 10-100 mg/l. The rate of decolourisation was higher for *Gordonia* sp. JC 51 under agitation conditions compared to static conditions. However, both agitation and static conditions reached similar maximum decolourisation percentages.

Decolourisation was also tested using cell-free systems of *Gordonia* sp. JC 51 such as broken cell preparations (crude or clarified), culture supernatant (extracellular) and cell lysate (intracellular). Although broken cell preparations (CS) provided higher biodegradation than cultures of *Gordonia* sp. JC 51 (UC) the differences was not significant. However, when broken cell preparations were heat-inactivated (CSH) they were unable to biodegrade Crystal Violet. The main enzyme involved in the decolourisation was triphenylmethane reductase, which was found in both the culture supernatant and cell lysate. The cell-free systems required NADH for enhanced decolourisation activity.

The purification of TMR by *Gordonia* sp. JC 51 was found to be difficult and therefore obtaining sufficient quantities of TMR for further characterisation studies was not possible. Unprocessed enzyme solutions provided a positive result for decolourisation in in-gel assays than partially purified enzyme prepared with acetone. The purification process may result in essential factors being lost and possibly disassemble or separate multiple-enzyme systems. The use of whole

cell biocatalyst would therefore be the best option for the decolourisation of CV, because the enzyme was difficult to isolate and requires expensive cofactors such as NADH.

The reaction products of CV decolourisation by *Gordonia* sp. JC 51 were determined by using UV/Vis spectrophotometry, TLC and LC/MS/MS analysis. UV/Vis spectral analysis showed that the reaction products were colourless as there were no peak at the visible region. The reaction products were also UV absorbing. TLC analysis confirmed that the reaction products after decolourisation were colourless and UV absorbing. TLC analysis also showed N-demethylated products at the initial stages of decolourisation. LC/MS/MS analysis was used to determine the structures of the reaction products. Crystal Violet was converted to LCV, *N*-demethylated products of CV and LCV, Michler's ketone and various other products. The degradation pathway of CV by *Gordonia* sp. JC 51 was elucidated and found to involve reduction of CV to LCV and subsequent *N*-demethylation to other Leuco-form compounds or cleavage to Michler's ketone and p-dimethylaminophenol. Coloured intermediates were also detected, which were the sequential *N*-demethylated forms of CV. Based on these results it is clear that the decolourisation of CV involves multiple pathways and may also involve other enzymes besides TMR. This evidence also highlights the fact that in order for *Gordonia* sp. JC 51 to grow in the presence of CV it should be able to decolourise and degrade CV.

Understanding the decolourisation and degradation pathway of CV by *Gordonia* sp. JC 51 was important in order to determine its potential use as a whole cell biocatalyst in the treatment of CV and related dyes in contaminated industrial wastewaters. CV decolourisation was not only caused by the conversion of CV to LCV but also by its subsequent degradation and mineralisation.

The next step would be to further develop *Gordonia* sp. JC 51 as a whole cell biocatalyst in the treatment of wastewater contaminated with Crystal Violet and other related dyes. Wastewater sourced from various industries (food, pharmaceutical, ink, chemical and textile industries) and dye contaminated sites should be tested using *Gordonia* sp. JC 51 for its decolourisation and degradation potential. The toxicities of the reaction products produced during various stages of decolourisation and after decolourisation should also be determined. Also the rates of appearance and disappearance, the stability and accumulation of these products should also be determined. In order to stabilise and recover whole cell biocatalysts for re-use it is recommended that *Gordonia* sp. JC51 be immobilised.

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Appendices

Appendix A: Characteristics of deep-sea actinobacteria studied

Table A1 Strain Descriptions

Tests	JC 18	JC 25	JC 50	JC 51	JC 70	JC 4	JC 24	JC 55	JC58	DSM 44693	AB 630
Genus and species[1]	*Gordonia* sp.					*Rhodococcus* sp.				*Williamsia maris*	*Pseudonocardia* sp.
Gram stain[2]	+	+	+	+	+	+	+	+	+	+	+
Appearance under microscope[2,3]	Rod-shaped with some filaments on the side									Short rods and coccoid-like elements	Filamentous
Growth and Maintenance Media[2]	ISP# 2 (YEME) Media (Standard Actinomyces growth media) Comments: No special nutrient requirements for growth										
Growth Conditions[2]	Temperature:30°C(Ambient); Aeration: 160rpm (Aerobic conditions); Pressure: Atmospheric; pH:7(neutral)										
Colony morphology on YEME[3]	White/cream /light yellow (matte)	Peach/orange (matte)			Light/milky peach (matte)	Orange (matte)			Yellow(matte)	Orange(glossy), round convex colonies	Brown basal mycelium and white aerial mycelium(matte)
Growth on YEME agar with 30 mg/l CV[3]	+	+	+	+	+	+	+	+	+	+	+
Oxidoreductase Enzyme Profile (High throughput Screen/Preliminary Screen)											
General Oxidoreductases Activity											
CV decolourisation[2]	+	+	+	+	+	+	+	+	+	+	+
RBBR decolourisation[2]		S	S	S	S	S	S	S	S	S	S
Poly R478 decolourisation[2]		S	S	+	+						
Specific Assays											

Tests	JC 18	JC 25	JC 50	JC 51	JC 70	JC 4	JC 24	JC 55	JC58	DSM 44693	AB 630
Laccase		+				+	+		+		+
Peroxidase		-	+	+		+	-	+	+		+
Polyphenol Oxidase		+				+	+	+		+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+

+: Positive, -: Negative Test result, S: Slight

Data obtained in this study and with acknowldgement to Dr Le Roes-Hill for providing additional data of strains.

