Biological Approach to Improving the Evaporation Rates of Mine Wastewater Desalination Brine Treated in Evaporation Ponds.

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WESTERN CAPE

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Being a scientist requires having faith in uncertainty, finding pleasure in mystery, and learning to cultivate doubt. There is no surer way to screw up an experiment than to be certain of its outcome.

−Stuart Firestein, Ignorance: How It Drives Science

Ever tried. Ever failed. No matter. Try again. Fail again. Fail better.

−Samuel Beckett in Failure: Why Science Is So Successful

KEY WORDS

Evaporation rate, evaporation ponds, desalination brine, halophilic bacteria, pigments, carotenoid biosynthesis, chemical mutagenesis, whole genome sequencing.

ABSTRACT

The disposal of brine effluent from inland wastewater desalination plants is a growing global problem with adverse economic and environmental implications because of the substantial cost associated with its disposal and the potential for polluting groundwater resources. Currently, the best and most economical option for brine disposal from inland desalination plants is the use of evaporation ponds, which concentrate the liquid until getting a solid waste that can be valued or directly managed by an authorized company. The effectiveness of these ponds is therefore dependent on the evaporation rate, which has previously been improved by the addition of dyes such as methylene blue. However, the addition of chemical dyes to the evaporation ponds poses a threat to the environment, wildlife, and humans. Several studies have shown that pigmented halophilic bacteria growing in solar saltern ponds increase the evaporation rate of the brine during commercial salt production. Thus, in this study, a biological approach using pigmented halophilic bacteria was assessed for use as an alternative method for increasing the evaporation rates of industrial wastewater brine discarded in an evaporation pond with the focus of investigating the effect of the reflected light spectrum of the pigment on the brine evaporation rate.

A novel orange pigment-producing halophilic bacterial strain, *Planococcus* sp. CP5-4, isolated from a salt crystallizer pond formed the basis of this study. Using chemical mutagenesis and genome sequence comparisons, 146 Kb and 3 Kb sequence deletions from the genomes of the pigment mutants assisted with the identification of a C_{30}-carotenoid biosynthetic pathway and the genes responsible for pigment synthesis by *Planococcus* sp. CP5-4. Carotenoid analysis showed that a glucosylated C_{30}-carotenoid fatty acid ester, methyl 5-(6-$C_{17:3}$)-glucosyl-5, 6'-dihydro-apo-4, 4'-lycopenoate was produced by *Planococcus* sp. CP5-4, while 5-(6-$C_{17:3}$)-glucosyl-5, 6'-dihydro-apo-4, 4'-lycopene was produced by a yellow mutant of *Planococcus* sp. CP5-4.

Inoculations of 20% (v/v) each of the wild type, yellow and unpigmented *Planococcus* sp. CP5-4 strains in 800 mL of mine wastewater desalination brine effluent from the eMalahleni water reclamation plant contained in laboratory-scale evaporation pans resulted in evaporation rate increments of 18.56, 10.31, and 6.19 %, respectively, compared to the control under laboratory conditions when using infrared lamps as a source of heat. Thus, indicating that the reflected light spectrum of the pigment produced by halophilic bacteria influences the rate at which the brine evaporates and that pigmentation is not a prerequisite for halophilic bacteria to affect an increment in the brine evaporation rate, but, a factor for causing even higher evaporation rate increments.

DECLARATION

I declare that **Biological Approach to Improving the Evaporation Rates of Mine Wastewater Desalination Brine Treated in Evaporation Ponds**, is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name: Anesu Conrad Moyo Date: 11/03/2021

Signed: 

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DEDICATION

To my mothers (I have five), who have been a source of inspiration, and gave me strength when I thought of quitting, who continually provide their moral, spiritual, and emotional support.

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LIST OF ABBREVIATION

-ve	Negative
°C	Degrees Celsius
+ve	Positive
AAI	Average amino acid identity
ANI	Average nucleotide identity
ANOVA	Analysis of variance
AOC	Assimilable organic carbon
ARDRA	Amplified ribosomal DNA restriction analysis
a_w	Water activity
B2G	Blast2Go
BHR	Broad host range
BHT	Butylhydroxytoluene
bp	Base pairs
C_{10}	Ten carbon
C_{20}	Twenty carbon
C_{30}	Thirty carbon
C_{40}	Forty carbon
C_{50}	Fifty carbon
CDS	Coding sequence(s)
CFU/mL	Colony forming units per milliliter
cm	Centimeter(s)
cm/h	Centimeter(s) per hour
cm^2	Square centimeter(s)
cm^3/h	Cubic centimeter(s) per hour
CNV	Copy number variants

COD	Chemical oxygen demand
Conc	Concentration
dH_2O	Distilled water
dNTPS	Deoxynucleoside triphosphates
DSBs	Double strand breaks
DTT	Dithriothreitol
DWAF	Department of water affairs
EC	Electrical conductivity
EC	Evidence code(s)
EWRP	eMalahleni water reclamation plant
FA	Fatty acid
FPP	Farnesyl pyrophosphate
FSS	First solvation shell
g L^{-1}	Gram(s) per liter
gi	Gene identifier(s)
GO	Gene ontology
InCQ	Incompatibility group Q
IR	Infrared
IS	Insertion sequence(s)
IVR	Internal vibrational redistribution
Kb	Kilo base pairs
kV	Kilovolts
kW/m^2	Kilo Watt(s) per square meter
L	Liter
LSD	Least significant difference
M	molar

m^{-1}	per meter
MANOVA	Multivariate analysis of variance
mg/L	Milligram(s) per liter
MIC	Minimal inhibitory concentration
Ml	Mega liter
mL	milliliter(s)
mM	Milli Molar
MMS	Methyl methanesulfonate
$mol.Kg^{-1}$	Mol(s) per kilogram
MS	Mass spectrometry
ms	Micro second
mS/cm	millisiemens per centimeter
NER	Nucleotide excision repair
NGS	Next generation sequencing
nm	nanometer
OD_{660nm}	Optical density at 660 nanometers
ORF	Open reading frame
PCR	Polymerase chain reaction
PLC	Preparative thin layer chromatography
PP	Pyrophosphate
ppm	Part(s) per million
PSU	Practical salinity unit
PVC	Polyvinylchloride
RH	Relative humidity
RO	Reverse osmosis
rpm	Revolutions per minute

rRNA	Ribosomal RNA
sdH$_2$O	Sterile distilled water
SNP	Single nucleotide polymorphism
SNT	Supernatant
STDEV	Standard deviation
SV	Structural variation(s)
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TOC	Total organic carbon
t_R	Retention time
TSB	Tryptic soy broth
TVC	Total viable count(s)
U	Enzyme unit(s)
UN	Unpigmented
UPLC-qTOF-MSE	Ultra-performance liquid chromatography coupled with an electrospray ionization quadrupole time-of-flight mass spectrometry operating in MS^E mode
UV/Vis	Ultraviolet/visible
V	Volt(s)
v/v	Volume per volume
VC	Vibrational cooling
W/m^2	Watt(s) per square meter
w/v	Weight per volume
WGS	Whole genome sequencing
WT	Wild type
YE	Yellow
λ_{max}	Absorption maximum

µF	Microfarad
µM	Micro molar
µm	Micrometer(s)
τ_{SC}	Shell cooling
τ_{SS}	Solute to solvent
ϒ	Gamma
Ω	Ohm(s)

Chapter 1

Introduction

1.1 Background

Because of the rising demands and diminishing freshwater supplies in most world regions, unconventional water resources historically thought to be a lost cause such as mine wastewater are being used as viable sources of potable water to augment rainfall and river runoff in an attempt to meet human demands. The eMalahleni water reclamation plant (EWRP), established as a joint initiative between Anglo Coal South Africa and BHP Billiton Coal Energy South Africa, treats polluted mine water from underground workings to deliver potable and/or process water to the eMalahleni municipality and mining operations (Kabuzire *et al.*, 2010). Feed water is received by the EWRP from the Greenside, Kleinkopje, and South Witbank collieries in the Witbank area as shown in Figure 1.1.

About 25 Ml/day of potable water is produced by the EWRP from the mine feed water using a Hi recovery Precipitating Reverse Osmosis (HiPRO) process technology developed by Keyplan (Randall, Nathoo and Lewis, 2011). The process has recovery rates above 99 % and generates a concentrated brine effluent with salinities rangning from 1.62 g/L – 4.53 g/L that has to be managed. An estimated 0.2 Ml of reject brine is produced per day while processing 25 Ml of mine feed water (Sekar *et al.*, 2014).

The reject brine is collected from the third stage of the reverse osmosis process and disposed of in an evaporation pond where it is concentrated into a solid (in the form of gypsum) that maybe landfilled *in situ* or collected and disposed of elsewhere (Kabuzire *et al.*, 2010).

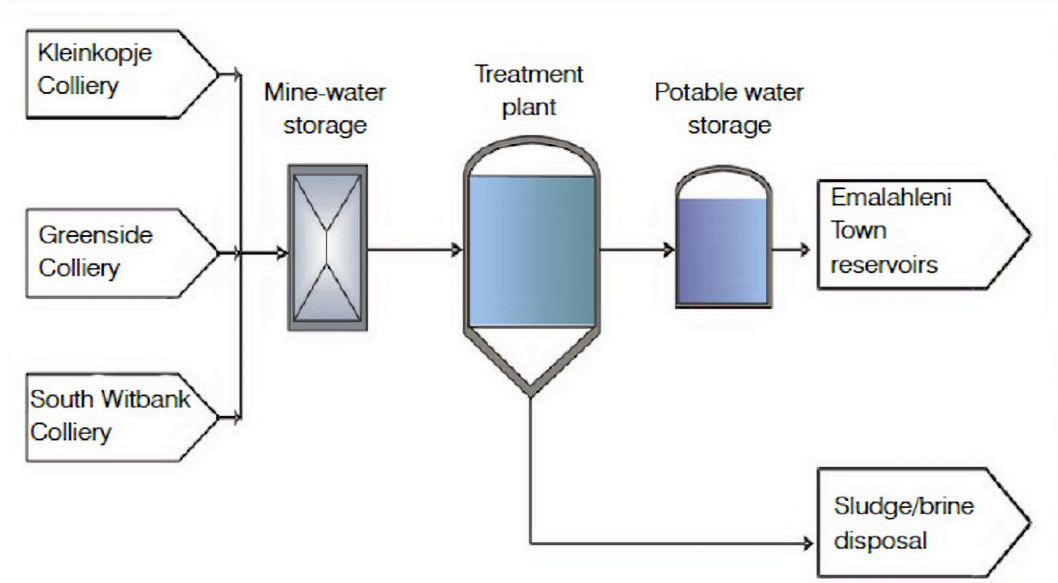

Figure 1.1 Feed water processing at the eMalahleni water reclamation plant (Gunther and Mey, 2006).

One problem with the utilization of evaporation ponds for reject brine disposal is that securing effective evaporation rates is challenging and requires awareness of how evaporation can be enhanced. Several studies have investigated methods to enhance evaporation rates of sodium chloride based brines (Bloch, Farkas and Spiegler, 1951; Kingdon, 1963; Winans, 1967; Barthakur and Arnold, 1995). Kingdon, (1963), explored the concept of weakening the hydrogen bonds of water by attaching foreign gas molecules to the surface of the water. The author suggested that this would increase the evaporation rate. The gases examined included helium, nitrogen, butane, and oxygen (Kingdon, 1963). Optimal results were achieved using butane. However, the use of large volumes of butane to enhance the evaporation rate in EWRP ponds is not feasible considering the cost and the potential environmental impacts associated with this method. Other researchers looked at the possibility of adding dyes to maximize the absorption of solar energy (Bloch, Farkas and Spiegler, 1951; Winans, 1967). A coloured solution absorbs more solar energy than an uncoloured solution resulting in an increase in the temperature of the solution. This lowers the surface tension of the water leading to a higher saturation vapor pressure, and a subsequent increase in the evaporation rate (Ahmed *et al.*, 2002).

However, the application of such dyes for increasing brine evaporation rates in evaporation ponds posses a threat to the environment, wildlife and humans because of their non-biodegradable and carcinogenic nature (Hassaan and Nemr, 2017). Halophilic microorganisms that grow in saltern evaporation ponds may offer a free and environmentally friendly option for enhancing desalination brine effluent evaporation rates. Not only are these microorganisms able to tolerate high salinities, but their pigments are also responsible for the orange to red colours seen in solar saltern ponds and for the absorption of solar radiation (Ma *et al.*, 2010; Menon *et al.*, 2019). The halophilic microorganisms' pigments transfer the absorbed solar radiation to the surrounding liquid or brine, which is thought to cause an increase in brine temperature and evaporation rate in the saltern ponds during salt production (Menon *et al.*, 2019). Since this study investigated pigmented halophilic bacteria, the discussions that follow will focus on bacterial pigments.

According to Balevičius *et al.* (2019), following the absorption of radiant energy by the pigment, the photo-excitation energy is dissipated by the process of internal vibrational redistribution (IVR) in which a small subset of optically excited pigment molecules relax following their initial excitation. Subsequently, energy is exchanged between the molecules and the bulk solution in a process called vibrational cooling (VC), leading to an increase in the temperature of the bulk solvent (the water fraction of the brine), and enhanced evaporation. As can be seen in Figure 1.2, this exchange can be interpreted as energy transfer from the pigment to the first solvation shell (FSS), and heat dissipation from the FSS to the bulk solvent. The latter processes constitute VC and are characterized by time scales τ_{SS} ("solute-to-solvent") and τ_{SC} ("shell cooling"), respectively (Balevičius *et al.*, 2019).

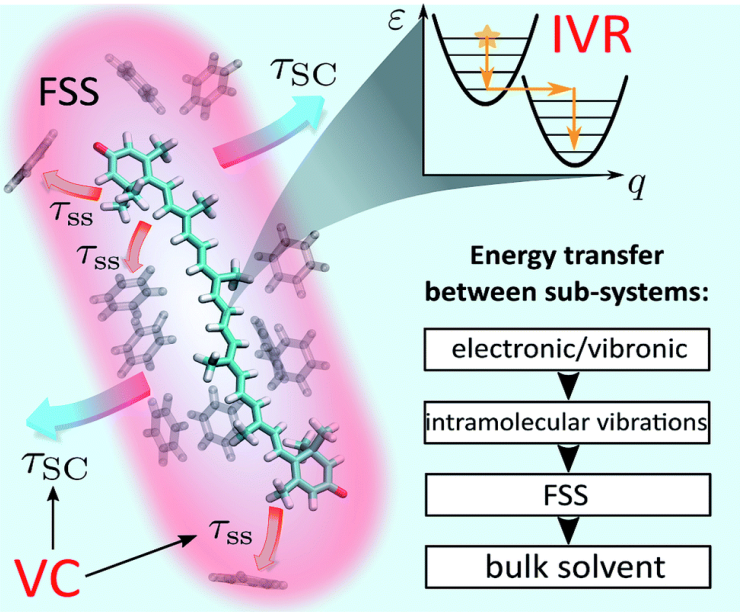

Figure 1.2 Schematic representation of heat transfer from halophilic bacterial pigment molecule to saltern brine solution (Balevičius *et al.*, 2019).

Thus in this thesis, a biological approach whereby pigmented halophilic bacteria are used to increase the brine evaporation rate in an evaporation pond set up was assessed. The assessment was done at a laboratory scale using 1 L volumes of the brine inoculated with pigmented halophilic bacteria, and the inoculated brine evaporated under infrared lamps. As such, the aims of the study were:

1. To determine the effect of reflected light spectrum (colour) of pigments produced by halophilic bacteria isolated from a salt crystalizer pond and from an industrial waste water reverse osmosis evaporation pond on the evaporation rate of industrial desalination brine effluent from the eMalahleni water reclamation plant, and
2. To identify and characterize the pigment(s) produced by the pigmented halophilic bacterial strain(s) affecting the highest increment in brine evaporation rate as well as
3. To characterize the pigment biosythetic pathway(s) of the halophilic bacterial strain(s) affecting the highest brine evaporation rates.

1.2 Scope

This study focuses on improving the evaporation rate of reverse osmosis desalination brine effluent disposed of in evaporation ponds. The use of evaporation ponds for desalination brine effluent disposal is effective and economical because of their ease of construction, low maintenance, and minor operator attention. However, the efficiency with which these ponds dry out the brine is limited by ineffective evaporation. Securing effective evaporation in these ponds is a challenge and requires an awareness of how RO brine evaporation can be enhanced in a cost-effective and environmentally friendly manner.

The biological approach for improving desalination brine evaporation rates described herein is intended to provide an environmentally friendly and economic solution to increasing industrial reverse osmosis brine evaporation rates in evaporation ponds. The amount by which the brine evaporation rate was increased under the controlled laboratory conditions was profoundly influenced by the reflected light spectrum of the pigment produced by the moderately halophilic bacterial strains used to inoculate the brine.

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Chapter 2

Literature Review

2.1 Desalination brine

Desalination brine, hereinafter referred to as brine effluent and/or brine, is the largest source of wastewater from a desalination process. According to El-naas (2015), brine effluent is concentrated saline water in the last stage of the desalination process that is discharged as wastewater. Not only is the brine effluent extremely saline, but it also contains residual chemicals introduced during the desalination operations. As a result the brine effluent may contain different concentrations of the following major process chemicals:

i. Sodium hypochlorite used for chlorination to prevent bacterial growth in the desalination facility;

ii. Chemical flocculants such as ferric chloride or aluminum chloride, for the removal of suspended matter from the water;

iii. Anti-scale additives such as sodium hexametaphosphate used to prevent scale formation on the pipes and on the membranes; and

iv. Ionized products of strong acids such as sulfuric acid or hydrochloric acid that adjust the pH of the brine.

It is necessary, therefore, to effectively dispose of the brine in a manner ensuring the reduction or elimination of the potentially harmful impacts of the brine to the environment. Common disposal options for brine are surface water discharge, deep-well injection, land application, and evaporation ponds (Subramani and Jacangelo, 2014). However, for many inland desalination plants located far from shorelines the option to dispose of brine, as surface water discharge into the sea does not exist. In such instances, the use of evaporation ponds is especially suitable for brine disposal from both an economic and environmental standpoint.

Compared to deep-well injection and land application, evaporation ponds are the least costly means of brine disposal, especially in areas with low land costs (Mickley *et al.*, 1993). However, securing effective evaporation rates when using evaporation ponds for brine disposal is a challenge and requires awareness of how evaporation can be enhanced in an economical and environmentally friendly manner.