Appendix B: Properties of Crystal Violet

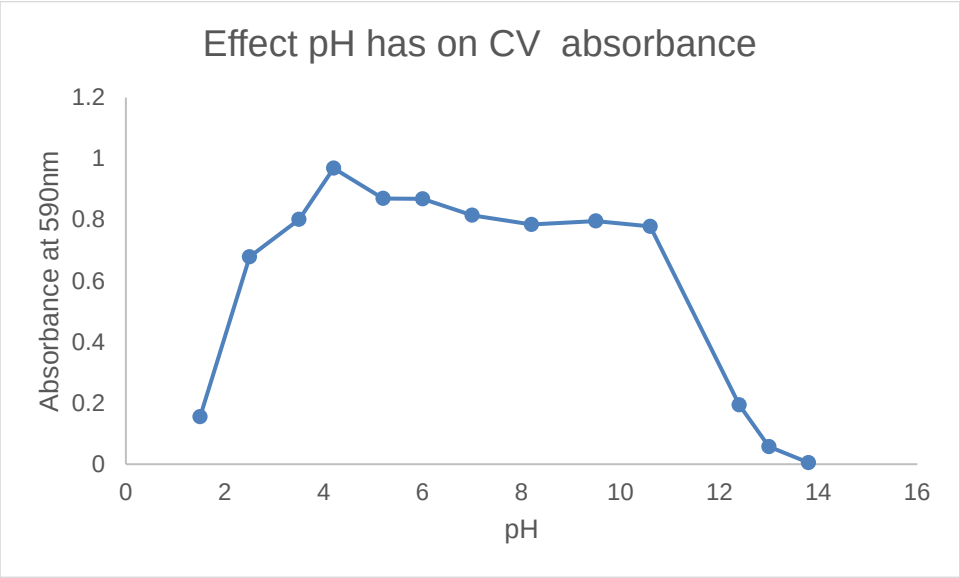


Figure B1 The effect pH has on the absorbance of 10 mg/l CV at 590 nm

At different pH the absorbance values of CV at 590nm changes. Different pH may therefore also influence the extinction coefficient for CV. Based on this pH tests studies were performed at a ranged between 5 and 10.

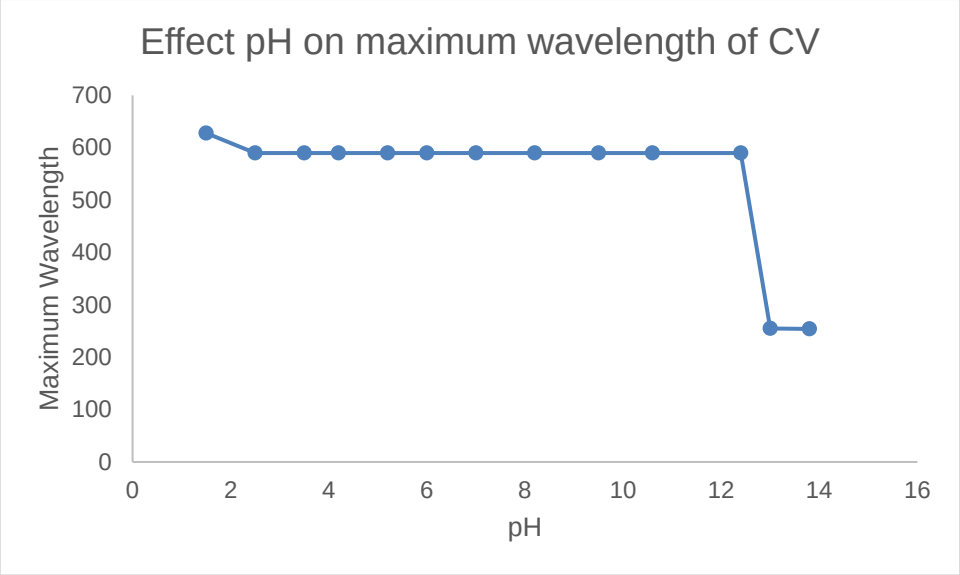


Figure B2 Effect pH has on the maximum wavelength of 10 mg/l CV

Since CV is a pH indicator it was important to determine the maximum wavelength of CV at different pH conditions. The graph shows that pH 2 -12 the maximum wavelength is constant at 590nm, however at pH 1.5 the peak shifts to 628. At pH 13 and 13.8 the maximum wavelength shifts to 255 and 254 nm respectively.