In this chapter, the literature on the characteristics of reverse osmosis (RO) desalination brine, the environmental impacts of desalination brine, brine management and the various brine disposal options available for inland desalination plants will be discussed. Furthermore, for the contextualization of brine evaporation rate and its importance for effective brine disposal in evaporation ponds, the literature on the use of evaporation ponds for brine disposal, the pros, and cons of evaporation ponds, and the factors affecting brine evaporation rates will be reviewed. Also reviewed will be literature on the ecology of desalination brine evaporation ponds, halophilic microorganisms, their classification, diversity, and adaptation mechanisms to high salinity and exposure to ultra-violet radiation in brine evaporation ponds. In conclusion, a review of the literature on bacterial pigments in context to halophilic bacteria and their effects on solar saltern brine and industrial RO brine evaporation will be presented.

2.2 Desalination brine characteristics

Reverse osmosis desalination brine characteristics are dependent on several factors, which include feed water quality, pre-treatment method, and the cleaning procedures and chemicals used during desalination. Several studies on RO desalination concur that not only do the brine effluents differ in the concentration of chemicals used in the desalination operations, but also in the concentration of organic and inorganic matter. The characteristics of brine generated from different feed water sources are summarized in Table 2.1 (modified from Pramanik, Shu and Jegatheesan, (2017)), and below are a number of examples detailing some of the characteristics.

Ersever *et al.,* (2007), found that brine effluent from a water reclamation plant in California had detectable levels of copper, manganese, mercury and selenium. They also found alkaline organic and inorganic salts that included calcium carbonate ($CaCO_3$; 500 – 1490 mg/L), ammonia (NH_3; 62 – 98 mg/L of nitrogen), chloride (800 – 1000 mg/L), and sulfate (1000 – 1480 mg/L) in the brine effluent.

In another study, brine effluent from the RO treatment of livestock wastewater was found to contain NH_3, humic substances, nitrate, phosphate and potassium (Yoon *et al.*, 2004). The phosphate concentration in the effluent was as high as 40 mg/L whereas in the feed water the phosphate concentration was 5 mg/L (Kumar, Badruzzman and Oppenheimer, 2007). In yet another study, Gomes *et al.*, (2007), found high levels of chemical oxygen demand (COD; up to 15 285 mg/L) in brine effluents from the RO treatment of textile finishing plant wastewater. While Subramani *et al.*, (2011), noted high concentrations of silica (>250 mg/L) and total organic carbon (TOC; >60 mg/L) in RO brine from the treatment of water produced during oil and gas production.

In a study on the use of activated carbon as a pretreatment process for RO brine treatment and recovery, Lee *et al.*, (2009), noted that the water quality of RO brine effluent from industrial sites differed from that of municipal brine effluent. In addition to metals, brine effluents from mine contaminated groundwater treatment sites had high concentrations of calcium (>1000 mg/L), silica (>200 mg/L), and sulfate (>4500 mg/L); while RO brine effluents from municipal wastewater treatment contained high concentrations of TOC in addition to the presence of sparingly soluble salts. Apart from the water quality, another characteristic of brine is high electrical conductivity (EC), which is as a result of high salt content (Lee, Ng, Ong, Tao, *et al.*, 2009). Randall, Nathoo and Lewis, (2011), noted that the conductivity level of brine from the mining industry was approximately equal to the EC levels of RO brine from desalination plants (22 000 mS/cm). The EC of brine from a municipal wastewater was reported to be as high as 23 mS/cm by Umar, Roddick and Fan, (2013).

Table 2.1 Characteristics of brine effluents from different feed water sources

	Composition												
Source	**EC mS/cm**	**TDS mg/L**	**pH**	**TOC mg/L**	**COD mg/L**	**DOC mg/L**	**Mg^{2+} mg/L**	**Na^{+} mg/L**	**Cl^{-} mg/L**	**SO_4^{2-} mg/L**	**K mg/L**	**Ca^{2+} mg/L**	**Ref.**
Sea water	15	19 200	-	50 - 70		-	320	7 200	-	-	-	480	Tunc and Groth, 2011
	85.20	79 660	-	-	-	-	1 290	10 800	19 400	2 708	392	411	Quist-Jensen, Macedonio and Drioli, 2016
	60.5	-	-	-	-	-	1 830	14 600	-	4 824	668	625	Melián-Martel, Sadhwani Alonso and Pérez Báez, 2013
	21 400	16 142	-	-	-	-	448	4 250	8 118	2 466	145	665	Ahmed *et al.*, 2001
Reclamation plant	-	39 65	-	40	-	-	-	-	900	1 240	-	-	Ahmed *et al.*, 2001
	1.70	1 129	-	18	66	-	7	203	256	217	62	65	Ersever, Ravindran and Pirbazari, 2007

Table 2.1 (Continued)

Source	Composition EC mS/cm	TDS mg/L	pH	TOC mg/L	COD mg/L	DOC mg/L	Mg^{2+} mg/L	Na^{+} mg/L	Cl^{-} mg/L	SO_4^{2-} mg/L	K mg/L	Ca^{2+} mg/L	Ref.
Brackish water	-	-	-	-	-	-	386	5 130	8 960	1 920	-	819	Badruzzaman *et al.*, 2009
	13.5		-	-	-	-	245	2 084	4 068	2 160	79	540	Korngold, Aronov and Daltrophe, 2009
	-	7 500	7.0	-	-	-	318	991	2 823	1 553		1 032	Macedonio *et al.*, 2011
	-	17 500	8.0	-	-	-	386	5 130	8 960	-	-	819	Martinetti, Childress and Cath, 2009
Coal seam gas	85.6	62 450	8.0	-	-	-	2 421	19 500	34 061	4 768	713	725	Martinetti, Childress and Cath, 2009
Synthetic wastewater													
	21.8	14 100	8.2			-	17	6 840	5 520	-	32	14	Duong *et al.*, 2015
	4.05		7.54	-	-	-	27	600	701	443	185	381	Tran *et al.*, 2012

2.3 Environmental impacts of desalination brine effluent

Although desalination potentially provides clean water, the brine effluent from desalination is a critical environmental issue. In light of this fact, the South African Department of Water Affairs (DWAF) rated desalination brine effluent as the second most harmful waste to be discarded into the environment in South Africa because of the high concentrations of chemical additives used in the pretreatment of the feed water (Kabuzire *et al.*, 2010).

Numerous studies have evaluated the environmental impact of brine disposal on soil, groundwater, and the marine environments. These studies showed that the discharge of brine from inland desalination plants could have potentially detrimental impacts on both the physicochemical and ecological attributes on the receiving environments, (Rao *et al.*, 1990; Areiqat and Mohamed, 2005; Al-Faifi *et al.*, 2010), which will be discussed in the following sections.

2.3.1 Impact of brine on soil and ground water

The improper disposal of brine effluent by inland desalination plants has the potential to pollute ground water resources, causing detrimental effects to subsurface soil properties. Many inland desalination plants dispose of their brine effluent in ponds, some of which are unlined. The disposal of brine effluent into unlined ponds increases the risk of saline brackish water intrusion into freshwater sources (El-naas, 2015). Lined ponds also have the risk of the lining being worn out or damaged after long periods of time thus resulting in the percolation of brine effluent into the ground water systems. A case study in India indicated that brine seepage into an underground aquifer caused an increase in the hardness of the ground water (Rao *et al.*, 1990).

Soil structure may also deteriorate due to the high salinity of the reject brine, when calcium ions are replaced by sodium ions in the exchangeable ion complex (Al-Faifi *et al.*, 2010). Replacement of Ca^{2+} ions by Na^{+} ions in soil results in the reduction of the infiltration rate of water and soil aeration (El-naas, 2015).

Although sodium does not reduce the intake of water by plants, it does change the soil structure and impairs the infiltration of water hence affecting plant growth (Areiqat and Mohamed, 2005). In addition, the elevated levels of sodium, chloride and boron associated with brine effluent can reduce plant productivity and increase the risk of soil salinization (El-naas, 2015). Moreover, heavy metals and inorganic compounds that build up in the soil and groundwater sources may cause long-term health problems (Mohamed, Maraqa and Handhaly, 2005).

2.3.2 Impact of brine on the marine environment

Because of the various concentrations of chemicals used in the pre- and post-cleaning operations of a desalination process and the salinity of the brine effluent, the potential damages of reject brine to the marine environment include eutrophication, pH fluctuations, increased salinity, proliferation of heavy metals, and sterilization of some marine species due to the characteristics of the disinfectants used in desalination (Giwa *et al.*, 2017). In recent years there has been worry in the Mediterranean countries over *Posidonia oceanica,* the most abundant sea grass species in the Mediterranean, considered a very important ecosystem (Cebrian and Duarte, 2001). In the last decade the 40 000 km^2 meadows of *P. oceanica* have undergone regression in several coastal areas due to brine discharge from desalination plants (Gacia *et al.*, 2007).

2.4 Brine management and disposal options for inland desalination plants

Due to the large amounts of brine effluent produced worldwide (51.7 billion m^3/year) (Jones *et al.*, 2019) and the potentially harmful environmental impacts posed by uncontrolled brine disposal, there is a need for the environmentally friendly and economical management of RO brine to ensure efficient disposal and/or reuse. Different management options are available at several scales to overcome the environmental problems related to RO brine discharge. Adoption of a particular brine management and/or disposal option is dependent on the location of the desalination plant as well as on the type of recovery process used. Critical factors such as the quantity and quality and composition of the brine, the physical and geographical location of the discharge point of the brine, public acceptance, capital and operating costs and the legalities of the discharge option also influence

the selection of a suitable method for a particular brine stream (Masnoon and Glucina, 2011; El-naas, 2015). The brine management and disposal options available for inland desalination plants consist of brine volume minimization, direct reuse, and direct disposal (Giwa *et al.*, 2017).

2.4.1 Direct disposal

Although direct disposal into open water bodies such as the ocean is a common way to dispose of RO brine, it is typically not cost-effective to transport the concentrate from inland regions to an ocean for disposal. In addition, RO brine effluent contains significant amounts of environmentally harmful substances that cannot be released into inland surface waters or onto soil surfaces except under special circumstances. Moreover, the disposal of RO brine effluent into local municipal sewage systems, as is practiced by some small desalination plants around the world, increases the total dissolved solids of the treated water thus making it unsuitable for irrigation. Also, large-scale desalination facilities do not have the option of disposing their brine effluent into municipality sewers because the large volume of waste brine could overwhelm the system (Petrik *et al.*, 2015).

An alternative option for brine disposal from inland desalination plants where surface water discharge is not viable or is very costly is deep well injection. In this method RO brine is injected into a number of disposal wells which channel the effluent into an acceptable, confined, deep underground aquifer that is not used for drinking water (Masnoon and Glucina, 2011). The system is designed to isolate the brine effluent from potential potable water aquifers and prevent migration of contaminants to the potable water.

Injection wells are; however, not ideal for areas subject to earthquakes or where faults that can provide a direct hydraulic connection between the receiving aquifer and an overlying potable water aquifer are present (Mickley and Associates, 2006). Therefore, prior to drilling any injection well, careful assessment of geological conditions must be conducted to determine the depth and location of suitable porous aquifer reservoirs (El-naas, 2011, 2015).

While deep well injection is a feasible option for RO brine disposal, the system still suffers from many drawbacks such as the need for selecting a suitable well site, the extra cost involved in conditioning the reject brine, corrosion and subsequent leakage of the well casing and seismic activity that could cause damage to the well and lead to ground water contamination (El-naas, 2011).

2.4.2 Direct reuse

Land application is a direct reuse method for RO brine in which the effluent is used for landscape and irrigation purposes. Under this method, the RO brine is used in spray irrigation, rapid infiltration, percolation ponds, and overflow applications. Amongst the land applications for RO brine, spray irrigation is the most common. However, spray irrigation is only applicable when the resulting irrigation operation does not harm crops or groundwater (El-naas, 2015; Petrik *et al.*, 2015). As a consequence, reject brine is used for the irrigation of salt tolerant grasses and vegetation in places such as parks or golf courses (Masnoon and Glucina, 2011). It is important to consider the long-term impacts of the increasing salinization of the soils due to the irrigation operations.

2.4.3 Brine minimization

According to Giwa *et al.,* (2017), brine volume minimization is accomplished through high water recovery from a desalination process through brine treatment. Water is recovered from the brine effluent through the use of pressure-driven and electrical potential-driven membranes, and through the use of thermal based technologies. Amongst these, the thermal based technologies are the most suitable and efficient for minimizing the volume of high salinity brines such as those produced after coalmine wastewater desalination. Thermal based technologies use either natural or mechanical evaporation to produce a solid end product from the brine effluent. This is normally in the form of precipitated salts and/or mineral slurry that is either disposed of in a landfill or sold if there are market possibilities. An example of a thermal based brine minimization technology is an evaporation pond.

2.5 Evaporation ponds

The use of evaporation ponds is a direct disposal method suitable for highly saline brine effluents from inland desalination plants. Evaporation ponds are shallow basins that expose their contents to the environment, reducing liquid volume by means of evaporation. Depending on the brine composition and other regulatory criteria, evaporation ponds may be lined with impervious PVC or hypalon liners or constructed on natural geologic confining layers to prevent infiltration of the saline effluent to underground aquifers (El-naas, 2011, 2015). Evaporation ponds are most effective in regions with low rainfall, and where climatic conditions are favorable for steady and relatively rapid evaporation rates (Ahmed *et al.*, 2000). The viability of this brine disposal option depends primarily on the availability of inexpensive land for the construction of the evaporation ponds. Advantages of evaporation ponds for desalination brine disposal over other disposal options include the ease of construction of the ponds, the low maintenance costs and the need for very little input from operators (Ahmed *et al.*, 2000; Mickley and Associates, 2006). No mechanical equipment other than the pump that conveys the wastewater to the pond is required. Furthermore, evaporation ponds are the least costly means of brine disposal, especially in areas with high evaporation rates and low land costs (Mickley and Associates, 2006). The need for large tracts of land is a concern in areas where the evaporation rate is low or when the disposal rate is high. There is also the potential of contaminating underlying water aquifers through seepage as a result of poorly constructed ponds (Ahmed *et al.*, 2000). Even though evaporation ponds are a simple and straightforward method for brine disposal, this option is limited for use in small desalination plants (producing less than 5 Ml/day) and generally restricted to arid climatic conditions (Ahmed *et al.*, 2000).

2.5.1 Evaporation rate and the factors that affect it

The fundamentals of water evaporation are widely evident in many aspects of nature and industrial processes; it is therefore necessary to have a good understanding of evaporation rate and the factors that affect it. Evaporation rate is the amount of water evaporated per unit surface area per unit time.

It can be expressed as the mass or volume of liquid water evaporated per unit area which equates to a depth (in mm or m) of liquid water evaporated per unit time (Petrik *et al.*, 2015). For water molecules to evaporate, they must be located near the surface, be moving in the proper direction, and have efficient kinetic energy to overcome the liquid-phase intermolecular forces holding them in place (Zheng, 2015). Consequently, the number of water molecules fulfilling these conditions determines the evaporation rate. Thus, the fewer the water molecules that meet these criteria, the lower the evaporation rate, and vice-versa (Zheng, 2015). Additionally, salinity, the meteorological conditions such as temperature, humidity, and the wind speed to which the brine is exposed, and the surface area from which the brine is evaporating from also determine the evaporation rate (Dama-Fakir and Toerien, 2009). However, salinity is the most important parameter amongst those affecting evaporation because salinity also changes how water responds to the prevailing meteorological conditions to which it is exposed (Oroud, 1995). The effects of salinity and meteorological conditions on RO brine evaporation, and how the brine responds to these meteorological conditions are discussed in the successive subsections.

2.5.1.1 Effects of salinity on evaporation rate

Salinity affects the evaporation rate of water negatively. According to Al-Shammiri (2002), an increase in salinity raises the boiling point of water, which in turn reduces its vapor pressure and evaporation rate. Vapor pressure is the pressure above a liquid resulting from the evaporation of that liquid (Meranda and Furter, 1977). Since water vaporizes when the vapor pressure is as high as or equal to that of the atmosphere, saline water evaporates at a slower rate than freshwater because it requires a larger amount of heat energy to get the vapor pressure equal to the atmospheric pressure (Meranda and Furter, 1977). Not only does salinity lower the vapor pressure, but it also lowers the latent heat of vaporization of the water, which is the amount of heat energy required to turn 1 g of a liquid into water vapor, without a rise in the temperature of the water (Abdelrady, 2013). Lowering the latent heat of vaporization also causes the saline

water to vaporize at high temperatures and to evaporate at a slower rate than that of freshwater.

2.5.1.2 Temperature

Temperature is the primary driver of the evaporation process. An increase in the surface temperature of the brine causes the kinetic energy of the water molecules to increase, which results in the water molecules moving faster. The faster the water molecules move, the more easily they can overcome the liquid-phase intermolecular forces holding them in place and escape from the water surface. While an increase in the temperature of saline water tends to increase the evaporation, brine is inefficient in converting radiant energy into latent heat because of the loss of a large percentage of the absorbed radiation through back-radiation and sensible heat exchange to the atmosphere (Oroud, 1997). Thus, with the same input of energy, the evaporation rate of brine is lower than that of freshwater (Mickley *et al.*, 1993).

2.5.1.3 Relative humidity

The effects of relative humidity (RH) on saline water evaporation have been known for some time. Early work on the effects of RH on saline water evaporation was conducted by Moore and Runkles, (1968) who found that the rate of saline water evaporation decreased as the RH increased, especially at high air temperatures, which contradicted the notion that warm air holds more water than cold air (Moore and Runkles, 1968). Additionally, Leaney and Christen, (2000) stated that dissolved salt composition influenced the level of RH at which the evaporation of saline water stopped. In their study, Leaney and Christen, (2000), found that water saturated with sodium chloride did not evaporate at RH above 70%, while water saturated with magnesium chloride stopped to evaporate at a lower RH percentage.

2.5.1.4 Solar radiation

The sun emits energy as many forms of electromagnetic radiation in varying quantities. About 43% of the emitted energy lies in the visible parts of the electromagnetic spectrum, while the bulk of the remaining energy lies in the near-infrared (49%) and ultraviolet region (7%) (Kushnir, 2000). The remaining 1% of emitted solar radiation is made up of x-rays, gamma waves, and radio waves (Kushnir, 2000). The sun's energy is transferred through space to the earth's atmosphere as radiation, which reaches the outer atmosphere at around 1 368 W/m^2 (Menon *et al.*, 2019). This amount is, however, reduced because most of the high-energy wavelengths that comprise the solar radiation are absorbed, reflected or scattered back to space. As seen from Figure 2.1 the amount of radiation reaching the earth's surface, and subsequently the water surface is low. Regardless, the amount of solar radiation that makes it through the atmosphere is more than adequate to drive evaporation from water bodies such as evaporation ponds.

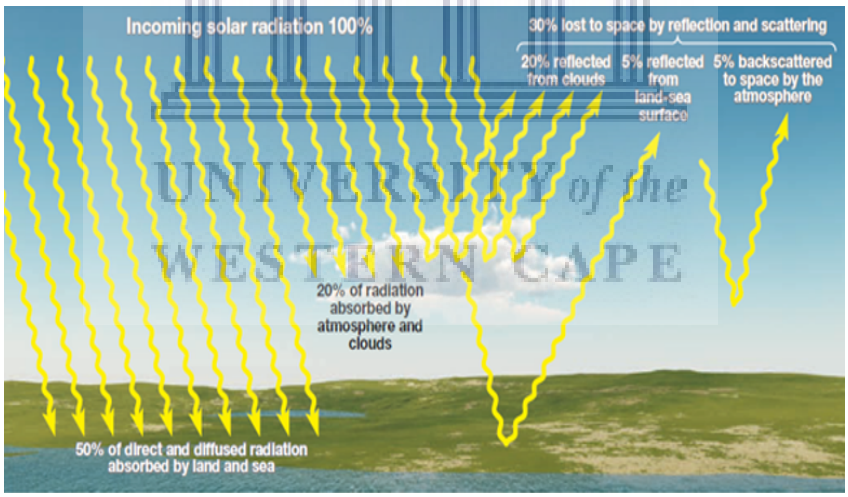

Figure 2.1 Average distribution of incoming solar radiation (Tarbuck *et al.*, 2005).

While the amount of solar radiation reaching the surface of evaporation ponds is more than sufficient to drive the evaporation process, its efficient absorption and attenuation for the evaporation of the saline water is limited by the low absorption coefficient of the water, which is – 0.00027 m^{-1}/practical salinity unit (PSU) at

near-infrared wavelengths, specifically at 715 nm (that of freshwater is 0.01 m^{-1}) (Pegau, Gray and Zaneveld, 1997; Menon *et al.*, 2019). Thus, a small fraction of the incident radiation is absorbed to cause volumetric heating of the bulk brine, which results in a lower temperature at the water surface, compared to a case where all the radiation is absorbed in the surface. As a result, the transient thermal response of the evaporation ponds is slow, which when coupled with the diurnal variation of solar radiation, results in low evaporation rates (Menon *et al.*, 2019).

2.5.1.1 Wind speed

The speed of the wind is another important factor that influences the evaporation rate. According to Yu, (2007) high wind speeds affect evaporation rate directly by carrying water vapor away from the water's surface to allow the re-establishment of the air-water humidity gradient at a faster rate, thus increasing evaporation. High wind speeds also affect evaporation indirectly by enhancing the wind-driven circular water currents at the basin of the pond which, in turn, drives more heat transport by boundary currents, raises the water surface temperature, and speeds up the evaporation rate (Yu, 2007; Fu, Charles and Yu, 2009).

2.5.2 Use of dyes for improving brine evaporation rates

As part of efforts to improving the brine evaporation rates in evaporation ponds under natural prevailing meteorological conditions, a number of studies looked at the possibility of using dyes to maximize the absorption of radiant energy by the brine (Bloch, Farkas and Spiegler, 1951; Keyes and Gunaji, 1966; Rajvanshi, 1981; Patel, Meena and Inkia, 2013). These studies theorized that the addition of dyes would increase the absorption of radiant energy by the brine, thus, increasing the brine temperature and subsequently the brine evaporation rate.

In their study, Keyes and Gunaji (1996) found that the addition of methylene blue dye increased the solar evaporation of the brine while the addition of congo red had little or no effect on brine evaporation.

Suffice to say, the colour of the dye played a vital role in the absorption of radiant energy with methylene blue mediating the absorption of more radiant energy than the congo red dye. Similarly, Patel *et al.* (2010), found that different coloured dyes (black, blue, and red) affected evaporation differently. Their results showed that the addition of a black dye to a single slope active solar still coupled to an evacuated glass tube solar collector increased distillate output by 30.4 % to which they concluded that black and blue dyes were most efficient at increasing evaporation rate.

In yet another study on the use of dyes to increase evaporation rates Rajvanshi (1981), observed that the addition of black naphthylamine at a concentration of 172.5 ppm increased the distillate output of a solar still compared to the addition of red carmosine and dark green dyes to the same final concentration. Despite these findings, the use of chemical dyes to enhance brine evaporation in brine ponds posses a threat to both the environment and human health because of their non-biodegradable and potential carcinogenic nature (Hassaan and Nemr, 2017). An alternative to increasing solar absorption by the desalination brine is to use pigmented halophilic microorganisms, which have long been known to inhabit saline environments like brine ponds.

2.6 Microbial ecology of man made saline environments

2.6.1 Microbial ecology of solar salterns

Multi-pond solar salterns provide a range of environments with different salinities, from that of seawater up to sodium chloride saturation and sometimes even higher. According to Pedrós-Alió (2004), these two extremes provide one of the most common habitats in the world (seawater) and one of the most extreme habitats in the world (calcium, and magnesium chloride saturated brines). From a microbial ecology perspective, the range of salinities existing in multi-pond solar salterns enables the selective elimination of large organisms and the proliferation of microbial communities in the highest salinities.

According to Larsen (1980), as the salinity gradient increases, the relative proportions of the salt components in solution also changes, which causes the number of species able to develop and the biological productivity of the ponds to decrease rapidly. As a consequence, the cyanobacteria (*Spirulina*, *Oscillatoria*, *Coccochloris*, *Lyngbya*, and others), the green alga *Dunaliella viridis*, and the brine ciliate, *Fabrea salina* are hindered from growing.

At the higher salt concentrations a considerable population (appromimately 10^8/mL) of bacteria develop, notably members of the genera *Halobacterium* and *Halococcus* which contribute to the red colouration of these brines (Davis, 1974; Larsen, 1980; Oren, Stambler and Dubinsky, 1992a). Also, *Dunaliella salina* may be present in the concentrated brines in such numbers that it contributes to the red colour, but the alga appears to be considerably hampered in its metabolism in concentrated salt solution (Larsen, 1980; Vo and Tran, 2014). Recent metagenomic studies have provided more detailed assessment of the microbial diversity and metabolic activities of microorganisms in solar salterns. Metagenomics studies on the Santa Pola saltern of two intermediate-salinity ponds with 13% and 19% NaCl, and two crystallizer ponds with 33% and 37% NaCl revealed the phyla *Euryarchaeota* and *Bacteroietes* to be present in the four ponds, with *Euryarchaeota* dominating as salinity increased (Ghai *et al.*, 2011; Ventosa *et al.*, 2015). Members of the class *Gammaproteobacteria* were abundant in the 13% and 19% salinity ponds. The microbial diversity in these intermediate salinity ponds was found to be large, containing representatives from 7 to 9 different taxa (Ghai *et al.*, 2011; Fernández *et al.*, 2014; Ventosa *et al.*, 2015). These studies also affirmed the dominance of the genus *Haloquadratum* at the highest salinities. According to Ventosa *et al.* (2015), the currently accepted model for community structure in saltern ponds is that the square archaeon *Haloquadratum waslbyi*, the bacteriodete *Salinibacter ruber,* and nonhaloarchaea are predominant members at high salt concentrations, while more diverse archaeal and bacterial taxa are observed in habitats with intermediate salinities.

2.6.2 Microbial ecology of industrial wastewater desalination brine ponds

Compared to solar salterns, industrial wastewater desalination brines are rich in anions other than chloride (for example, sulphate) and frequently in cations other than sodium such as calcium and magnesium (Demergasso *et al.*, 2004). As such, the microbial ecology of industrial wastewater desalination brines is quite different from that of solar salterns that contain concentrated seawaters with NaCl as the major salt (Demergasso *et al.*, 2004; Clegg and Gajardo, 2009). A bacterial profiling metagenomic study on brine samples from the eMalahleni water reclamation plant revealed five taxonomic groups comprising *Aerobacillus pallidus*, *Arthrospira plantesis*, *Halomonas* sp., *Thauera* sp., and uncultured bacteria (Sekar *et al.*, 2014).

According to Sekar *et al.* (2014), the cultured species found in brine samples collected from 14 stages of the desalination operation, including the brine pond, were mostly halotolerant or halophilic comprising of *Actinobacteria*, *Bacteriodetes*, *Cyanobacteria*, *Dienococcus-Thermus*, *Firmicutes*, *Planctomycetes*, and *Gammaproteobacteria*. In their study, Sekar *et al.* (2014), observed changes in the microbial diversity of the process water sampled from different stages of the desalination process due to differences in the physicochemical parameters of the water samples. Samples ranged from being lightly alkaline to highly alkaline (pH 7.40–10.91) throughout the various treatment stages, with the salinity ranging from 1.62 to 4.53 g L^{-1} and dissolved oxygen concentrations ranging from 7.47 to 9.12 mg L^{-1} (Sekar *et al.*, 2014). Phenotypic switching was found to occur due to these physicochemical parameters. Suffice to say; the final brine effluents' physicochemical characteristics determine the bacterial community structure in the brine pond.

Unlike solar saltern ponds, the microbial ecology of industrial RO desalination brine ponds has not been studied extensively. To date, the study by Sekar *et al.* (2014) is the only one to have profiled the bacterial community of an industrial wastewater desalination brine pond.

Other researchers have studied the microbial ecology of RO desalination plants focusing on the microbial communities associated with RO membrane biofouling (Ridgway *et al.*, 1983; Chen *et al.*, 2004; Bereschenko *et al.*, 2007, 2008; Nguyen, Roddick and Fan, 2012; Nagaraj *et al.*, 2017).

2.7 Halophilic microorganisms

It is evident from the description of the microbial community structures in both saltern ponds and industrial wastewater RO desalination brine ponds in section 2.6 that halophilic microorganisms are the predominant organisms in these environments. Halophilic microorganisms are 'salt-loving' organisms found as normal inhabitants of saline environments.

More precisely, halophilic microorganisms are organisms able to grow optimally at salt concentrations of 50 g L^{-1} [equivalent to 0.85 M sodium chloride (NaCl)] or higher and tolerate at least 100 g L^{-1} salt (equivalent to 1.7 M NaCl) (Oren, 2008). These extremophilic microorganisms can balance the osmotic pressure of the environment in which they reside, thereby resisting the denaturing effects of the salts (Oren, 2002). Also referred to as halophiles, halophilic microorganisms include a variety of heterotrophic and methanogenic archaea, photosynthetic, lithotrophic, and heterotrophic bacteria and photosynthetic and heterotrophic eukaryotes (Oren, 1999; Ma *et al.*, 2010).

2.7.1 Classification of halophilic microorganisms

Different classification schemes for halophilic microorganisms have been proposed over the years. All such schemes are to some extent artificial as they do not take into account the continuum of properties within the world of microorganisms This continuum dictates that there will always be organisms that cannot be classified unambiguously within any defined group (Ventosa, Nieto and Oren, 1998).

The most widely accepted and used of these schemes is that of Kushner (1978), depicted in a slightly modified form in Table 2.2. According to Kushner (1978), microorganisms can be classified as being extreme halophiles, moderate

halophiles, slight halophiles or non-halophiles based on their dependence or tolerance for salts (Kushner, 1978). In addition, halotolerant microorganisms exist that, while not requiring high salt concentrations for growth, are able to grow at high concentrations of NaCl and of other salts (Oren, 1999). The term 'salts' in this case refers to a saline mixture akin to that of the sea or saline lakes. The ionic composition of hypersaline and saline environments consists of a mixture of salts (Ma *et al.*, 2010) as exemplified by the EWRP reject brine, which is a sodium (Na_2SO_4) based brine.

Table 2.2 Classification of microorganisms according to their response to salt

Category	**Properties**	**Examples**
Extreme Halophiles	Grow best at salt concentrations of 2.5M – 5.2 M	*Halobacterium salinarium, Hortaea werneckii*
Moderate Halophiles	Grow best at salt concentrations of 0.5M – 2.5M	*Salinivibrio costicola, Flavobacterium gondwanense, Marinococcus halophilus* (formerly *Planococcus halophilus*).
Slight Halophiles	Grow best at salt concentrations of 0.2M – 0.5M	Most marine bacteria
Non – Halophiles	Grow best at salt concentrations less than 0.2M	Most fresh water Microorganisms
Halotolerant	Non- halophiles that tolerate salt; if the growth range extends above 2.5M salt, it is considered extremely halotolerant.	*Staphylococcus aureus*, *Dunaliella*

2.7.2 Phylogenetic diversity of halophilic microorgansims

The phylogenetic diversity of halophiles is staggering. Halophiles are found in all three domains of life Archeae, Bacteria and Eucarya. Figure 2.2 shows branches in the phylogenetic tree of life in which organisms able to grow at salt concentrations above 100 g L^{-1} have been identified (Madigan *et al.*, 2012).

In most cases the halophiles and their non-halophilic relatives are grouped together in the phylogenetic tree. To this end, many genera, families and orders have representatives with vastly different salt requirements and tolerance.

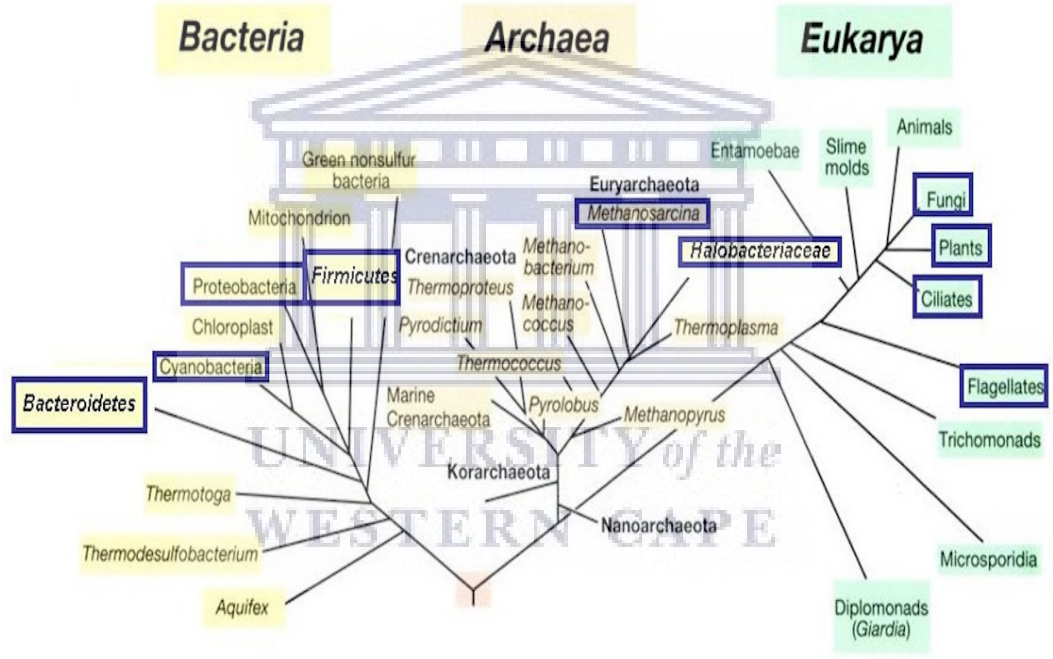

Figure 2.2 Distribution of halophilic microorganisms with in the universal phylogenetic tree of life. Grouping is based on small subunit rRNA gene sequences. Groups marked with blue boxes contain at least one halophilic representative (for example, the *Bacteroidetes*, of which *Salinibacterruber* is the sole halophilic member described to date); others such as the *Halobacteriales* consist entirely of halophiles (Doan Van, 2009).

Microorganisms requiring the most salt for growth are found within the Archaea in the order *Halobacteriales*. This order contains a single family, the *Halobacteriaceae* (Ventosa & Nieto 1995; Oren 2008). The *Halobacteriaceae* are halophiles *par* excellence requiring over 100 - 150 g L^{-1} salt for growth and

structural stability (Oren, 2002). Members of this family are the main component of the microbial biomass of environments such as the Dead Sea, the hypersaline soda lakes such as Lake Magadi in Kenya and the saltern crystallizer ponds (Oren, 2008).

Halophilic microorganisms are also found within the methanogenic branch of the *Euryarchaeota* within the class *Methanothermea* (*Methanococci*), order *Methanosarcinales* and consist of the genera *Methanohalophilus* and *Methanohalobium*. No halophilic microorganisms have yet been identified in the *Crenarchaeota* (Yadav, Singh and Nupur, 2014).

In contrast to the Archaea, halophilic representatives are scarce within the domain Eucarya; in fact, the green alga *Dunaliella* is the only halophilic microorganism of importance in this domain. This alga is the main or only primary producer in the Dead Sea and in many other hypersaline lakes and ponds (Oren 2008). *Dunaliella* is halotolerant rather than trully halophilic. Most strains can grow over a broad range of salt concentrations, and relativey low concentrations (in the order of 1M for the most salt requiring types) can support growth (Oren, 1999; Ma *et al.*, 2010). Halophilic representatives are also found amongst the fungi. Fungal halophiles require high salt environments and are able to grow in salt concentrations approaching saturation. Examples are the ascomycetous black yeasts *Hortaea werneckii* and *Phaeotheca triangularis*, and the meristematic fungus *Trimmatostroma salinum* (Zalar, De Hoog and Gunde-Cimerman, 1999; Kogej *et al.*, 2004).

Many species of halophilic and halotolerant microorganisms are contained within the domain Bacteria. These halophilic and halotolerant representatives are spread over a large number of phylogenetic subgroups. Most of the halophilic bacteria characterized to date belong to the γ – subdivision of the Proteobacteria. Other subgroups of the Proteobacteria also contain moderate halophilic bacterial representatives such as the low G+C and the high G+C Gram-positive bacteria (Firmicutes and actinobacteria respectively), the cyanobacterial branch, the *Flavobacterium* branch, and the Spirochetes (Ventosa, Nieto and Oren, 1998).

Halophiles are found both within the aerobic branch (*Bacillus* and related organisms) and within the anaerobic branches of the Gram – positive bacteria (Firmicutes) (Oren, 2002). The order Halanaerobiales, consisting of two families (the Halanaerobiaceae and the Halobacteroidaceae) comprises solely of halophilic anaerobic microorganisms (Zhilina *et al.*, 2001; Oren, 2002, 2006).

Generally, most halophiles that are classified as members of the domain Bacteria are moderate rather than extreme halophiles (Ventosa, Nieto and Oren, 1998; Oren, 2002). However, a few representatives of the halophilic bacteria resemble the archaeal halophiles of the family Halobacteriaceae in their salt requirements and tolerance. Notable examples are several photosynthetic purple bacteria of the genus *Halorhodospira* (γ – Proteobacteria), the actinomycete *Actinopolyspora candidatus* and the recently discovered *Candidatus* Salinibacter (Antón *et al.*, 2000, 2002; Oren, 2002).