Table B1 Absorbance peaks and absorbance values of CV at various pH conditions in aqueous solution

pH	**Maximum Wavelength (λ max) in nm**	**Absorbance**
Control (distilled water and dye)	590	0.871
1.5	628 426 590	0.2312 0.0625 0.1552
2.5	590	0.6788
3.5	590	0.8011
4.2	**590**	**0.9688**
5.2	590	0.8699

6	590	0.8686
7	590	0.8147
8.2	590	0.7846
9.5	590	0.796
10.6	590	0.7783
12.4	590	0.1939
13	255	0.2909
	590	0.0575
13.8	254	0.272
	590	0.0051

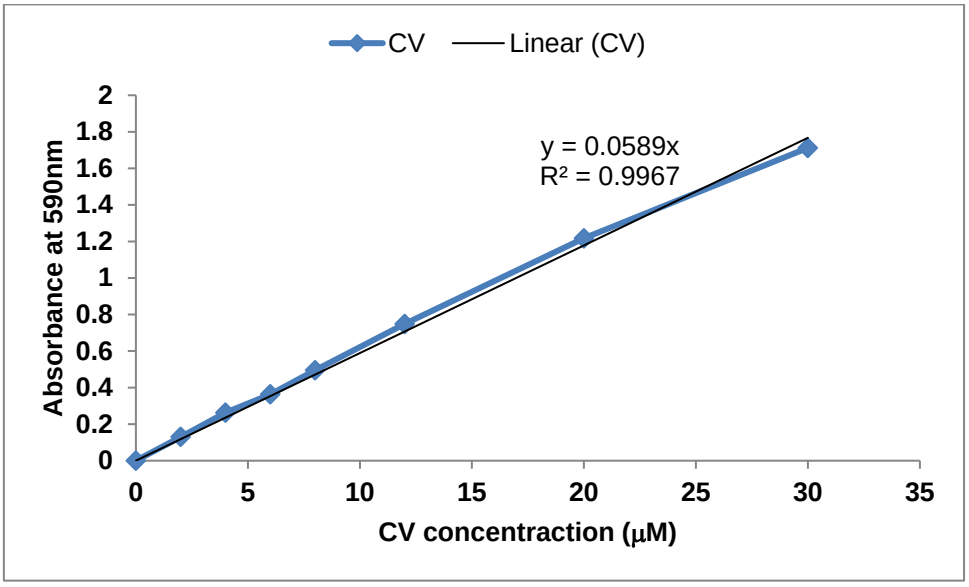


Figure B3 Relationship between Absorbance at 590nm and CV concentration

The relationship between the absorbance at 590nm and CV concentration was determined. This was used to determine the values of unknown concentrations of CV in reaction solutions.

Appendix C

Formulae

CV decolourisation

C1

Zone of clearing = Area of clearing - Area of colony

C2

$$\textbf{D}\boldsymbol{ecolourisation}\,\% = \frac{\boldsymbol{A_0 - A_1}}{\boldsymbol{A_0}} \times \mathbf{100}$$

Where A_0 is the initial absorbance and A_1 is the final absorbance.

C3

$$\boldsymbol{Biodegradation}(\%) = \frac{\boldsymbol{A_0 - A_1 - A_2}}{\boldsymbol{A_0}} \times \mathbf{100}$$

Where A_0 is the initial absorbance and A_1 is the final absorbance. A_2 is the absorbance of the extracted dye from the cell pellet at the final time point.

Protein concentration

Standard BSA protein concentration Curve equation

$$\boldsymbol{y = 0.0025x}$$

y= Absorbance at 595nm

x= Protein concentration (μg/ml)

C5

$$\boldsymbol{Protein\ concentration\ of\ sample}\ (\mu\mathbf{g/ml}) = \frac{\boldsymbol{Absorbance\ at\ 595nm}}{\mathbf{0.0025}}$$

Total protein

C6

$$\boldsymbol{Total\ protein}(\mu g) = \frac{\boldsymbol{Protein\ concentration}\ (\mu g/ml)}{\boldsymbol{Total\ volume\ of\ sample}\ (ml)}$$

Enzyme Activity

C7

$$\boldsymbol{Dilution} = \frac{\boldsymbol{total\ reaction\ volume}\ (ml)}{\boldsymbol{Volume\ of\ sample}\ (ml)}$$

C8

$$\boldsymbol{Factor} = \text{Dilution} \times \frac{1}{\varepsilon} \times 1000$$

C9

$$\boldsymbol{Activity}\left(\frac{U}{ml}\right) = \frac{\Delta \boldsymbol{Absorbance}}{\boldsymbol{min}} \times \boldsymbol{factor}$$

C10

$$\boldsymbol{Total\ Activity}(U) = \text{Activity}\left(\frac{U}{ml}\right) \times \boldsymbol{Total\ volume\ sample}\ (ml)$$

C11

$$\boldsymbol{Specific\ Activity}\left(\frac{U}{\mu g}\right) = \frac{\boldsymbol{Total\ activity}\ (U)}{\boldsymbol{Total\ protein}\ (\mu g)}$$

C12

$$\boldsymbol{Yield}(\%) = \frac{\boldsymbol{Activity\ in\ solution}}{\boldsymbol{Activity\ in\ crude\ extract}} \times 100$$

C13

$$\boldsymbol{Fold\ purification} = \frac{\boldsymbol{Specific\ activity\ in\ solution}}{\boldsymbol{Specific\ activity\ in\ crude\ extract}}$$

TLC

C14

$$Rf = \frac{distance\ traveled\ by\ component}{distance\ traveled\ by\ solvent}$$

Appendix D: TMR Activity determination

Table D1. Example of TMR activity determination of three independent trials

Independent trials	Per Reaction of 1ml			Per Sample of 1ml		
	-ΔAb/min	Reaction volume/Sample Volume	Sample dilution	Ab/min/ml	μmol/min/ml	nmol/min/ml
					U/ml	mU/ml
Trial 1	0.010	4	1	0.04	0.000	0.36
Trial 2	0.015	10	1	0.15	0.001	1.35
Trial 3	0.020	10	1	0.2	0.002	1.80
			Average	0.13	0.001	1.17
			STD	0.08	0.001	0.74
			CV	63.0	100.000	63.0

STD: Standard deviation

CV: Coefficient of Variation

Appendix E: Conversions of CV

Table E1 Conversions of Crystal Violet concentrations

g/l	mg/l	mM	μM
0.5	500	1.266	1266
0.01	10	0.025	24.5
0.012	12.24	0.030	30
0.02	20.4	0.050	50
0.03	30	0.074	73.5
0.04	40.8	0.100	100
0.20	204	0.500	500
0.41	407.98	1.000	1000
0.82	815.96	2.000	2000
0.01	10	0.025	24.5
0.03	30	0.074	73.5
0.06	60	0.147	147.1
0.1	100	0.245	245.1
0.3	300	0.735	735.3

Appendix F: Preparations of reagents and solutions for Protein electrophoresis and analysis

Polyacrylamide-gel electrophoresis

10% Ammonium persulphate (AMPS) (5ml): Dissolve 0.5g AMPS in to a final volume of 5ml distilled water. Store at 4^{o}C and discard after 2 weeks.

30% Acrylamidemix (100ml): Dissolve 29g acrylamide and 1g N, N'-methylenebisacrylamide in 50ml distilled water. Adjust volume to 100ml with distilled water. Store at 4^{o}C and cover container with foil. Caution: Wear gloves and a mask when making acrylamide as it is a neurotoxin.

10% SDS (50ml): 5g SDS dissolve in 30ml distilled water. Adjust volume to 50ml with distilled water. Filter sterilize.

1M DTT (20 ml): 3.085g DTT dissolve in 20ml 10mM Sodium acetate (pH 5.2). Filter sterilize. Divide in aliquots of 1ml and store at -20^{o}C.

5x Loading sample buffer (10ml): 2.1 ml 1.5 M Tris-HCl (pH 6.8), 5ml glycerol, 0.125 ml 1% bromophenol blue and 2.77ml distilled water.

> Denaturing (50μl):10 μl loading sample buffer, 5 μl 1M DTT, 10 μl 10%SDS, 25 μl Sample
>
> Non-denaturing (50 μl): 10 μl loading sample buffer, 15 μl distilled water,25 μl Sample

Running Buffer (1L):Denaturing : Dissolve 10ml 10% SDS, 14.4g Glycine, 3.03g Tris in 800ml. Adjust to 1L with distilled water. Store at room temperature. For non-denaturing (native gels) omit SDS.

1.5M Tris-HCl (pH 8.8) (100ml)**:** 18.2g Tris Base in 40ml distilled water. Adjust pH with HCl and add water to 100ml.Autoclave

1.5M Tris-HCl (pH 6.8)(100ml)**:**18.2g Tris Base in 40ml distilled water. Adjust pH with HCl and add water to 100ml.Autoclave

Resolving/separating gel:Denaturing

Table F1: The preparation of the resolving or separating gel

% gel	Reagents	Volume of reagents(ml) per gel mould volume of	
		25ml	**50ml**
10%	dH_2O	9.9	19.8
	30% Acrylamide mix	8.3	16.7
	1.5 M Tris (pH 8.8)	6.3	12.5
	10% SDS*	0.25	0.5
	10% AMPS	0.25	0.5
	TEMED	0.01	0.02
12%	dH_2O	8.2	16.50
	30% Acrylamide mix	10	20
	1.5 M Tris (pH 8.8)	6.3	12.5
	10% SDS*	0.25	0.5
	10% AMPS	0.25	0.5
	TEMED	0.01	0.02
18%	dH_2O	3.19	6.48
	30% Acrylamide mix	15	30
	1.5 M Tris (pH 8.8)	6.3	12.5
	10% SDS*	0.25	0.5
	10% AMPS	0.25	0.5
	TEMED	0.01	0.02

*****SDS omitted for non-denaturing and replaced with distilled water.

Stacking gel: Denaturing

Table F2: The preparation of stacking gel

% gel	Reagents	Volume of reagents(ml) per gel mould volume of	
		5ml	10ml
5%	dH_2O	3.4	6.8
	30% Acrylamide mix	0.83	1.7
	1.5 M Tris (pH 6.8)	0.63	1.25
	10% SDS*	0.05	0.1
	10% AMPS	0.05	0.1
	TEMED	0.005	0.01

*SDS omitted for non-denaturing and replaced with distilled water.

Staining and Destaining Solutions

Coomaasie Blue Staining Solution (500ml): Dissolve 1.25g "Coomaasie Brilliant Blue R-250" in 225ml methanol, 50ml acetic acid and 200ml distilled water. Adjust volume to 500ml with distilled water.

Coomaasie Blue Destaining solution (1L): Mix 450ml ethanol, 400ml distilled water and 100ml acetic acid. Adjust the volume to 1L with distilled water.