2.7.3 Osmoadaptation

The term osmoadaptation refers to both the physiological and genetic manifestations of adaptation to a low water activity (a_w) environment to avoid water loss by osmosis (Galinski, 1995; Grant, 2004). Two strategies of osmoadaptation have evolved in halophiles to cope with osmotic stress. These strategies are the salt-in-cytoplasm and the organic osmolyte production or accumulation strategies (Galinski, 1995; Ventosa, Nieto and Oren, 1998; Grant, 2004) for both the extremely halophilic and moderately halophilic bacteria, respectively. Considering the literature on the measured salinity of the eMalahleni water reclamation plant RO reject brine sampled at the final stage of the desalination process, which ranges from 4.32 to 4.53 g L^{-1} (Mabovu, 2011; Sekar *et al.*, 2014), the organic osmolyte production or accumulation strategies will be discussed further as they relate more to the types of halophilic bacteria expected in the brines used in this study.

Accumulation of compatible solutes

Since the intracellular ion concentrations measured in the moderate halophiles are generally insufficient to provide osmotic balance with the external medium (Ventosa, Nieto and Oren, 1998), these organisms produce or accumulate low molecular mass organic compounds that have osmotic potential (Grant, 2004) called compatible solutes. Compatible solutes are polar, normally zwitterionic compounds under conditions experienced in the cell that belong to several classes of compounds that include the following:

i. Polyols such as glycerol, arabitol, mannitol, sugars or sugar derivatives such as trehalose, sucrose, sulphotrehalose and glucosylglycerol;

ii. Betaines (trimethylammoniumcompounds) and thetines (dimethyl sulfonium compounds);

iii. Amino acids, including proline, glutamate and glutamine;

iv. N-acetylated amino acids such as Nδ-acetylornithine;

v. Glutamine amide derivatives such as Nα-carbamoyl glutamine amide; and

vi. Ectoines, notably ectoine and β-hydroxyectoine (Grant, 2004).

Amongst the several classes of compatible solutes found in halophilic microorganisms betaines, ectoines, proline, N-acetylated amino acids and glutamine amide derivatives (carboxyamides) were found to occur predominantly in moderately halophilic bacteria (Trüper and Galinski, 1986; Severin, Wohlfarth and Galinski, 1992; Galinski, 1993). These organic osmolytes are often accumulated to cytoplasmic concentrations well above 1 mol. Kg^{-1} water without interacting with proteins or resulting in the disruption of vital cellular processes such as DNA repair, DNA-protein interactions and the cellular metabolic machinery (Grant, 2004). This is because compatible solutes are strong water-structure formers and as such are excluded from the hydration shell of proteins (Galinski and Trüper, 1994). This "preferential exclusion" explains their function as effective stabilizers of enzyme function, providing protection against salinity (Welsh, 2000) as shown in Figure 2.3.

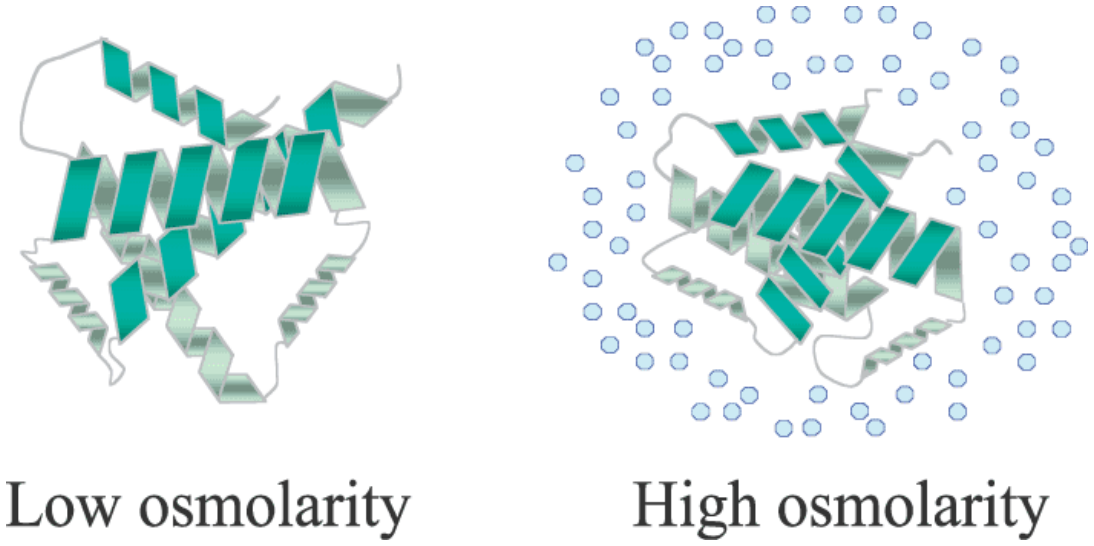

Figure 2.3 Structure stabilization of halotolerant enzymes at elevated osmolarity. Preferential exclusion of compatible solutes (blue circles) from the protein surface helps to maintain enzyme structure at elevated osmolarity, while also helping to increase cell volume (Sleator and Hill, 2002).

2.7.4 Adaptation to high levels of ultra violet radiation exposure

In addition to high salt concentrations, halophiles have to contend with high levels of ultraviolet (UV) exposure in their environments. As a consequence these microorganisms have evolved mechanisms to render them highly resistant to the damaging effects of UV light exposure. Resistance to UV radiation is due in part to robust UV DNA repair systems, including photo reactivation and nucleic excision repair (NER) as well as other photo protective strategies that include carotenoid pigments in the cell membranes of the halophilic bacteria (Baxter *et al.*, 2007). Carotenoid pigments have long been known to provide protection to microbes from intense UV damage by quenching reactive oxygen species and triplet state photosensitizers like protoporphyrin IX and heme (Castenholz and Garcia-Pichel, 2000).

2.8 Pigments of halophilic bacteria

Bacteria produce quite a number of different pigments. Among the pigments produced are carotenoids, melanins, flavins, quinones, monascins, violacein, phycocyanin etc.

These pigments vary in colour from red, yellow, orange, purple etc., and have been a subject of a lot of research not only for their colourful nature but also for the roles they play in photosynthesis, pathogenesis, protection and survival. Halophilic bacteria mainly produce carotenoid pigments. Carotenoids are naturally occurring pigments originating from the terpenoid biosynthetic pathway (Jones and Baxter, 2016) and consist of isoprene residues and a polyene chain of conjugated bonds (Vachali, Bhosale and Bernstein, 2012). Structurally and functionally, carotenoids can be classified as: (1) carotenes, which are hydrocarbons that are either linear or cyclized at one or both ends, and (2) xanthophylls, the oxygenated derivatives of carotenes (Sajilata, Singhal and Kamat, 2008). Some examples of carotenoids are shown in Figure 2.4.

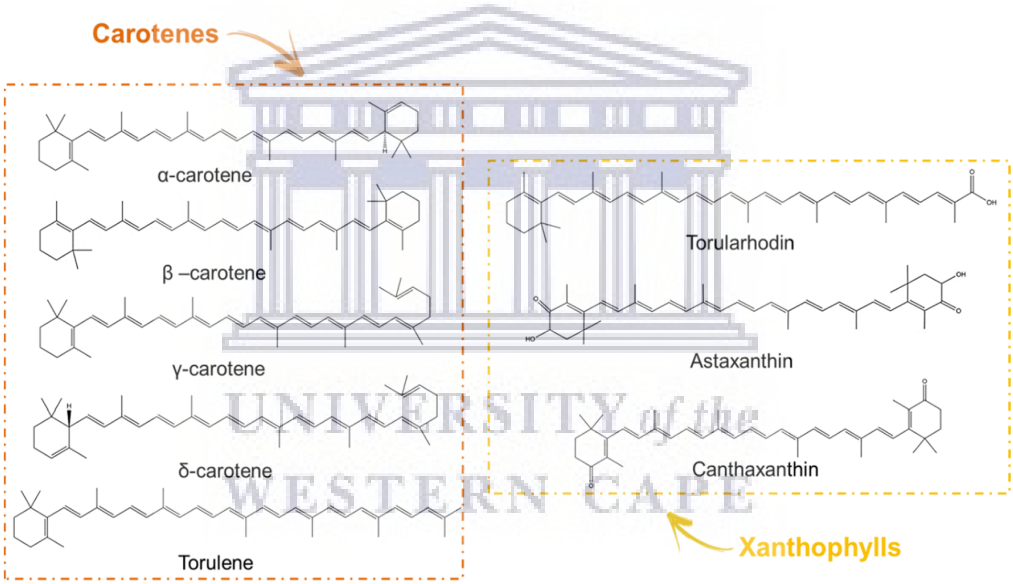

Figure 2.4 Examples of carotenoids.

These pigments are responsible for the wide variety of orange to red colours seen in solar saltern ponds (the natural environment of halophilic bacteria) and absorb light in the wavelength range of 300 – 600 nm (Vachali, Bhosale and Bernstein, 2012). The absorbance is directly related to the number of conjugated double bonds and functional groups present in the structure. Phytoene, for example, contains only three conjugated double bonds and absorbs ultraviolet light, whereas

ζ -carotene and more highly unsaturated carotenoids possess seven or more conjugated double bonds and thus absorb visible wavelengths (Armstrong, 1997).

2.8.1 Carotenogenesis in halophilic microorganisms

Carotenoid biosynthesis in halophilic bacteria is a well-regulated mechanism dependent on the biochemical make-up of the organism's environmental conditions and stress incurred during growth. Halophilic bacteria often accumulate these compounds as either 50- or 40- or 30-carbon (C_{50}, C_{40}, and C_{30}) carotenoid glycosides with polar end groups spanning through the membrane (Valery M. Dembitsky, 2004; Köcher *et al.*, 2009) where they influence membrane fluidity and stability as well as prevent oxidative damage to the membrane due to their antioxidant properties (Britton, 1995; Woodall, Britton and Jackson, 1997; Köcher *et al.,* 2009).

The synthesis of either one of these carotenoid compounds is dependent on the type of the first hydrocarbon-carotenoid produced during biosynthesis (Misawa, 2010). Fifty-carbon and C_{40} carotenoids are biosynthesized from the hydrocarbon phytoene, while the C_{30} carotenoids are biosynthesized from 4,4'-diapophytoene (Figure 2.5) (Mercadante *et al.*, 2004; Dogbo *et al.*, 2006; Misawa, 2010; Yatsunami *et al.*, 2014).

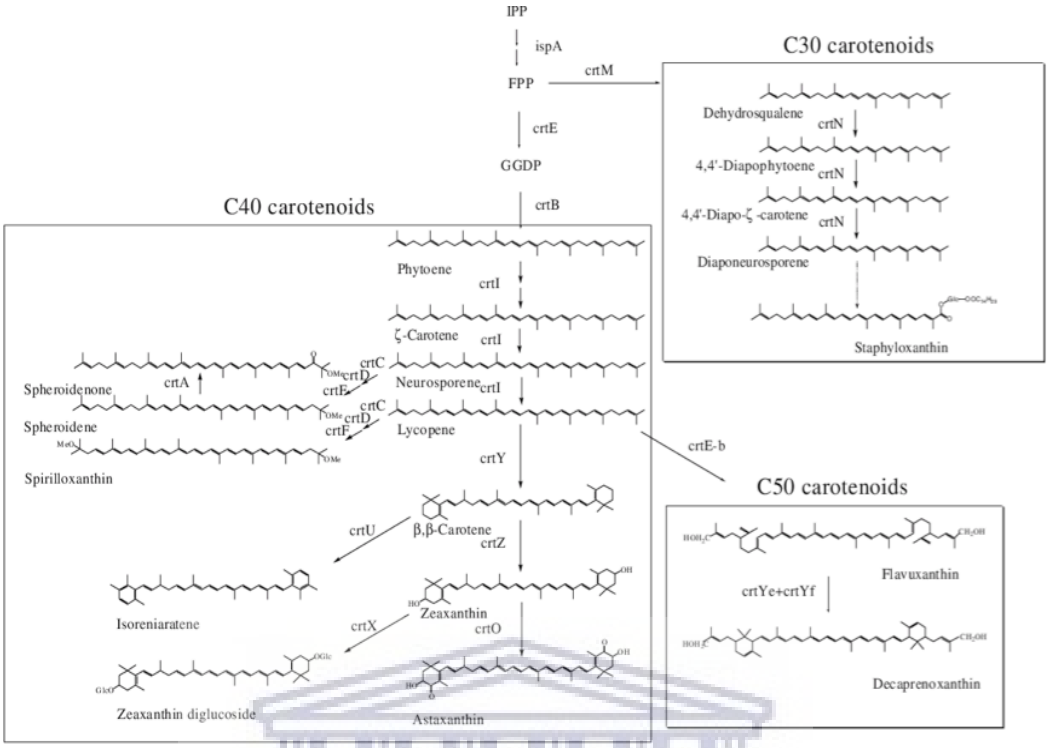

Figure 2.5 Biosynthetic pathways for C_{30}, C_{40}, and C_{50} carotenoid compounds in bacteria (Schmidt-Dannert, Lee and Mijts, 2006)

Phytoene and 4,4'-diapophytoene are generated from the condensation of two molecules of geranylgeranyl diphosphate (pyrophosphate) (GGPP) or farnesyl diphosphate (FPP) by either phytoene synthase (CrtB) or 4,4'-diapophytoene synthase (CrtM), respectively (Misawa, 2010).

The hydrocarbon precursors are subsequently desaturated (dehydrogenated) to form conjugated double bonds, which are subjected to a series of modifications that result in the production of a large variety of C_{50}, C_{40}, and C_{30} carotenoid structures (Mercadante *et al.*, 2004; Dogbo *et al.*, 2006; Köcher *et al.*, 2009; Misawa, 2010; Yatsunami *et al.*, 2014). Amongst the C_{50}, C_{40}, and C_{30} carotenoid compounds, C_{40} compounds are the most common in nature followed by the less common C_{30} carotenoids, and the even lesser common C_{50} carotenoids, which are derived from the C_{40} structure by the addition of two 5-carbon (C_5) isoprene units (Mercadante *et al.*, 2004; Yatsunami *et al.,* 2014).

The biosynthesis of C_{50} and C_{40} compounds in halophilic bacteria such as *Salinibacter ruber* (de Lourdes Moreno *et al.*, 2012) and *Halobacterium salinarum* (Yang *et al.*, 2015), proceeds when phytoene is converted to lycopene (a C_{40} acyclic carotenoid) through desaturation. This desaturation step is a multistep process varying between microorganisms. The most common of all is the four-step desaturation process mediated by the bacterial type phytoene desaturase (CrtI) (Tian and Hua, 2010; Vachali, Bhosale and Bernstein, 2012). Depending on the final structure of the carotenoid compound, lycopene can be modified to produce either acyclic or cyclic carotenes or xanthophylls (Armstrong, 1997; Tian and Hua, 2010; Vachali, Bhosale, and Bernstein, 2012).

Several halophilic bacteria, which include *Staphylococcus aureus* (Marshal and Wilmoth, 1981; Kim and Lee, 2012), *Rubritella squalenifaciens* (Shindo *et al.,* 2007; Shindo and Misawa, 2014), *Planococcus maritimus* (Shindo *et al.*, 2008, 2014; Shindo and Misawa, 2014) and *Halobacillus halophilus* (Köcher *et al.,* 2009; Osawa *et al.,* 2010a, 2010b) synthesize C_{30} carotenoids. The C_{30} carotenoid products of carotenoid pathways in these bacteria include staphyloxanthin derived from diaponeurosporene, a methyl glucosyl apolycopenoate derivative (Shindo et al., 2008, 2014; Osawa *et al.,* 2010a, 2010b) and acylglycosyl monoesters (Shindo and Misawa, 2014) or diacylglycosyl diesters both derived from 4,4′-diapolycopene (Shindo *et al*., 2007).

Amongst the bacterial genera and species that synthesize C_{30} carotenoids, *S. aureus* has the best-studied gene cluster for the C_{30} pathway wherein all genes involved in staphyloxanthin synthesis were functionally assigned (Pelz *et al.*, 2005).

According to Pelz *et al.*, (2005), Misawa, (2010), and Kim and Lee, (2012), five genes and enzymatic reactions are involved in the biosynthesis of staphyloxanthin, which begins with the condensation of two molecules of farnesyl diphosphate to form dehydrosqualene mediated by CrtM (Figure 2.5). The dehydrosqualene thus produced is converted to 4,4′-diaponeurosporene through a sequential desaturation reaction that is mediated by the enzyme 4,4’-diapophytoene desaturase (CrtN), a homolog of CrtI.

Oxidation of the terminal methyl group of 4,4'-diaponeurosporene by the mixed function oxidase, 4,4'-diaponeurosporene oxygenase (CrtP), results in the formation of 4,4′-diaponeurosporenic acid, which is then followed by esterification of glucose at the C_1'' position with the carboxyl group of 4,4′-diaponeurosporenic acid to yield glucosyl-4,4′-diaponeurosporenoate; the esterification step is mediated by a glucosyltransferase (CrtQ) enzyme. Staphyloxanthin is then produced from the glycosyl-4, 4′-diaponeurosporenoate by the esterification of glucose at the C_6'' position with the carboxyl group of 12-methyltetradecanoic mediated by an acyltransferase enzyme.

2.8.2 Effects of carotenogenesis on brine evaporation

Evaporation ponds present an environment with increasing salinity in which different halophilic organisms are found along a salinity gradient created as evaporation progresses. An assessment by Saju *et al.,* (2011) on the impact of halophilic bacteria on the overall evaporation rate in salt works primary concentrator ponds in India revealed the average evaporation percentage to be higher than that for a control pond devoid of halophilic bacteria (Saju *et al.*, 2011). The average evaporation percentage in the pond inoculated with halophilic bacteria was found to be 16.8 ± 1.33 % whereas that of the control pond was 13.68 % during March 2004 (Saju *et al.*, 2011). The development of colour in the pond inoculated with halophilic bacteria was thought to be responsible for the observed difference in evaporation percentage.

It has long been known that dense communities of heterotrophic halophilic archaea and bacteria contribute to the orange to red colouration of saltern ponds (Baas-Becking, 1931; Oren, Stambler and Dubinsky, 1992b). The pigments produced by these microorganisms absorb radiant energy in the 300 – 600 nm wavelength range of the electromagnetic spectrum, which is thought to increase the brine temperature and enhance the evaporation rate (Davis, 1974; Children, 2001; Oren, 2002; Bhosale, 2004). Without this natural colouration, evaporation proceeds only with great difficulty (Davis, 1974).

Considering the nature of industrial RO desalination brines, and the role played by halophilic bacteria in enhancing evaporation rates in solar saltern ponds, a biological process utilizing pigmented halophiles to enhance industrial RO brine evaporation rates offers advantages in terms of cost-effectiveness, biodegradability, and reduced environmental impact.