Activity Stains for non-denaturing gels

73.5μM Crystal violet staining solution (500ml): Add 750μl 2% (w/v) Crystal Violet to 500ml 20mM Sodium Phosphate Buffer (pH7). NADH added for triphenylmethane reductase activity

APPENDIX G: Media and Reagents

G1) YEME (Yeast extract malt extract) media (1L):4g/L Yeast extract, 10g/L malt extract, 4g/L glucose. Dissolve in water. Adjust pH with Sodium Hydroxide to pH 7. For agar plates add 20g/L Bacteriological Agar. (autoclave)

G2) 50% glycerol stock (100ml):50ml 100% glycerol in 50ml distilled water. (autoclave / filter sterilize)

G3) 2% Crystal Violet stock (100ml):2g Crystal Violet in 100ml autoclaved distilled water.(filter sterilize)

G4) 60% Tween (50ml):30ml 100% Tween 80 in 20ml autoclaved distilled water. Filter sterilize

G5) Carbon utilization media (ISP #9) (1L):2.64g $(NH_4)_2SO_4$(Ammonium sulphate), 2.38g KH_2PO_4 (Potassium dihydrogen phosphate), 5.65g $K_2HPO_4 \cdot 3H_2O$ (di-potassium hydrogen orthophosphate), 1g $MgSO_4 \cdot 7H_2O$ (Magnesium sulphate), 1ml Trace salts. Dissolve in water. For agar plates add 20g/L Bacteriological Agar.(autoclave)

G6) Trace Salts (100ml):0.64g Cu $SO_4 \cdot 5H_2O$, 0.11g $FeSO_4 \cdot 7H_2O$, 0.79g $MnCl_2 \cdot 4H_2O$, 0.15g $ZnSO_4 \cdot 7H_2O$. Dissolve in Distilled water. (autoclave)

G7) 0.003% Crystal violet (1L):1.5ml 2% CV in 1L autoclaved media

G8) 0.006% Crystal violet (1L):3ml 2%CV in 1L autoclaved media

G9) 1% Sasol waste (500ml):5ml 100% Sasol waste in 500ml media (autoclave)

G10) 0.1% Tween 80 (250ml):417µl 60% Tween 80 in 250ml media.

G11) 0.1M Sodium Acetate Buffer (pH 5) (500ml):6.8g sodium acetate trihydrate. Dissolve in 400ml distilled water. Adjust pH with glacial acetic acid. Adjust volume to 500ml with distilled water. (autoclave)

G12) 0.2M Sodium phosphate, mono-sodium salt (500ml):Dissolve 13.8g $NaH_2PO_4 \cdot 1H_2O$ in 250ml distilled water. Adjust to 500ml with distilled water. (autoclave)

G13) 0.2M Sodium phosphate, di-sodium salt (500ml):Dissolve 26.81g $Na_2HPO_4 \cdot 7H_2O$ in 250ml distilled water. Adjust to 500ml with distilled water. (autoclave)

G14) 0.1M Sodium phosphate, mono-sodium salt (500ml):Dissolve 5.99g NaH_2PO_4(anhydrous) in 250ml distilled water. Adjust to 500ml with distilled water. (autoclave)

G15) 0.1M Sodium phosphate, di-sodium salt (500ml): Dissolve 7.09g Na_2HPO_4 (anhydrous)in 250ml distilled water. Adjust to 500ml with distilled water. (autoclave)

G16) 50mM Sodium phosphate buffer (pH 7) (500ml): 48.75ml 0.2M mono-sodium phosphate salt.76.25ml 0.2M di-sodium phosphate salt. Adjust volume to 500ml with distilled water. (autoclave)

G17) 0.2M Potassium phosphate, mono- potassium salt (500ml):Dissolve 13.6g $KH_2PO_4 \cdot 1H_2O$ in 250ml distilled water. Adjust to 500ml with distilled water. (autoclave)

G18) 0.2M Potassium phosphate, di- potassium salt (500ml):Dissolve 17.4g $K_2HPO_4 \cdot 7H_2O$ in 250ml distilled water. Adjust to 500ml with distilled water. (autoclave)

G19) 50mM Potassium phosphate buffer (pH 6) (500ml):109.625ml 0.2M mono-potassium phosphate salt.15.375ml 0.2M di-potassium phosphate salt. Adjust volume to 500ml with distilled water. (autoclave)

G20) 100mM Potassium phosphate buffer (pH 7) (200ml):39ml 0.2M mono-potassium phosphate salt.61ml 0.2M di-potassium phosphate salt. Adjust volume to 250ml with distilled water. (autoclave)

G21) 0.05M H_2O_2 (Hydrogen Peroxide) (50ml): 76.5μl H_2O_2 (1.11g/ml density) in distilled autoclaved water.

G22) 5 mM ABTS (50ml):0.1372g ABTS in 50ml distilled autoclaved water. Cover with foil.

G23) 10mM L-Dopa (50ml):0.0986g in 50ml 50mM Potassium phosphate buffer. Cover with foil.

G24) 5 mM Guaicol (10ml):50 μl Guaicol in 10ml distilled autoclaved water. Cover with foil.

G25) DNS Solution A (500ml):5g 3, 5-Dinitrosalicyclic acid, 0.25g sodium sulphate, 5g sodium hydroxide. Make up to 500ml with distilled water. Cover with foil.

G26) 40% Potassium sodium tartrate (Solution B):40g Potassium sodium tartrate in 100ml distilled water.

G27) 0.5mM catechol (50ml):0.0275g catechol in 50ml 50Mm Potassium Phosphate buffer pH 7. Cover with foil.

G28) 1mg/ml BSA Stock (50ml): 0.05g BSA in 50ml autoclaved distilled water

Appendix H : Standard Calibration Curves

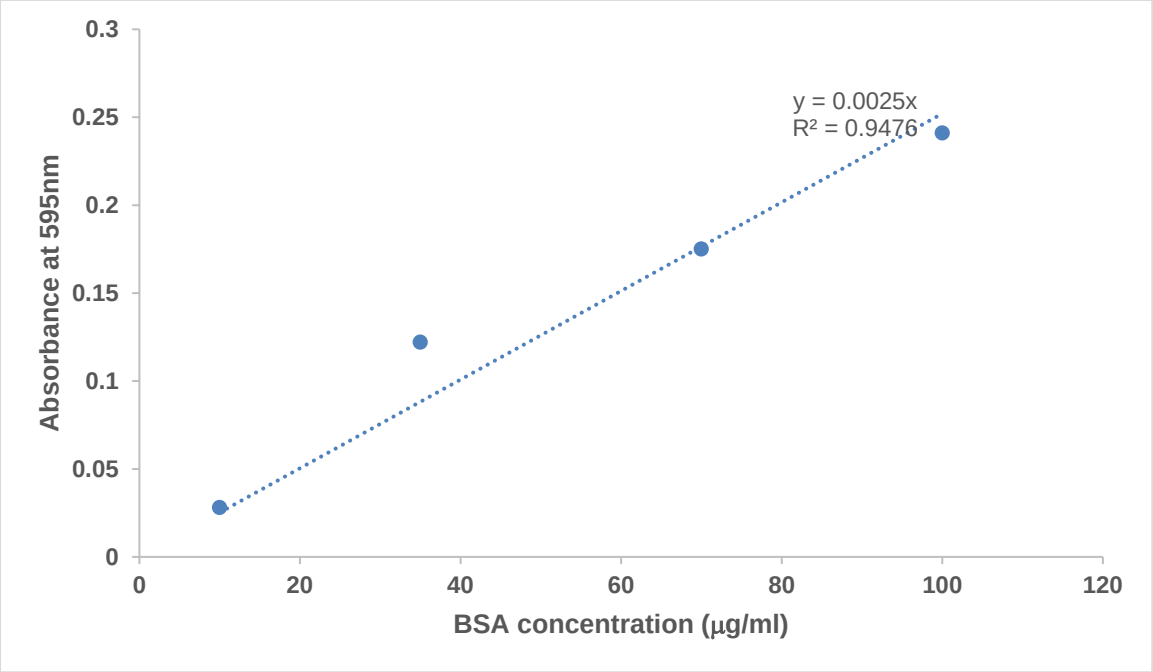


Figure H1: Standard Bradford BSA Protein Determination curve

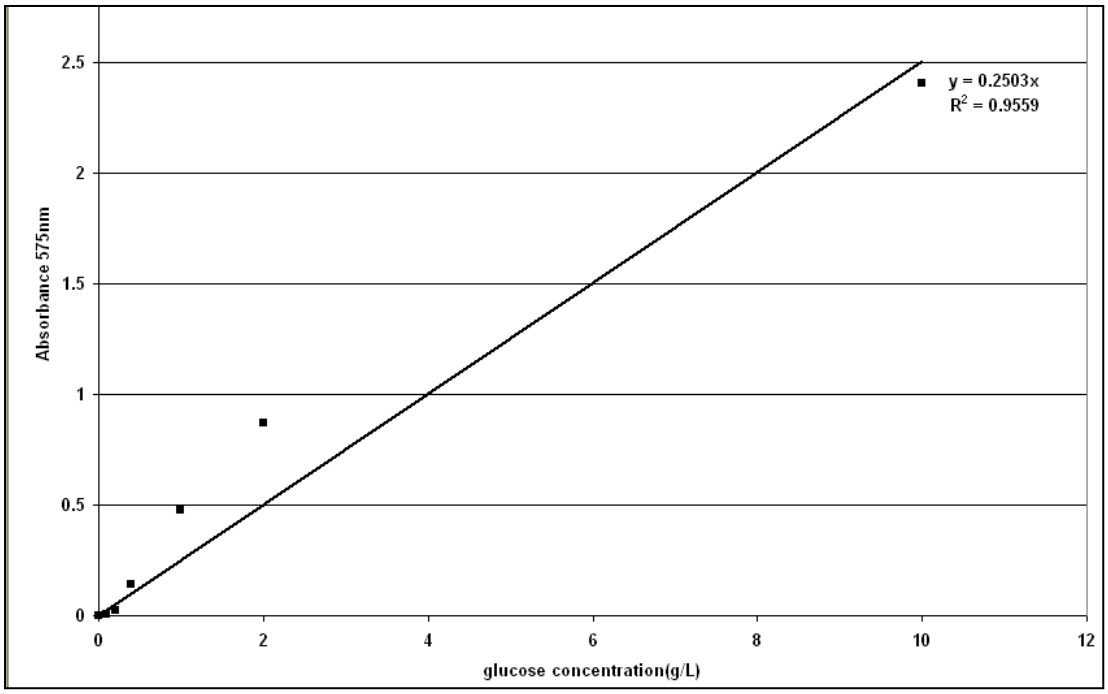


Figure H2: Standard Glucose Concentration Calibration Curve

Appendix I : Extinction coefficients (ε)