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Chapter 3

Isolation and characterization of halophilic bacteria strains, and their effects on coal mine wastewater desalination brine evaporation

3.1 Introduction

Halophilic bacteria that grow in saltern ponds have long been known to enhance the evaporation process in these ponds during salt production. The literature reviewed indicated that the growth and pigment production by these microorganisms in solar saltern ponds leads to enhanced evaporation rates during salt production. The premise being that coloured brine solutions absorb more radiant energy from the sun than clear solutions and that the bacterial pigments are responsible for the increased absorption of radiant energy which results in increased brine temperature, reduced brine surface tensions, increased brine vapor pressure, and ultimately increased brine evaporation rates in solar saltern ponds.

Considering the role played by pigmented halophilic microorganisms in enhancing brine evaporation rates in solar saltern ponds, they may play a similar role in desalination brine ponds leading to enhanced evaporation rates of the brine contained therein. To this end, the effects of pigmented halophilic bacteria and the extracted pigment on the evaporation rate of a synthetic brine formulated according to the chemical composition of the EWRP brine and industrial RO desalination brine supplied by NuWater Global, Epping, South Africa, were investigated by Khumalo, (2018) and Silva-Castro *et al.*, (2019), respectively.

The work presented in this chapter contributes to, and continues, the work reported by Khumalo, (2018) and Silva-Castro *et al.*, (2019), and focuses on the assessment of the effects of the reflected light spectra of the pigments produced by the halophilic bacteria on the evaporation rate of a mine wastewater RO desalination brine from the EWRP.

To assess the effect of the reflected light spectrun of the pigment produced by halophilic bacteria on the brine evaporation rate, PCR amplification and sequence BLASTn analysis were used for the identification of the isolate affecting the highest increment in the evaporation rate of the synthetic brine, while chemical mutagenesis was used to generate pigment mutants of this isolate. The pigment mutants were used together with the wild type strain to test the hypothesis that the reflected light spectrum of the bacterial pigment determined the brine evaporation rate increment and the time taken for complete brine evaporation.

Although the popularity of chemical mutagenesis has reduced in recent years because of the perception that mapping chemically induced mutations is arduous, this method was used because of its cost-effectiveness, efficiency, scope, lack of bias during phenotypic screening and most importantly, because of the lack of genetic tools for this bacterium. Also, chemical mutagenesis was used because mapping and variant calling can now be done on a large scale due to next-generation sequencing (NGS) technology and NGS data analysis tools such as Geneious (https://www.geneious.com, no date) and CLC Genomics workbench (https://www.qiagenbioinformatics.com, no date). The evaporation experiments for testing the study's hypothesis were carried out in an access-controlled laboratory to minimize fluctuations in the temperature, humidity, and wind speed, and to correlate the outcome to the pigment colour being assessed.

3.2 Materials and methods

3.2.1 Brine samples

Brine samples used in this study were collected from the eMalahleni Water Reclamation pond situated in the Mpumalanga province, South Africa (S 25°56′41.4, E 29°11′67.0); and from five Cerebos crystallizer salt ponds in Velddrif, Western Cape, South Africa (S 32°47′10,632, E 18°10′9,499). Isolation of bacteria from the Cerebros crystallizer ponds was conducted to increase the chances of obtaining pigmented halophilic bacteria in the event that none were obtained from the eMalahleni brine samples. Also, because of the wide salt tolerance range of some halophilic bacteria (Kushner, 1978) it was expected that

isolates from the Cerebros samples would be able to tolerate the salilnty gradient of the eMalahleni brine during the evaporation rate studies. The composition of the eMalahleni brine was determined by Bemlab (South Africa) (see Appendix A).

A synthetic brine was formulated based on the composition of the eMalahleni brine, with the following composition (w/v): 3497 mg L^{-1} Na^{+}; 758 mg L^{-1} K^{+}; 223 mg L^{-1} Mg^{2+}; 964 mg $L^{-1}Ca^{2+}$; 762 mg L^{-1} Cl^{-} and 10 255 mg L^{-1} SO_4^{2-}. The use of the synthetic brine was necessitated by the difficulties of attaining and storing the brine and the need to reduce potential brine composition variations from using different batches of the brine. The composition of any desalination brine effluent batch is dependent on several factors, which include the quality of the feed water, the permeate water grade, and the chemicals used during the cleaning-in-place operations. These factors are not static and change regularly, thus, resulting in the production of brines with different compositions (Katal *et al.*, 2020).

3.2.2 Isolation of halophilic bacteria

To isolate bacteria from the eMalahleni desalination brine, 1 L of the desalination brine was vacuum filtered through a 0.22 µm nitrocellulose Millipore™ membrane. The membrane was washed, and the cells suspended in 10 mL of sterile distilled water (sdH_2O). One gram of each of the Cerebros brine slurry samples was resuspended in 10 mL of sdH_2O and mixed by vortexing. A serial dilution was prepared from the bacterial suspensions up to 10^{-3} and 0.1 mL from each dilution inoculated by spreading onto TSB agar (17 g L^{-1} pancreatic digest of casein; 3 g L^{-1} enzymatic digest of soya bean; 5 g L^{-1} sodium chloride; 2.5 g L^{-1} dipotassium hydrogen phosphate; 2.5 g L^{-1}glucose; 14 g L^{-1} agar) plates prepared using sterile eMalahleni brine instead of dH_2O. The plates were incubated at room temperature for a period of 5 days. Pigmented isolates growing on the plates were picked and streaked on TSB-brine agar.

3.2.3 Evaporation rate studies and isolate growth kinetics

The effect of the isolates on desalination brine evaporation rate was assessed in deep well glass petri dishes with 200 mL of the synthetic brine inoculated with an overnight culture of the isolates. A control containing methylene blue at a concentration of 200 mg L^{-1} in the synthetic brine was also set up.

This methylene blue concentration was selected based on the increment in the evaporation rates of synthetic brine after the addition of different methylene blue concentrations in a previous study (see Appendix B).

The addition of methylene blue to a final concentration between 200 and 300 mg L^{-1} resulted in the most volume of liquid lost during the experiment; these results showed no significant difference (p = 0.05) between them, therefore 200 mg L^{-1} concentration was selected to compare with the biological treatment. The brine was evaporated using 240-Watt infrared lamps situated approximately 40 cm above the surface of the cultures as a source of heat. These lamps emit IR light that travels through air like normal light and heats up any material it comes in contact with. The overnight cultures were standardized to an optical density of 1 at 660 nm. The amount of brine lost was measured at 6-h intervals, until the pan was completely dry. The rate of evaporation of the brine was calculated as shown in Equation 3.1.

Equation 3.1. Brine evaporation rate (Ahmed *et al.*, 2000),

$$\text{Evaporarion rate } (\text{cm/h}) = \frac{\text{Volume of brine lost over time } (\text{cm}^3/\text{h})}{\text{Brine surface area } (\text{cm}^2)}$$

Parallel to this, the growth kinetics of the isolates in the eMalahleni water reclamation desalination brine effluent was investigated. Inocula for the brine was prepared from 24-hour cultures of the isolates grown in 10 mL of TSB broth by standardizing against a 0.5 M McFarland standard to give a turbidity of a culture containing 1.5 x 10^8 cells/mL. One milliliter from each of the standardized cultures was used to inoculate 20 mL of eMalahleni brine vacuum sterilized

through a 0.45 μm membrane and then through a 0.22 μm membrane filter. The inoculated brine was incubated at room temperature with shaking at 150 rpm.

One milliliter of sample was drawn from the brine at 12 hour intervals for the measurement of growth at $OD_{660nm.}$ To follow pigment production by the isolates in the brine, cells in the 1 mL samples drawn at the 12 hour intervals were pelleted by centrifugation at 3214 x g for ten minutes, washed, and the pigment extracted from the cell pellets by mixing with methanol followed by incubation at 60 °C for 15 minutes. After incubation, the cell suspensions were centrifuged at 3214 x g and the absorption of the coloured SNTs measured at the respective λ_{max} of each pigment extract following a spectrophotometric scan of the absorption spectra of each extract from 200 nm to 700 nm.

3.2.4 Characterization and identification of the isolates

The isolates affecting the highest increase in synthetic brine evaporation rate and exhibiting good growth in eMalahleni water reclamation desalination brine devoid of nutrient supplementation were characterized and identified by analyzing their 16S rRNA gene sequences. Genomic DNA was extracted from cells pelleted from 5 mL cultures of the isolates using a modified version of the method by Wang *et al.* (1996). The following modifications were made: lysozyme concentration was increased to 25 mg/mL, 0.2 mg/mL (final concentration) of proteinase K was added to the lysis buffer and cells were incubated overnight at 37 °C in the lysis buffer. Following extraction and RNAse A digestion, DNA pellets were re-dissolved in 100 μL of Tris-EDTA (TE) buffer and stored at 4 °C. PCR amplification was performed in 50 μl volumes which contained 100 ng template DNA, 1.25 units of Thermo Fisher Scientific Dream Taq polymerase, 10X Dream Taq PCR buffer, 0.2 mM of deoxynucleoside triphosphates (dNTPS) and 0.1 μM of each primer. The universal primer set E9F (Hansen *et al.*, 1998) and U1510R (Reysenbach *et al.*, 1995) was used for amplification. The following thermo cycling conditions were used: initial denaturation at 95 °C for 5 min (1 cycle); 30 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 s, extension at 72 °C for 90 s and final extension at 72°C for 5 min.

After gel purification, using a Nucleospin kit (Machery-Nagel Inc, USA), Sanger sequencing of 16S rRNA gene fragments was carried out by the Central Analytical Facility at Stellenbosch University using an ABI PRISM 377 automated sequencer and the sequence compared with the NCBInr database using BLASTn to identify the isolates. The maximum likelihood phylogenetic tree was constructed using MEGA 7 software (Kumar, Stecher and Tamura, 2016).

3.2.5 Chemical mutagenesis

Chemical mutagenesis was employed to generate a mutant library with the hope that a mutation resulting in a loss of pigment production would arise, and the mutant used to study the effect of pigment on brine evaporation. Mutagenesis was carried out on cells that had grown to the mid exponential phase (OD_{660nm} 0.7 – 0.75) in TSB medium supplemented with 5 % (w/v) NaCl and 0.5 M sorbitol (final concentration) (TSB-salt-sorbitol medium). The cells were harvested by centrifuging at 3214 x g for a period of 10 minutes. After harvesting, the cells were washed once with, and re-suspended in, 50 mL of TSB-salt-sorbitol medium. The re-suspended cells were concentrated 50-fold by centrifugation and re-suspension in 1 mL of quarter strength Ringer's solution (Sigma-Aldrich, Steinheim, Germany). To 1 mL of cell suspension 12.75 µL of MMS (Sigma-Aldrich, Steinheim, Germany) were added and mixed vigorously to give a final concentration of 0.15 M of the mutagen. After 5 min of incubation at room temperature in a fume hood, the dosed wild type cells were transferred to a 50 mL falcon tube containing 30 mL Ringer's solution, sealed with para-film and centrifuged for 10 minutes at 3889 x g in a Eppendorf 5810R (Hamburg, Germany) refrigerated bench top centrifuge (fitted with the F-34-6-38 high-speed fixed-angle rotor) set at 20 °C. This step was repeated thrice and the washed pellet re-suspended in 1 mL TSB containing 15 % (v/v) glycerol and 5% (w/v) NaCl to make the mutant library, which was then stored at -80° C until needed. Screening of the library was conducted by spread plating a one in one thousand dilution of the library in Ringer's solution on TSB-salt-sorbitol agar plates. Colonies were then visually inspected for pigmentation differing from that of the wild type after incubation at 28 °C for 48 hrs.

3.2.6 Mutation frequency determination

The proportion of mutant bacteria present in the library was determined using a modified version of the *p*0 method (Luria and Delbrück, 1943; Pope *et al.*, 2008) as follows: Twenty five parallel experiments of 10 mL cultures inoculated using an inoculum of 1.5×10^8 cells from the mutant library were set up and grown to saturation at 28 °C with shaking at 150 rpm. At the end of the growth period (5 days), the number of mutants in each culture was determined by serially diluting each culture up to the 10^{-4} dilution and spread plating 100 µL of this dilution onto TSB-salt-sorbitol agar plates.

The plates were incubated at 28 °C until visible growth of colonies was observed. Only the number of mutant phenotypes (i.e, non-orange colonies) present in each culture were counted and tabulated as shown in Table 3.1. This is because when using this method for the determination of the mutation frequency there is no need to enumerate the total number of colonies and this simplifies the process (Luria and Delbrück, 1943; Foster, 2006; Pope *et al.*, 2008).

Table 3.1 Number of mutants recorded in each plate

Number of mutants observed in each culture (*r*)	**Number of cultures observed to have mutants**
0	15
1	0
2	1
3	2
4	0
5	3
6	4
7	0
8	0
9	0
10	0
Total number of cultures	**25**

Because the number of mutations (not mutants) per culture is Poissonally distributed, $p0$, the proportion of cultures without mutants was the zero-term of this Poisson distribution given by:

Equation 3.2 Zero - term of Poisson distribution (Luria and Delbrück, 1943; Rosche and Foster, 2000):

$$p0 = e^{-m}$$

Equation 3.2 was rearranged to give the number of mutational events as follows:

Equation 3.3. Number of mutational events (Rosche and Foster, 2000).

$$m = - \ln p0$$

Where $p0 = \frac{Number\ of\ cultures\ with\ mutants}{Total\ number\ of\ cultures}$, and

m = mutational frequency.

3.2.7 Mutant culture purity check

The purity or identity of the mutant strains was confirmed through amplified ribosomal DNA restriction analysis (ARDRA) of their 16S rRNA genes. The 16S rRNA genes of the wild type and mutant strains were amplified using colony PCR and the amplicons digested using four base cutter restriction enzymes to generate ARDRA profiles for each of the strains. These profiles were then compared for similarities. Amplification of the 16S rRNA gene from colony PCR was done as follows: A colony was picked from a 48-hour culture and suspended in 20 µL of a 0.5 mg/mL proteinase K + 20 mM dithriothreitol (DTT) lysis buffer. The suspension was incubated at 37°C for 10 minutes followed by deactivation of the proteinase K enzyme at 95°C for 5 minutes. The suspension was cooled and centrifuged for 30 seconds to pellet the cell debris. One micro litre of the supernatant was used as template for 16S rRNA gene PCR. PCR amplification was performed in 50 µL volumes which contained 1 µL of supernatant as template

DNA, 1.25 units of Dream Taq polymerase, 10× Dream Taq PCR buffer, 0.2 mM of deoxynucleoside triphosphates (dNTPS) and 0.1 μM of each primer.

The universal primer set E9F (Hansen *et al*., 1998) and U1510R (Reysenbach *et al.,* 1995) was used for amplification. The thermo cycling conditions used were identical to those used for the amplification of the 16S rRNA gene for the identification of the isolates through sequence BLASTn analysis in section 3.2.4. The PCR-amplified reaction mix was analysed on a 1.2 % (w/v) agarose gel stained with 0.5 μg/mL ethidium bromide to check the integrity of the amplicons. Electrophoresis was conducted in Tris-acetate-EDTA (TAE) buffer at a constant 90 volts for 1 hour.

The four base pair restriction enzymes AluI, HaeIII and RsaI were used to generate ARDRA profiles from the 16S rRNA amplicons of the mutant and the wild type phenotype. Restriction digests were performed in 25 μL containing: 0.5 units of restriction enzyme, 250 ng of PCR product and 1X NEB Cutsmart buffer. The restriction digestion reactions were incubated 37 °C for 15 minutes as per the NEB time saver protocol. After which the digest products were loaded onto a 2.5 % (w/v) agarose gel stained with 1.5 μg/mL ethidium bromide, electrophoresed in 1X TAE buffer at 80V for 2 hours, and the resulting pattern compared for similarities.

3.2.8 Effect of chemical mutagenesis on strain growth

Before determining the effects of the pigment produced by the strain(s) on brine evaporation rate, the growth rates of the mutant strain(s) were compared to that of the wild type strain in eMalahleni brine to establish whether the chemical mutagenesis had caused mutations to the genes responsible for metabolism leading to altered growth rates. The growth rate comparisons were conducted in 50 mL of filter-sterilized eMalahleni brine inoculated with 1 mL of inoculum containing 1.5 x 10^8 CFU of either the wild type or pigment mutant strain, which were incubated at room temperature on an orbital shaker set at 150 rpm. One hundred microliter samples were drawn from each culture at 6-hour intervals, for a period of 48 hours, and spread platted onto TSB-salt-sorbitol agar plates for the

determination total viable counts (TVCs). The TSB-brine agar plates were incubated at 28 °C until visible colonies could be seen.

Total viable counts were calculated according to:

Equation 3.4. Number of bacterial colony forming units per mL of medium

$$\frac{\text{cfu}}{\text{mL}} = \frac{\text{number of colonies x dilution factor}}{\text{volume of culture plated}}$$

3.2.9 Determination of the effect of pigments on brine evaporation

The effects of the pigment on the evaporation rate and the duration of evaporation of the brine were determined in glass evaporation pans measuring 27 cm in length, 20 cm in width and 7.5 cm in height. The brine was sterilized by pressure filtration through 0.45-micron and then a 0.2-micron nylon membrane filter, and stored in 2 L sterile Schott bottles until use. Inocula to be added to the brine was prepared from the wild type and pigment mutant strains cultured on TSB-salt-sorbitol agar plates. Single colonies from each of the wild type and mutant cultures growing on TSB-salt-sorbitol agar plates were transferred to 10 mL of TSB-salt-sorbitol broth and the liquid cultures incubated at 28 °C for 48 hours with shaking at 150 rpm. At the end of the incubation period, the cultures were standardized against a 0.5 M McFarland standard to give an inoculum size of approximately 1.5 x 10^8 CFU/mL. One milliliter from each of the standardized cultures was used to inoculate separate 199 mL of TSB-salt-sorbitol broth and the cultures incubated for 48 hours under the same conditions mentioned above.

At the end of the 48-hour incubation period, 800 mL of brine and 200 mL of wild type, and mutant culture were added to the autoclaved evaporation pans. Two control pans per experiment were included: i) one containing 1 L of brine, and ii) the other containing 800 mL of brine mixed with 200 mL of sterile TSB-salt-sorbitol medium. The brine was evaporated using 240-Watt infrared lamps placed approximately 40 cm above the liquid surface as a source of heat (see Appendix C). The amount of brine lost through evaporation was measured at 12-hour intervals until the pans were completely dry.

The evaporation rate of the brine at each 12-hour interval was calculated using Equation 3.1. The evaporation rates calculated for each 12-hour period were multiplied by a pan correction factor of 0.75. This was done because water evaporates faster from an evaporation pan than from a large water surface such as an evaporation pond.

Thus, when using pan evaporation rates they must be multiplied by a correction factor of 0.75 to better approximate evaporation losses (Liu and Kang, 2007). The corrected evaporation rates were used for statistical analysis to determine whether there were any differences in the evaporation rate of the brine as influenced by the different strains and by the TSB-salt-sorbitol. Also, the duration of brine evaporation from each pan was determined using regression analysis, and the estimated mean durations subjected to statistical analysis to determine whether there were any differences in the duration of brine evaporation as influenced by the different pigment mutant and wild type strains, and by the TSB-salt-sorbitol. Furthermore, the temperature on the surface of the brine in each pan was monitored on an hourly basis until the end of the experiment using iButton temperature loggers included in the pans. The temperature data was downloaded from each iButton temperature logger and plotted.

3.2.10 Statistical analysis

The evaporation rate experiments were conducted in triplicate and descriptive statistics computed. Mean scores, standard errors of mean scores, and percentage losses from the total were calculated for the volume of brine evaporated per 12-hour period. Normality and homogeneity of variance were checked for in the calculated evaporation rate data (see Appendix D) and one-way multivariate analysis of variance (MANOVA) with Fisher's least significant (LSD) post hoc test performed. One-way MANOVA with LSD post hoc test was performed to determine whether there were any significant differences in the brine evaporation rates per 12-hour period during the experiment. Given that research has shown that evaporation rate has an impact on the duration of evaporation, a one-way analysis of variance (ANOVA) with Fisher's LSD post hoc test was performed to determine whether there were any significant differences in the duration of brine

evaporation as a consequence of the bacterial strains and TSB. All statistical analyses were performed using IBM® SPSS® Statistics (ver. 23.0, IBM® Corp., USA).

3.3 Results

3.3.1 Bacterial isolation and selection of canidates for evaporation study

Six unique bacteria were isolated from both the eMalahleni Water Reclamation plant pond and Cerebos salt crystallizer ponds based on colony morphology and colour. The effect of the isolates on synthetic brine evaporation rate was compared to those of TSB medium prepared with synthetic brine, and 200 mg L^{-1} of methylene blue dye added to synthetic brine**.** The evaporation trials were conducted in 200 mL volumes to identify isolates that could potentially improve evaporation rates. Inoculation with isolates CP5-4, EP1 and EP3 had a positive effect on the evaporation rate after 24 h, and all performed better than the 200 mg L^{-1} of methylene blue (Table 3.2).

Table 3.2 Evaporation rates of the synthetic brine affected by the isolates

Isolate	*NaCl conc (%)	Evaporation rate (cm h^{-1})			
		0 – 24 h	**24 – 48 h**	**48 – 56 h**	**56 – 72 h**
Brine	-	0.027±0.002	0.028±0.002	0.036±0.002	0.041±0.000
Brine + TSB	-	0.034±0.003	0.038±0.001	0.068±0.005	0.045±0.001
MB	-	0.035±0.006	0.048±0.002	0.038±0.001	0.038±0.001
EP1	10	0.043±0.003	0.054±0.003[a]	0.030±0.019	0.029±0.000
EP2	5	0.044±0.003	0.043±0.002	0.048±0.003	0.040±0.004
EP3	5	0.043±0.002	0.085±0.005[a]	0.002±0.000[a]	0.000±0.000
CP2-2	10	0.037±0.005	0.041±0.001	0.056±0.004	0.039±0.007
CP5-4	10	0.039±0.004	0.050±0.001[a]	0.094±0.007[a]	0.005±0.000
CP5-7	5	0.037±0.003	0.046±0.003	0.045±0.001	0.035±0.001

Mean ± Standard deviation.
* Percent (w/v) of NaCl in which the isolates grew best.
[a] Significant difference in the evaporation rates of the uninoculated synthetic brine and synthetic brine inoculated with each of the isolates using Multivariate analysis of variance (MANOVA) with LSD (α = 0.05).

Synthetic brine inoculated with isolate EP3 experienced a high evaporation rate of 0.085 cm per hour between 24-48 h, while that inoculated with isolate CP5-4 experienced the highest evaporation rate of 0.094 cm per hour after 48 h.

These results showed significant difference with respect to the synthetic brine control and 200 ppm methylene blue (LSD test; $p = 0.05$), hence these isolates were selected for further study. The salt tolerance of the isolates was tested in R2A broth supplemented with different NaCl concentrations. The isolates were able to grow in concentrations ranging from 0 to 30 % (w/v) NaCl. EP3 and CP5-4 had optimal growth at 5 % and 10 %, respectively (Table 3.2), making these moderate halophiles (Ventosa, Nieto and Oren, 1998; Ma *et al.*, 2010).

3.3.2 Growth and pigment production by isolates EP3 and CP5-4 in eMalahleni brine.

An initial characterization of the pigment(s) produced by EP3 and CP5-4 was performed using synthetic brine amended with 100 % strength TSB. As seen in Figure 3.1, the UV-visible spectra of the red-pink and orange pigments produced by isolates EP3 and CP5-4 had absorption maxima at 495 and 475 nm, respectively.

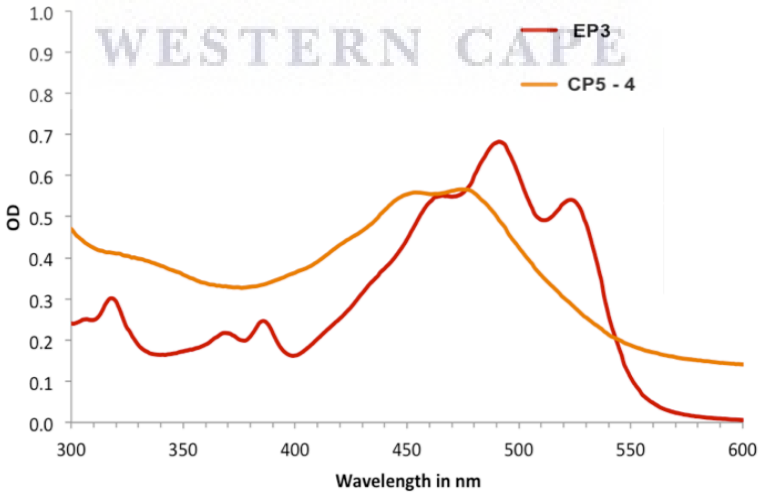

Figure 3.1 UV-Vis absorption spectra of pigments produced by isolates EP3 and CP5-4 in synthetic brine medium.

The production of these pigments was hypothesized to be the main contributors to the increased evaporation rates observed. To assess the ability of these isolates to increase the evaporation rate of real world coalmine wastewater desalination brine as opposed to the synthetic version, growth and pigment production by the two isolates was assessed in eMalahleni coalmine wastewater desalination brine effluent. Isolate CP5-4 maintained good growth and pigment production in eMalahleni desalination brine (Figure 3.2), a characteristic that made this isolate an attractive candidate for evaporation rate studies as this was indicative of the adaptability of isolate CP5-4 to some of the evaporation pond conditions. Furthermore, the ability of isolate CP5-4 to grow and produce pigment in the brine indicated the reduced need for additional nutrient supplementation during the course of the evaporation studies.

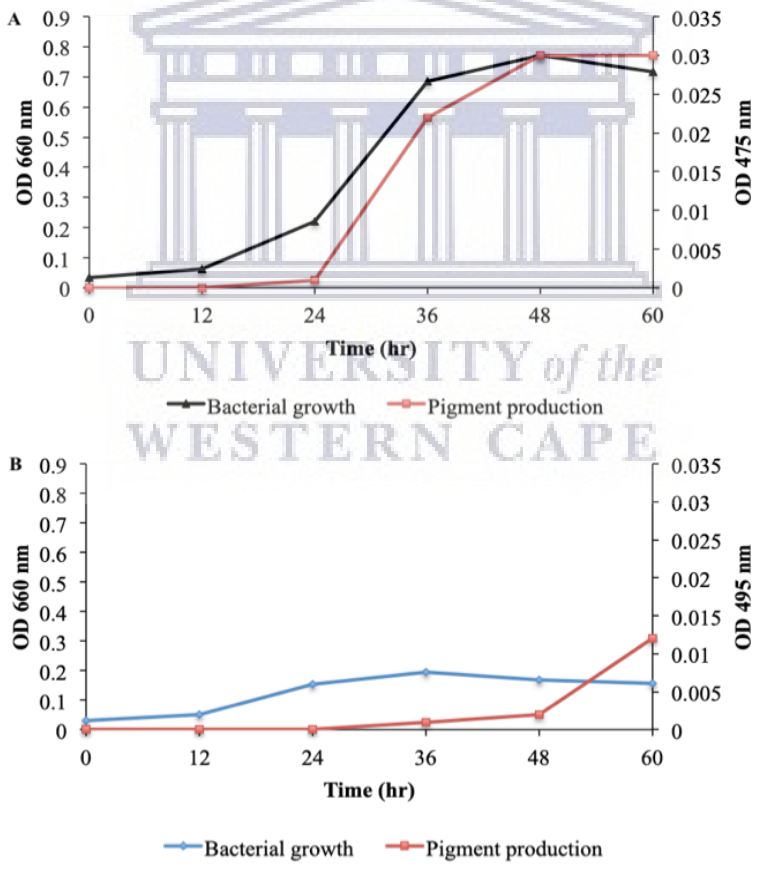

Figure 3.2 Growth and pigment production by isolates CP5-4 (A) and EP (B) in eMalahleni brine.

A query of the 16S rRNA sequence of isolate CP5-4 against the curated NCBI 16S rRNA sequence database showed this sequence to have 97 % similarity to that of *Planococcus maritimus* (NR_025247.1) over a query cover of 98 % using 1423 bp of the query sequence. The CP5-4 16S rRNA sequence was subsequently submitted to the GenBank database under accession number KY859787. Also, alignment scores generated from comparing the 16S rRNA gene sequence of CP5-4 to those of related *Planococcus* strains were used to construct the phylogenetic tree shown in Figure 3.3. The CP5-4 isolate is possibly a novel species based on % sequence similarity of the 16S rRNA sequence and the evolutionary distance of this strain from the closely related members.

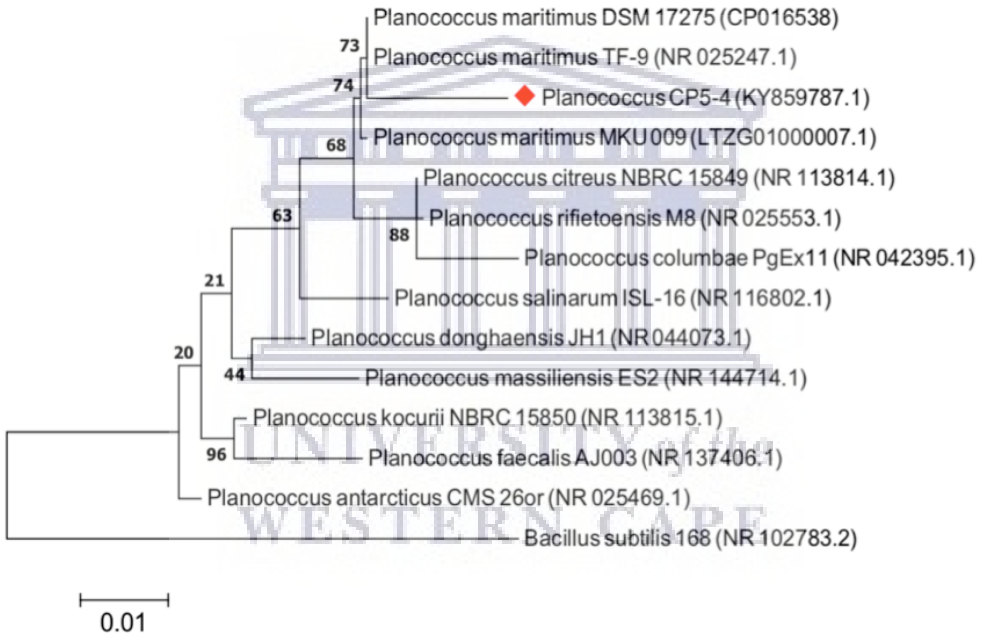

Figure 3.3 Phylogenetic relationship of *Planococcus* sp. strain CP5-4 to that of related *Planococcus* strains. The tree was constructed by the Maximum Likelihood method based on the alignment of the 16S rRNA gene sequences of *Planococcus* sp. strain CP5-4 (1432 bp) to those of 12 closely related *Planococcus* strains. The 16S rRNA gene sequence for *Bacillus subtilis* 168 was used as an out-group. Numbers on branches indicate bootstrap values after 1000 re-samplings. Accession numbers of the bacterial species are mentioned in parenthesis. All gaps and missing data were eliminated from the alignment. There were a total of 1392 positions in the final dataset.

3.3.3 Chemical mutagenesis

Chemical mutagenesis of *Planococcus* sp. CP5-4 was employed to generate a mutant library with the hope that a mutation resulting in the loss of pigment production would arise, and the mutant used as a control to study the effect of pigment on brine evaporation. Two mutant strains, one yellow and the other unpigmented, were generated and designated *Planococcus* sp. CP5-4 YE for the yellow and *Planococcus* sp. CP5-4 UN for the unpigmented mutant. The three *Planococcus* sp. CP5-4 strains are shown in Figure 3.4.

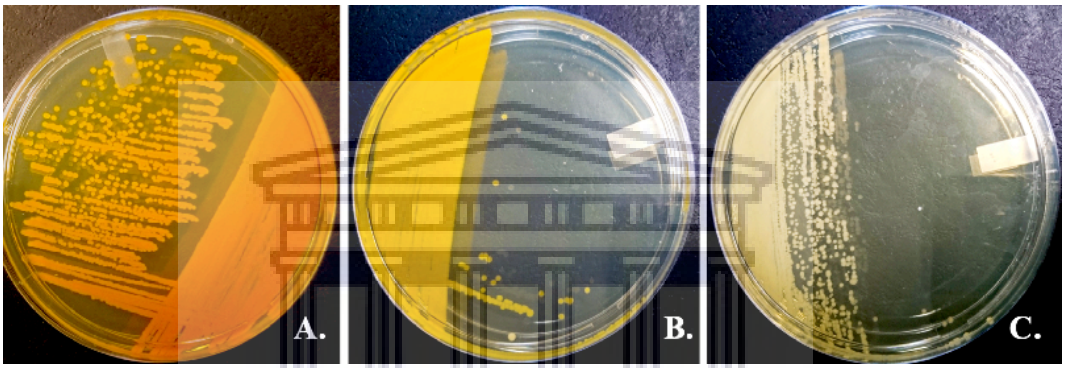

Figure 3.4 *Planococcus* sp. CP5-4 wild type (A), yellow (B), and unpigmented (C) strains.

The *p*0 method developed for determining mutation rates from spontaneous, and induced mutations in bacterial cultures (Rosche and Foster, 2000), was applied to determine the mutation rates in the *Planococcus* sp. CP5-4 cultures dosed with MMS. Fifteen out of the 25 cultures tested had no mutants (Table 3.1), giving a calculated *p*0 value of 0.6. From Equation 3.3, the mutation frequency of the experiment (m) was calculated to be 0.511. This mutation frequency was an estimation of the probability of a mutation occurring per cell division following mutagenesis. In other words, the calculated mutation frequency represents the proportion of mutant bacteria present in the *Planococcus* sp. CP5-4 MMS mutant library.

All three *Planococcus* sp. CP5-4 strains were used for studying the effects of pigment on brine evaporation. However, before the commencement of the study, the identity of the mutant strains was confirmed through restriction digestion of their 16S rRNA genes and comparison of the generated restriction patterns to that of the wild type strain. Matching restriction profiles were generated for all three strains following digestion with AluI, HaeIII, and RsaI restriction enzymes. The generated profiles are shown in Figure 3.5.

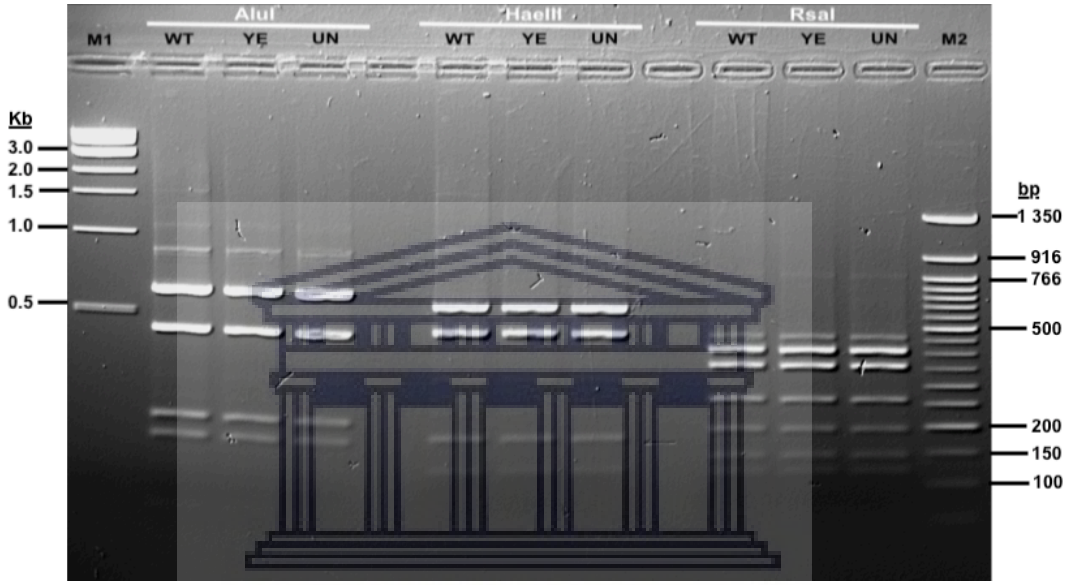

Figure 3.5 ARDRA profiles for *Planococcus* sp. CP5-4 wild type and mutant strains. M1 – 1 kb DNA marker, WT – wild type, YE – yellow mutant, UN – unpigmented mutant, and M2 – 50 bp DNA marker.

The matching restriction profiles showed that the two mutant strains, and the *Planococcus* sp. CP5-4 wild type strain were of the same genotype. ARDRA profiles are a quick and robust way to cluster bacterial communities into genotypic groups or for strain typing (Sklarz *et al.*, 2009), and in this case the technique was valuable for confirming that the mutants were indeed *Planococcus* sp. CP5-4 strains and not contaminants.

3.3.4 Effect of mutagenesis on strain growth

Since the bacterial growth rate and the level of gene expression are intertwined, it was necessary to establish whether chemical mutagenesis had affected the growth rates of the *Planococcus* sp. CP5-4 mutant strains compared to that of the wild type strain in eMalahleni brine. The three strains showed a similar growth pattern in the eMalahleni brine (Figure 3.6) suggesting that either none of the genes and regulatory pathways involved in cell proliferation were affected by the MMS treatment, or that the DNA repair mechanisms of the *Planococcus* sp. CP5-4 mutant strains had rectified the affected DNA of the genes involved in cell proliferation. Nonetheless, the similarity in the growth rate of the mutant strains to that of the wild type strain facilitated their use in determining the effect of bacterial pigment on brine evaporation rate.