I1) ε_{420} **(ABTS)** = 36000 $M^{-1}cm^{-1}$

I2) ε_{470} **(guaiacol)** = 26600 $M^{-1}cm^{-1}$

I3) ε_{475} **(L-Dopa)** = 3600 $M^{-1}cm^{-1}$

I4) ε_{400} **(*p*-cresol)** = 1433 $M^{-1}cm^{-1}$

I5) ε_{285} **(veratryl alcohol)** = 17000 $M^{-1}cm^{-1}$

I6) ε_{460} **(*o*-dianisidine)** = 11300 $M^{-1}cm^{-1}$

I7) ε_{510} **(phenol/4-aminoantipyrine)** = 7100 $M^{-1}cm^{-1}$

I8) ε_{260} **(catechol)** = 1750 $mol^{-1}cm^{-1}$

I9) ε_{375} **(catechol)** = 44000 $mol^{-1}cm^{-1}$

Appendix J: Assays

J1) Bradford Assay

Table J1: Experimental set-up of Bradford Assay

Reagents	**Blank**	**Test**
Bradford Reagent (Sigma)	1ml	1ml
Distilled water	100μl	-
Sample	-	100μl
Total	1ml	1ml

The above solutions were placed in 2ml plastic cuvettes and the colour was left to develop for at least 5min (not more than 30min).The absorbance was read at 595nm. Absorbance readings were converted to μg/ml using a standard curve prepared using BSA (bovine serum albumin) (see Appendix H Figure H1 and Appendix C Formula C5) (Bradford, 1976).

J2) Glucose Assay

The solutions that were used in this assay was Solution A (see Appendix G:G25) and Solution B (see Appendix G: G26).Place 750μl cell free spent media (cell filtrate/ supernatant) and 750 μl Solution A in eppendorf tube and incubate in a 90^{o}C waterbath for 15min. Add 250 μl Solution B and leave at room temperature to cool. Transfer solution in 2ml plastic cuvette. The absorbance was read at 575nm. Water was used as a blank (Miller, 1959).

J3) Laccase Assay

Table J2: Experimental set-up of Laccase Assay

REAGENTS	**Experimental set-up**			
	J3.1	**J3.2**	**J3.3**	**J3.4**
0.1M Sodium Acetate, pH 5	2.5 ml	850μl	Master Mix 750 μl	Master Mix 1ml
5mM ABTS/Guaiacol	330 μl	112μl		
Sample(test)/distilled water(blank)	100μl	38 μl	250μl	1ml
Volume Total	2.930ml	1ml	1ml	2ml

The above solutions were placed in either 4ml plastic cuvettes (if volume above 2ml) or 2ml plastic cuvette (if volume less than 2ml).The absorbance was monitored at 470nm for 3min when guaiacol was used as substrate and for ABTS the increase in absorbance was monitored at 420nm for 80seconds.

J4) Tyrosinase Assay

Table J3: Experimental set-up of Tyrosinase Assay

REAGENTS	**Experimental set-up**			
	J4.1	**J4.2**	**J4.3**	**J4.4**
10mM L-Dopa in 50mM Potassium phosphate buffer, pH 6	2.9 ml	966μl	Master Mix 750μl	Master Mix 1ml
Sample(test)/distilled water(blank)	100μl	34 μl	250μl	1ml
Volume Total	3ml	1ml	1ml	2ml

The above solutions were placed in either 4ml plastic cuvettes (if volume above 2ml) or 2ml plastic cuvette (if volume less than 2ml).The absorbance was monitored at 475nm for 5min.

J5) Peroxidase Assay

Table J4: Experimental set-up of Peroxidase Assay

REAGENTS	Experimental set-up	
	J5.1	J5.2
100mM Potassium phosphate buffer, pH 7	400µl	Master Mix 1ml
Substrate	200µl	
0.05M H_2O_2	200µl	
Sample(test)/distilled water(blank)	200µl	1ml
Volume Total	1ml	2ml

The substrate can either be 1mM ABTS, 1mM L-Dopa or 20mM guaiacol, which can be monitored at 420,475,470nm respectively. Samples were placed in plastic 2ml cuvettes.

J6) Catechol dioxygenase Assay

Table J5: Experimental set-up of Catechol dioxygenase Assay

REAGENTS	Experimental set-up	
	J6.1	J6.2
0.05mM catechol in 50mM Potassium phosphate buffer, pH 7	2.9ml	Master Mix 750µl
Sample(test)/distilled water(blank)	100µl	250 µl
Volume Total	3ml	1ml

The above samples were placed in quartz cuvettes (4ml and 2ml respectively). For the determination of Catechol 1,2 dioxygenase the increase in absorbance was monitored for 60-80 seconds at 260nm. For the determination of Catechol 2,3 dioxygenase the absorbance was monitored at 375nm for 60-80seconds.