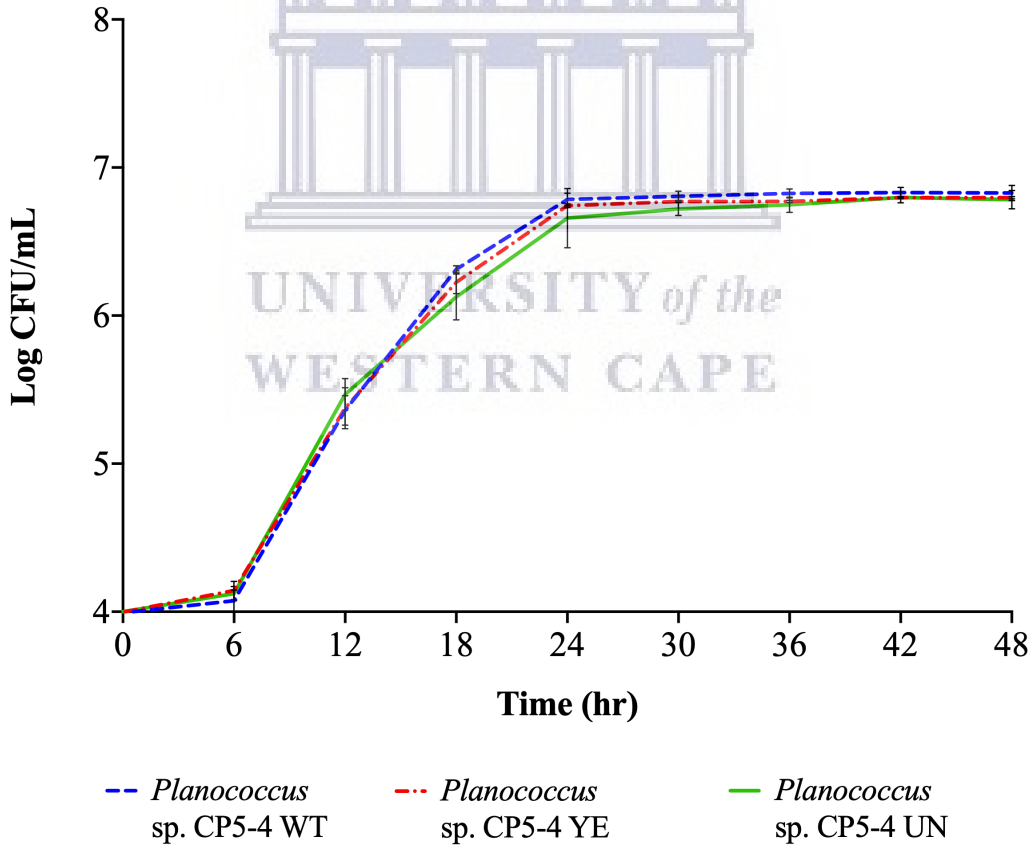

Figure 3.6 Growth rates of the *Planococcus* sp. CP5-4 yellow (YE) and unpigmented (UN) mutant strains vs. that of the wild type (WT) strain.

3.3.5 Effects of pigment on brine evaporation

Changes in the surface temperatures of the brine inoculated with the wild type, yellow, and unpigmented *Planococcus* sp. CP5-4 strains, including changes in the temperatures on the surfaces of the brine control and the brine mixed with TSB-salt-sorbitol medium, are shown in Figure 3.7. Brine inoculated with the wild type-orange *Planococcus* sp. CP5-4 strain experienced high surface temperatures followed by brine inoculated with the yellow strain, unpigmented strain, uninoculated brine control, and the brine mixed with the TSB-salt-sorbitol medium, respectively. The brine surface temperatures from the evaporation experiments support the notion that bacterial pigments increase the amount of thermal energy absorbed by the brine, thereby causing increments in the brine temperature, and subsequently the evaporation rate.

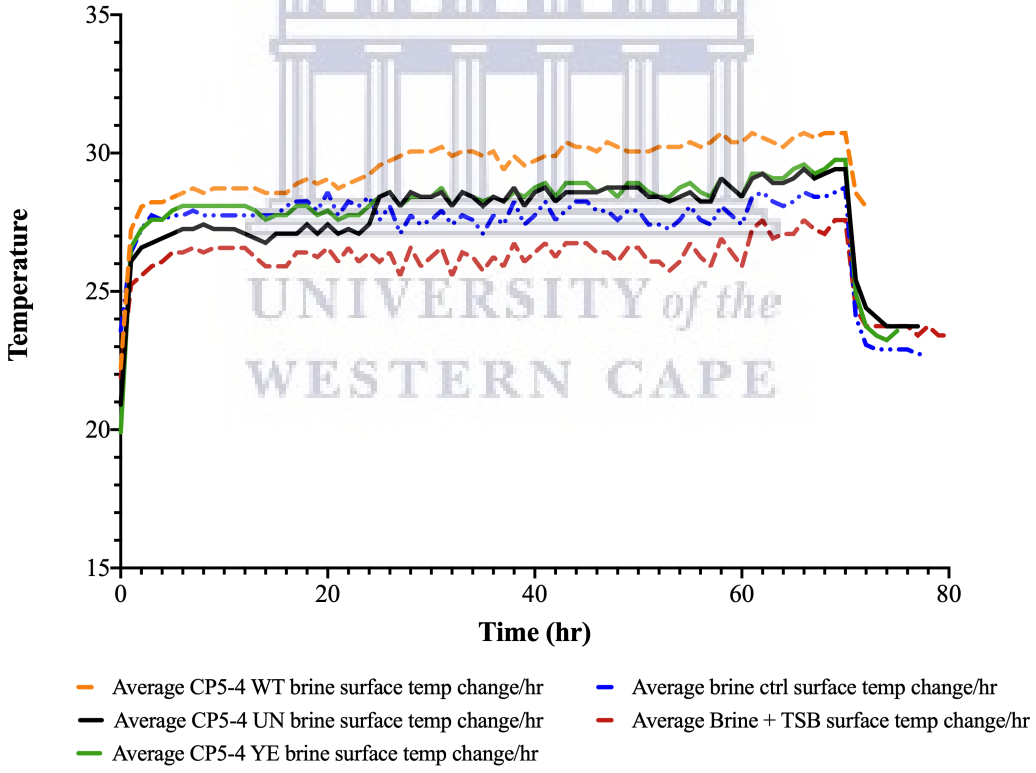

Figure 3.7 Changes in brine surface temperatures during the course of the evaporation experiments. The temperatures were automatically recorded at 1 hour intervals by iButton® temperature loggers.

The effects of the bacterial pigment on brine evaporation were two fold, i.e., brine inoculated with the pigmented *Planococcus* sp. CP5-4 strain experienced high evaporation rates and shorter durations of evaporation compared to the un-inoculated brine, brine mixed with TSB-salt-sorbitol medium, and the brine inoculated with the unpigmented *Planococcus* sp. CP5-4 strain (Table 3.3). It is worth mentioning at this point that during the experiment there was growth of a contaminating microorganism that formed a film on the surface of the brine with TSB-salt-sorbitol medium, which is not unexpected given that the system was exposed and could be easily seeded by organisms in the air . No attempt was made to identify the contaminating organism as it was not pigmented. We propose that the film formed on the surface caused the reduced brine evaporation rate and prolonged brine evaporation compared to the uninoculated brine control (see Appendix C). As such the recorded evaporation rates from the brine with TSB-salt-sorbitol medium pan(s) were excluded from the successive analyses.

Increments of between 10 % and 19 % in brine evaporation rate when inoculated with the orange *Planococcus* sp. CP5-4, and yellow *Planococcus* sp. CP5-4 strains were observed. There was a statistically significant difference in the duration of brine evaporation according to the one-way ANOVA analysis (F (3, 8) = 64.89; $p < 0.001$) (see Appendix F for ANOVA output and Fisher's LSD post hoc comparisons tables).

Table 3.3 Percentage increments in brine evaporation rates

Additive	Evaporation rate (cm/hr)			Duration of Evaporation (hr: mins: sec)
	Mean	Difference	Percent increase	
None	0.0194 ± 0.0051	0.0000	0.00	78: 36: 00 ± 00: 37: 30[a]
Planococcus CP5-4 WT	0.0230 ± 0.0022	0.0036	18.56	71: 38: 20 ± 00: 17: 36[a]
Planococcus CP5-4 YE	0.0214 ± 0.0039	0.0020	10.31	74: 10: 40 ± 00: 32: 42[a]
Planococcus CP5-4 UN	0.0206 ± 0.0057	0.0012	6.19	76: 27: 40 ± 00: 56: 21[a]

[a] Significant difference in the times for the complete evaporation of the uninoculated eMalahleni water reclamation plant desalination brine and eMalahleni water reclamation plant desalination brine inoculated with each of the *Planococcus* sp. CP5-4 strains using one way ANOVA and LSD post hoc test ($\alpha = 0.05$; p – values < 0.05).

Furthermore, a one-way MANOVA revealed significant differences in the means of the brine evaporation rates for each 12-hour interval, F (18, 8.97) = 6.910, $p < 0.0005$; Wilk's $\lambda = 0.0005$, partial $\eta^2 = 0.92$ (see Appendix G for multivariate comparisons table). Additional univariate analysis of variance (ANOVA) tests of between-subject effects showed that the evaporation rates per 12-hour period were significantly different as a result of the added *Planococcus* sp. CP5-4 strains (see Appendix G for univariate ANOVA table).

Compared to the un-inoculated brine control, the additives (the *Planococcus* sp. CP5-4 strains) had a statistical effect on the evaporation rates from 0 to 12 hours (F (3, 8) = 37.60; $p < 0.001$, partial $\eta^2 = 0.93$) and from 72 to 84 hours (F (3, 8) = 16.71; $p < 0.001$, partial $\eta^2 = 0.86$), which contributed to the observed increments in the brine evaporation rate. The evaporation rates of the brine as a consequence of the additives during the different 12-hour intervals are shown in Table 3.4.

Table 3.4 Brine evaporation rates per 12-hour interval

	Evaporation rate (cm/hr)						
Additive	**0 – 12 h**	**12 – 24 h**	**24 – 36 h**	**36 – 48 h**	**48 – 60 h**	**60 – 72 h**	**72 – 84 h**
None	0.014 ± 0.001	0.026 ± 0.001	0.022 ± 0.002	0.015 ± 0.002	0.020 ± 0.002	0.010 ± 0.002	0.010 ± 0.001[a]
Planococcus CP5-4 WT	0.022 ± 0.001[a]	0.021 ± 0.002	0.025 ± 0.001	0.023 ± 0.001	0.024 ± 0.001	0.000[a] ± 0.000	0.000 ± 0.000[a]
Planococcus CP5-4 YE	0.016 ± 0.001[a]	0.024 ± 0.001	0.024 ± 0.002	0.019 ± 0.001	0.024 ± 0.000	0.008 ± 0.000	0.001 ± 0.001
Planococcus CP5-4 UN	0.014 ± 0.000	0.019 ± 0.001	0.025 ± 0.001	0.019 ± 0.003	0.026 ± 0.004	0.009 ± 0.001	0.003 ± 0.002

[a] Significant difference in brine evaporation rates for the inoculated eMalahleni water reclamation plant desalination brine, and un-inoculated eMalahleni water reclamation plant desalination brine using one way Multivariate analysis of variance (MANOVA) with LSD post hoc test ($\alpha = 0.05$; p – values < .05).

3.4 Discussion

Halophilic bacteria were isolated from brine samples obtained from the eMalahleni Water Reclamation plant pond and from the Cerebos salt crystallizer ponds. However, the culturable bacterial diversity from these samples was low. Much diversity may have been missed due to the limitations of the culture media used for isolation (Sekar *et al.*, 2014). Choosing appropriate media and growth conditions is important because when working with environmental samples harbouring communities of novel microbial populations, the media and growth conditions chosen will bias the enrichment of certain populations (Schneegurt, 2012). Thus, the choice, preparation, and use of hypersaline media presents challenges unique to high salinities, as well as, some of the same concerns inherent with any microbial culture system (Schneegurt, 2012). Also, bacteria from hypersaline environments are fastidious in their nutrient requirements and to the salinity gradient to which they are subjected to during isolation (Rajendran, 2015). Although many can tolerate a wide salinity range, most cannot survive if moved from hypersaline to less saline or non-saline environments, which was likely the case in this study when washing the membranes and suspending the dislodged cells in sterile distilled water during isolation. This could therefore have contributed to the observed low culturable diversity.

During the evalutaion of the effect the halophilic bacteria had on synthetic brine evaporation, the increase in synthetic brine evaporation rate affected by EP3 and CP5-4 was speculated to be attributed to the red-pink and orange pigments produced by these isolates. Saltern crystallizer ponds and hypersaline lakes approaching NaCl saturation are generally coloured orange-red because of the presence of dense communities of halophilic microorganisms, which contribute to increased evaporation rates during salt production (Davis, 1974; Oren, Stambler and Dubinsky, 1992b; Ma *et al.*, 2010). According to Davis, (1970), and Zhiling and Guangyu, (2009), the pigments produced by these microorganisms increase the amount of thermal energy absorbed by the brine from the sun thereby causing an increase in the brine temperature and subsequently the evaporation rate.

The red-pink and orange pigments produced by isolates EP3 and CP5-4 were tentatively identified as carotenoids based on their respective UV-Vis spectra, which showed the typical 'three-finger' pattern spectrum that is characteristic of well known carotenoid pigments such as astaxanthin, beta-carotene, lycopene, etc (Chemat-Djenni *et al.*, 2010; Farci *et al.*, 2016; Takaya, Anan and Iwata, 2018). Such spectra show a maximal absorbance in the blue region (~450 nm) and strong shoulders at approximately 440 to 490 nm of the electromagnetic spectrum depending on the carotenoid pigment (Britton, Liaaen-Jensen and Pfander, 1994; Mercadante *et al.*, 2004). Generally, halophilic microorganisms produce carotenoids in response to dissolved oxygen and oxidative stress, nitrogen limitation (nutrient depletion), exposure to damaging ultraviolet light, temperature and salt stress, production of which help these microorganisms adapt to their environments (de Lourdes Moreno *et al.*, 2012; Sanchez *et al.*, 2013; Sowmya and Sachindra, 2015).

Switching the growth medium from the TSB supplemented synthetic brine to the eMalahleni brine resulted in the reduced growth of isolate EP3. According to Bahlaoui, Baleux, and Frouji, (1998), factors such as dissolved oxygen concentration, assimilable organic carbon (AOC), total nitrogen, pH, and the presence of pre- and post-cleaning operation chemicals, may have affected the growth rate of isolate EP3 in the eMalahleni brine. Also, residual antiscalants (organic compounds such as polyacrylamides and organophosphates) in the brine may have been more assimilable by isolate CP5-4 and provided nutrients and substrates that promoted the growth of isolate CP5-4. In light of this, isolate CP5-4 was selected for studying the effects of pigment on brine evaporation rate. Isolate CP5-4 was tentatively identified as a new species in the *Planococcus* genus based on the calculated evolutionary distance of *Planococcus* sp. CP5-4 from that of closely related strains (Figure 1.3), and the shared 16S rRNA gene sequence similarity of 97 % to that of *P. maritimus* (NR_025247.1).

According to proposed guidelines for bacterial classification, two strains are considered as belonging to different species if they share <97 % sequence similarity in their 16S rRNA genes (Janda and Abbott, 2007).

However, this cutoff value at the species level was later reevaluated to be 98.65% by Kim *et al.* (2014), following the absence of universal agreement about the percentage similarity required to assign a sequence to a particular species or genus. Several cut-off similarity levels have been suggested, ranging from 98.2 to 99.0 % for the species level (Stackebrandt and Jonas, 2006; Meier-Kolthoff *et al.*, 2013). However, according to Kim *et al.* (2014), the 98.65 % 16S rRNA gene sequence similarity can be used as the threshold for differentiating two species based on a twofold cross-validation statistical test of the level at which the 16S rRNA gene sequence similarity corresponds to currently accepted whole-genome average nucleotide identity (ANI) threshold for species demarcation from over one million comparisons. Thus, *Planococcus* sp. CP5-4 shared less than 98.65 % 16S rRNA sequence similarity with *P. maritimus*, prompting the conclusion that these two strains belonged to two different species. A more extensive analysis of this is presented in Chapter 4 (section 4.3.2).

Although the 16S rRNA gene has been a mainstay of sequence-based bacterial analysis for decades, dependence on the use of this single marker gene is known to be limited by PCR and sequencing errors, short read lengths, and the poor discrimination power it provides some genera, which has been responsible for its poor performance for Gram-negative rods, with only 23.7% of species level identification (high proportion of *Campylobacter* spp. (44%)) (Clarridge, 2004; Mignard and Flandrois, 2006; Poretsky *et al.*, 2014; Johnson *et al.*, 2019). However, as whole-genome sequencing has become more widely accessible due to the introduction of cost-effective high-throughput DNA sequencing technology, the use of whole-genome sequence comparisons have become the gold standard taxonomic parameter for the accurate identification of novel bacterial species based on computational similarity indices such as relative evolutionary divergence (RED) and Average Nucleotide Identity (ANI) for establishing taxonomic ranks (Parks *et al.*, 2018, 2020).

Following identification, chemical mutagenesis was used to alter the phenotype of the orange *Planococcus* sp. CP5-4 strain to evaluate the effect of culturing the different coloured strains in the brine on brine evaporation. Consequently, yellow

and unpigmented mutant phenotypes were generated following mutagenesis. Methyl methanesulfonate methylates DNA on N^7-deoxyguanine and N^3-deoxyadenine to form N^7-methylguanine (N^7-MeG) and N^3-methyladenine (N^3-MeA) causing base mispairing and replication blocks, which are repaired by either bypass repair, recombination repair or by base excision repair (BER). During this repair process, mutations arise due to the loss of DNA bases (Lindah and Wood, 1999). Reports in the literature on the elucidation of pigment biosynthesis pathways in halophilic bacteria provide support for the production of white (or unpigmented) and yellow mutant phenotypes and the resulting DNA damage and repair of the genes associated with pigment biosynthesis following chemical mutagenesis using DNA alkylating agents (Köcher *et al.*, 2009; Osawa *et al.*, 2010a, 2010b). The characterization and elucidation of the pigment biosynthetic pathway(s), the mutation(s) resulting in the altered phenotypes as a consequence of MMS mutagenesis, and the type of pigment(s) produced by *Planococcus* sp. CP5-4 will be presented in chapter 4.

Although the potential of pigmented halophilic bacteria as a biological treatment for desalination brine effluent through increasing the brine evaporation rate was demonstrated (Table 3.2), the extent to which the increase in evaporation rate was due only to the pigment could not be established using the different isolates because of variations in their growth rates and metabolic capabilities. It is conceivable that differences in the bacterial cell wall structure (i.e., the thickness of the peptidoglycan layer and presence or absence of the outer lipid membrane) and the high-level accumulation, either through synthesis or uptake from the environment, of organic osmolytes, could also have contributed, in part or in full, to increasing the evaporation rate. Thus, the best way to eliminate as many variables as possible, and to show the specific contribution of biological pigment production on evaporation rate, would be to utilize a set of pigment mutants of one bacterial isolate.