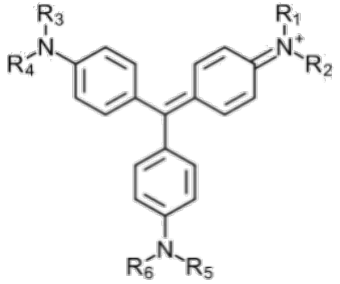
J6) Crystal violet decolourisation Assay

Table J6: Experimental set-up of Crystal violet decolourisation Assay

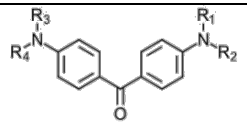
Blank	**Negative Control**	**Test**
1ml YEME	1ml 0.003% CV in YEME	1ml sample (cell free supernatant/filtrate)

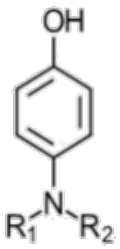
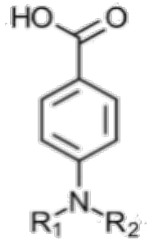
For a fixed reading at 590nm or scan from 450-650nm 2ml plastic cuvettes were used. For the scan across absorbance spectra 200-700nm a 2ml quartz cuvette was used. The decolourisation % of Crystal Violet was determined with C1 (Appendix C).

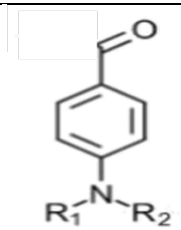
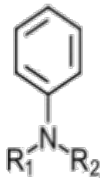
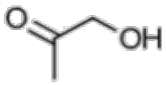
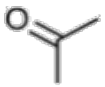
Appendix K: Properties of the reaction intermediates and products of CV decolourisation and degradation

Entry	Name	Synonyms	Abbreviations	Structure		Mass spectrum ions (m/z)
1	*N*,*N*,*N*′,*N*′, *N*″,*N*″-hexamethylpararosaniline	Crystal Violet	CV	$R_{1\text{-}6}$=CH_3	 **Pararosanilines**	372
2	*N*,*N*-dimethyl-,*N*′,*N*′-dimethyl-*N*″-methylpararosaniline		DDMPR	$R_{1\text{-}5}$=CH_3 R_6=H		358
3	*N*,*N*-dimethyl-,*N*′-methyl-*N*″-methylpararosaniline		DMMPR	$R_{1\text{-}3,6}$= CH_3 $R_{4\text{-}5}$=H		344
4	*N*,*N*-dimethyl-,*N*′,*N*′-dimethyl pararosaniline		DDPR	$R_{1\text{-}4}$= CH_3 $R_{5\text{-}6}$=H		344
5	*N*-methyl-,*N*′-methyl-*N*″-methyl pararosaniline		MMMPR	$R_{1,4\text{-}5}$= CH_3		330

				$R_{2\text{-}3,6}$=H		
6	*N,N*-dimethyl-,*N′*-methylpararosaniline		DMPR	$R_{1\text{-}3}$= CH_3 $R_{4\text{-}6}$=H		330
7	*N*-methyl-*N′*-methylpararosaniline		MMPR	$R_{1,4\text{-}6}$ =CH_3 $R_{2\text{-}3}$=H		316
8	*N,N*-dimethylpararosaniline		DPR	$R_{1\text{-}2}$ =CH_3 $R_{3\text{-}6}$=H		316
9	*N*-methylpararosaniline		MPR	R_1= CH_3 $R_{2\text{-}6}$=H		302
10	Pararosaniline		PR	$R_{1\text{-}6}$=H		288
11	4-(*N,N*-dimethylamino)-4'-(*N′,N′*-dimethylamino) benzophenone	Michler's ketone	MK	$R_{1\text{-}4}$=CH_3		269.05

12	4-(*N,N*-dimethylamino)-4'-(*N'*-methylamino) benzophenone		DMBP	R_{1-3} =CH_3 R_4=H	 **Aminobenzophenones**	255
13	4-(*N*-methylamino)-4'-(*N'*-methylamino) benzophenone		MMBP	R_{1-2} =CH_3 R_{3-4}=H		240.92 (240.98)
14	4-(*N,N*-dimethylamino)-4'-aminobenzophenone		DBP	$R_{1,4}$=CH_3 $R_{2,3}$=H		240.98 (240.98)
15	4-(*N*-methylamino)-4'-aminobenzophenone		MBP	R_1=CH_3 R_{2-4}=H		226.84 (226.96)
16	4,4'-Bis-aminobenzophenone		BP	R_{1-4}=H		213.06 (212.86)
17	4-(*N,N*-dimethylamino)phenol		DP	R_{1-2}=CH_3		138.16
18	4-(*N*-methylamino)phenol		MP	R_1=CH_3 R_2=H		124.03

19	4-aminophenol		AP	$R_{1\text{-}2}$=H	 **Aminophenols**	110.14
20	4-dimethylaminobenzoic acid		DBA	$R_{1\text{-}2}$=CH_3	 **Aminobenzoic acids**	184
21	4-methylaminobenzoic acid		MBA	R_1=CH_3 R_2=H		168
22	4-aminobenzoic acid		ABA	$R_{1\text{-}2}$=H		152
23	4-dimethylaminobenzaldehdye		DB	$R_{1\text{-}2}$=CH_3		165
24	4-methylaminobenzaldehdye		MB	R_1=CH_3 R_2=H		151

25	4-aminobenzaldehdye		AB	$R_{1\text{-}2}$=H	 **Aminobenzaldehyde**	137
26	*N,N*-dimethylaminobenzene				 **Aminobenzenes**	121
24	*N*-methylaminobenzene					107
25	Aminobenzene					93
27	1-hydroxy-2-propanone					74
28	Acetic acid					60

Data obtained from various sources (Chen et al., 2008; Chen et al., 2007a; Li et al., 2014) and this study.