The generation of the yellow and unpigmented mutant phenotypes of the orange *Planococcus* sp. CP5-4 strain (Figure3.4) in this study made it possible to evaluate the effects of the colour of the pigment on brine evaporation. Noteworthy is the

fact that the two pigment mutant *Planococcus* sp. CP5-4 strains exhibited comparable growth rates to that of the wild type strain in eMalahleni brine (Figure 3.6). Brine inoculated with the orange, yellow, and unpigmented *Planococcus* sp. CP5-4 strains experienced increments of 18.56, 10.31, and 6.19 % in evaporation rate, respectively, in comparison to the uninoculated brine control. The finding that brine inoculated with the unpigmented *Planococcus* sp. CP5-4 strain also experienced an increase in evaporation, albeit not as much as that of the brine inoculated with the pigmented strains suggests that pigmentation may not necessarily be the principal factor for halophilic bacteria to cause an increase in the brine evaporation rate, but is important for attaining even higher brine evaporation rate increments.

To explain the processes leading to the brine increased evaporation as a consequence of inoculating the brine with the *Planococcus* sp. CP5-4 strains, it is important to reiterate that infra red (IR) lamps were used as a heat source for driving the process of evaporation in this study. IR lamp bulbs are designed to produce IR light that flows through the air like normal light, but warms up any object it comes in contact with (Deshmukh, 2005). Depending on the temperature of the emitting body, the wavelength of the peak of the infrared radiation ranges from 780 nm to 1 mm (Gabbott, 2008). The IR lamps used in this study-emitted 240W of energy that resulted in a heat flux flow of 4.44 kW/m^2 of heat to the surface of the brine. As the IR radiation penetrated the brine it was absorbed by the microorganisms and reflected by the pigment to the surrounding brine.

There are more than a thousand types of pigments and each has a unique reflected light spectrum (colour) based on a specific pattern of reflectance or absorption of visible light (Herbst *et al.*, 2004). Like-wise, each pigment has distinct IR-reflective characteristics (Coser *et al.*, 2015). Enhanced IR-reflectance keeps pigments cooler than they would be compared to pigments with less IR-reflectance as such low reflectance pigments absorb the most heat energy and transfer it to surrounding surfaces (Canlas, 2016).

The current chapter demonstrates how the reflected light spectrum (colour) of the pigment produced by halophilic bacteria affects the evaporation rate based on the pigment's IR-reflectance characteristic.

Low IR-reflectance pigments (in this case, the orange pigment of *Planococcus* sp. CP5-4-WT) absorb more radiant energy (generated by the IR-lamps) than high IR-reflectance pigments (the yellow pigment produced by the *Planococcus* sp. CP5-4-YE mutant strain) (Canlas, 2016). Also demonstrated was the fact that pigmentation is not a prerequisite for halophilic bacteria to affect an increase in brine evaporation rate, but an important factor for affecting higher brine evaporation rate increments. Davis, (1974), states that without this natural colouration, brine evaporation proceeds with great difficulty as observed for the brine inoculated with the *Planococcus* sp. CP5-4-UN strain when compared to the brine inoculated with the pigmented strains.

In this study, the addition of TSB to the brine did not increase the brine evaporation rate as observed during a pilot study using NuWater desalination brine conducted by Moyo *et al.*, (2018). In both studies, contaminating microorganisms grew in the brine. However, the contaminating microorganisms in this study were unpigmented and formed a biofilm on the surface of the brine. Microorganisms from the environment may have contaminated the brine because the evaporation experiments were carried out under non-sterile conditions, and the formation of the biofilm on the brine surface during colonization slowed the rate of brine vaporization.

Since the effects of temperature, wind speed, humidity, and varying pond depth in conjunction with pigment colour were not assessed in this study, the attained results cannot be extrapolated to a real-world bring pond system. Regardless, the current study demonstrated how the colour of the pigments (and ultimately, the IR reflectance characteristics of the pigment) produced by halophilic bacteria might influence brine evaporation rates in real-world pond systems.

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Chapter 4

Structure and biosynthesis of *Planococcus* sp. CP5-4-WT pigments

4.1 Introduction

Among the microbes, bacteria have immense potential to produce diverse bio-products and one such bio-product is pigments (Venil, Zakaria and Ahmad, 2013). Bacterial pigments are organic secondary metabolites that are not directly involved in the normal growth and proliferation of the bacteria. Pigments however, play an important role in the physiology and molecular processes of the bacteria by acting as solar radiation protectants, mediating adaptation to extreme environments and the process of photosynthesis (Sutthiwong *et al.*, 2014). Bacterial pigments have been used as a taxonomic tool for the identification and classification of bacteria for several decades because of their diversity, which is dependent on their chemical structures and the presence of specific chromophores (Schippers-Lammertse, Muijsers and Klatser-Oedekerk, 1963; Koch, Schumann and Stackebrandt, 1995). Currently, besides the role in taxonomic study, bacterial pigments have been investigated widely for application in the food, cosmetics, pharmaceutical, textile, and desalination industries (Dufossé *et al.*, 2005; Higuera-Ciapara, Félix-Valenzuela and Goycoolea, 2006; Venil and Lakshmanaperumalsamy, 2009; Moyo *et al.*, 2018; Silva-Castro *et al.*, 2019).

Bacteria produce several pigment classes that include, but are not limited to, carotenoids, porphyrins, melanins, etc., (each with distinct IR-reflective characteristics) through the expression of genes commonly organized as biosynthetic clusters. The development of genomic sequencing technology has facilitated the mining of these bacterial secondary metabolite biosynthetic gene clusters allowing for their characterization and heterologous expression for enhanced pigment production. Although strains belonging to the *Planococcus* genus have been widely reported as pigment producers, information on the identification and characterization of the genes responsible for pigment biosynthesis in this genus is not exhaustively reported in the literature.

Therefore, this chapter presents an analysis of the sequenced genomes of the three *Planococcus* sp. CP5-4 strains from chapter 3, for the taxonomic classification of the wild type strain, the identification and characterization of the pigment biosynthesis genes, and the determination of sequence variants resulting in the production of the yellow and unpigmented phenotypes following mutagenesis. Also presented in this chapter is the characterization of the pigment(s) produced by the *Planococcus* sp. CP5-4-WT strain for the elucidation of the pigment biosynthetic pathway, and the structure of the pigment(s) produced.

4.2 Materials and methods

4.2.1 DNA extraction and genome sequencing

Genomic DNA was extracted from 5 mL cultures of the 3 *Planococcus* sp. CP5-4 strains as described in chapter 3, section 3.2.4. The quality of the DNA was checked by agarose gel electrophoresis as follows: 5 µL of DNA sample (4 µL DNA plus 1 µL of 5x Qiagen GelPilot® DNA loading dye) were loaded, parallel to a 1 Kb Quick – Load® DNA ladder from New England Biolabs, onto a 0.8 % (w/v) agarose gel stained with 0.5 µg/mL ethidium bromide and electrophoresed for 90 minutes at 100 volts in TAE buffer. At the end of electrophoresis, the genomic DNA on the gel was visualized using an Alpha Imager UV transilluminator (Alpha Imager 2000, Alpha Inno tech, USA). The amount of extracted DNA was then quantified using a Qubit 2.0 fluorometer prior to paired end library preparation and whole genome sequencing. Paired end libraries of the DNA were prepared using the Nextera™ DNA sample preparation kit and the genomes sequenced using the MiSeq v2 reagent kit (2 x 150 bp) on an Illumina MiSeq sequencing platform. This was conducted at the IMBM Next generation Sequencing facility, University of the Western Cape.

4.2.2 Sequence assembly

The fastq files from the MiSeq platform were imported into Geneious ver. 11.1.5 (https://www.geneious.com, no date; Kearse *et al.*, 2012) and processed in the program as follows: Firstly, reads were paired using the 'Set Paired Reads' option with an insert size of 300 bp. Datasets produced by the Nextera™ DNA sample preparation kit typically have a 40 – 565 bp insert size range using the high molecular weight buffer, and 300 bp represented the approximate midpoint of the size range expected for the data sets generated from the *Planococcus* sp. CP5-4 strains gDNA. Secondly, DNA bases of low quality were trimmed from the ends of the reads using the Geneious trimmer ('Trim Ends') with an error probability limit of 0.05. The data sets were further processed using 'Trim using BBDuk' (https://sourceforge.net/projects/bbmap/, n.d.), using default settings. As a result, low quality reads with a minimum average quality score less than 20 or lengths shorter than 10 bp were removed. Also, reads that extended past the start of their mate were trimmed to ensure complete adapter removal. The processed paired read sets were then assembled using the Geneious *de novo* assembler. Default settings ('Medium sensitivity/Fast') were used.

4.2.3 Taxonomic classification of Planococcus sp. CP5-4-WT

To obtain an accurate taxonomic classification of the *Planococcus* sp. CP5-4 wild type strain, the *de novo* assembled contiguous (contig(s)) sequences (in fasta format) were queried in the Genome Taxonomy Database Toolkit (GTDB-Tk; Chaumeil *et al.*, 2019) in the Kbase web portal (*http://kbase.us*, no date; Arkin *et al.*, 2018). The GTDB-Tk was used in preference to the NCBI (Federhen, 2012), SILVA (Yilmaz *et al.*, 2014), RDP (Cole *et al.*, 2014), Greengenes (McDonald *et al.*, 2012), and EzTaxon (Yoon *et al.*, 2017) databases because of the the inconsistent taxonomies with many polyphyletic groupings reported to being produced for bacteria by these databases (Parks *et al.*, 2018, 2020; Chaumeil *et al.*, 2019).

4.2.4 Gene prediction and annotation

For gene prediction and functional annotation, the *de novo* assembled contig sequences (in fasta format) were imported into Blast2Go (B2G) ver. 5.2.2 (Conesa *et al*., 2005; Götz *et al*., 2008, 2011). The gene prediction operation in B2G was performed using the 'Prokaryotic Gene Finding' option, which uses the Glimmer algorithm to locate genes using the ab initio (single run model) or 'hint based' (iterative run model) methodology on a prokaryotic query genome. Following gene prediction, functional annotation based on Gene Ontology (GO) data mining using the predicted gene sequences was done in B2G as follows: Briefly, the NCBI QBlast algorithm was used to find homologs to the fasta formatted gene query sequences in the non-redundant nucleotide (nr/nt) collection database. The setting to list the top 20 blast hits was used. Gene ontology terms together with their evidence codes (EC) were then extracted for each hit by mapping to existent annotation associations using Blast hit gene identifiers (gi), and gene accessions.

After mapping each query sequence, a set of candidate annotations from different hits of diverse similarity levels and various annotation sources were gathered, and an annotation rule applied to find the most specific annotations for the obtained ontologies. Default specificity and stringency options were selected for the annotation process.

The annotations for the pigment biosynthesis gene(s) obtained by B2G were validated by querying the translated gene sequences in the UniProtKB/Swiss-Prot database (*https://www.uniprot.org/uniprot/*, no date) for homologous sequences. The UniProtKB/Swiss-Prot database was used because it contains manually-annotated records with information extracted from literature and curator-evaluated computational analysis that brings together experimental results, computed features and scientific conclusions (Boutet *et al.*, 2007, 2016), as opposed to the computationally analyzed records awaiting full manual annotation obtained by the NCBI QBlast query in the non-redundant nucleotide (nr/nt) collection database.

4.2.5 Bioninformatic deduction of type of pigment produced by Planococcus sp. CP5-4-WT

Because the UV-Vis spectrum (Chapter 3, Figure 3.1) of the extracted pigment of *Planococcus* sp. CP5-4-WT resembled that of carotenoid pigment, bioinformatics guided deduction of the type of carotenoid pigment produced by this strain was conducted according to a method by Klassen (2010). Briefly, carotenoid biosynthetic enzymes with known functions were identified from the literature and their corresponding amino acid sequences retrieved from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). A multi FASTA file was created from the retrieved amino acid sequences and a multi-sequence alignment conducted in MEGA 7 (Kumar, Stecher and Tamura, 2016) using CLUSTALW. Subsequently, a phylogenetic analysis on the aligned sequences was conducted and a maximum likelihood tree constructed from the sequence alignment using MEGA 7.

4.2.6 Extraction of pigment from Planococcus sp. CP5-4-WT

Following the prediction and annotation of the pigment biosynthesis gene(s) in the *de novo* assembled contigs of *Planococcus* sp. CP5-4-WT, pigments were extracted from 5 day old, 1 L cultures, of the wild type and yellow mutant strains grown in TSB salt-sorbitol broth at room temperature with shaking at 150 rpm. Pigment from the *Planococcus* sp. CP5-4 yellow mutant strain was extracted to identify the effects of the mutation(s) induced by MMS on the structure of the pigment.

To extract pigment from the cells, the cells were first pelleted by centrifugation at 4 629 x *g* for 15 minutes in a Beckman Coulter Avanti J – HC™ refrigerated centrifuge (Indiana, USA) set at 4 °C, and washed three times with sterile water. Following washing, the cell pellets were dried and a mixture of acetone:methanol (7:3 v/v) containing 0.1 % butylhydroxytoluene (BHT) as antioxidant added. The resulting cell suspensions were subjected to freeze – thaw cycles in liquid N_2 to facilitate extraction of the pigment.

The extracts were then centrifuged at 12857 x *g* for 10 minutes in a Hermle Z446 high-volume universal centrifuge (Labnet international, Edison, New Jersey, USA) set to a temperature of 4 °C, and the coloured supernatant pipetted to foil-covered 50 mL Falcon tubes for protection against photo oxidation. Successive extractions were carried out on the cell pellets until both the solvent and the cells were colourless. The solvent phases were pooled together and evaporated to dryness under a N_2 gas stream.

Duplicate extractions were conducted for the *Planococcus* sp. CP5-4 wild type culture, and one set of the extracts saponified concurrently with the *Planococcus* sp. CP5-4 YE mutant extract. Saponification is the alkaline hydrolysis of fatty acid chains attached to pigment molecules, usually carotenoids, to facilitate the the removal unwanted lipids, which may interfere with the chromatographic separation (Schweiggert *et al.*, 2005; Saini and Keum, 2018). Saponification of the extracts was done as described by Cardinault *et al*. (2008). Briefly, the dried extracts were saponified with 5 mL of potassium hydroxide (KOH (55 mg mL^{-1})) in methanol for 1 h at room temperature and the process stopped by adding 10 mL of diethyl ether : water (1:1 v/v). The saponification mixture was centrifuged (10 min, 4629 x *g*, 4 °C) and the upper orange or yellow phase collected, washed with sterile water and recollected before being evaporated under N_2 gas to produce an 'oily' red or yellow extract. The lower phase containing the de-esterification products of carotenoid esters was discarded, and the 'oily' red and yellow residues stored together with the dried unsaponified extract under nitrogen at –80 °C until further processing. Extraction and saponification procedures were conducted in the dark.

4.2.7 Characterization of pigment extracts from Planococcus sp. CP5-4-WT

The dried unsaponified and saponified pigment extracts were re-dissolved in 500 µL of acetone containing 0.1 % BHT and loaded onto preparative thin layer chromatography (PLC) silica gel 60 F_{254} 2 mm plates measuring 4 cm x 14 cm (Merck, Darmstadt, Germany).

The dried PLC plates were developed in the dark in a chamber containing hexane: acetone (70:30) as mobile phase, and each separated pigment fraction collected. The collected silica powder containing the pigment fraction was suspended in 1 mL of acetone and centrifuged for 15 minutes at 12 857 x *g* in a Labnet prismR™ (Woodbridge, New Jersey, USA) refrigerated bench top centrifuge set at 4 °C. The coloured SNTs were collected, dried under N_2 stream, and their relative molecular masses measured. A Waters Synapt G2 High-Definition (HD) quadrupole time-of-flight (qTOF) mass spectrometer (MS) connected to a Waters Acquity ultra-performance liquid chromatograph (UPLC) (Waters, Milford, MA, USA) was used for UPLC-MS analysis. Five microliters of sample was injected into the mobile phase at equilibrium starting conditions. This conveyed the sample plug onto a Waters BEH C_{18}, 2.1 × 100 mm, 1.7 µm column thermostatted at 45 °C where separation of compounds took place. The mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid as solvent B. Elution from the column was carried out using a gradient of 30% solvent A and 62% solvent B for 5 mins, then a step to 100% (solvent B) for 25 mins. The column was returned to the initial conditions (30% solvent A and 62% solvent B) and equilibrated over 30 mins. A flow rate of 0.4 mL/min was employed.

After emerging from the column, the flow stream passed first to a photodiode array detector (Waters eλ PDA) set to measure in the 200 nm – 600 nm wavelength range, and then to the qTOF MS where data was acquired by scanning from m/z 150 to 1500 m/z in MSe positive mode. Data was acquired using two separation functions; F1: at a low collision energy (4 V) and F2: using a collision energy ramp (25−60 V) to simultaneously acquire both unfragmented and fragmented data. Leucine enkaphalin was used as lock mass (reference mass) for accurate mass determination and the instrument was calibrated weekly with sodium formate. The following MS settings were used: cone voltage of 15 V, desolvation temperature of 275 °C, desolvation gas at 650 L/h, and the rest of the MS settings optimized for best resolution and sensitivity. Data acquisition, processing, and elemental composition calculations were conducted using the Targetlynx module of MassLynx (v 4.1, Waters, Milford, USA).

4.2.8 Read mapping and variant calling for identification of mutations

After the identification and characterization of the pigment biosynthesis gene(s) in the *de novo* assembled draft genome of the *Planococcus* sp. CP5-4-WT strain, and the characterization of the pigment extracts from both the wild type and the yellow mutant strain, the effects of the MMS mutagenesis on the genomes of the yellow and the unpigmented *Planococcus* sp. CP5-4 strain were investigated.

For the identification of the mutations, the *de novo* assembled and annotated *Planococcus* sp. CP5-4-WT contigs were first ordered against the *Planococcus* species genome having the highest % ANI (% fraction of genome shared) with species CP5-4 as determined by the GTDB-Tk classification in section 4.2.3. The Mauve Contig Mover (MCM) software (Darling *et al.*, 2004; Rissman *et al.*, 2009) was used for the ordering of the contigs to produce the draft genome used as a reference during reading mapping and variant calling operations. Following this step, read mapping and variant calling were done in Geneious ver. 11.1.5 (https://www.geneious.com, no date; Kearse *et al.*, 2012).

Sequence reads were paired as described in section 4.2.2, then DNA bases of low quality were trimmed from the ends of the reads using the 'Trim with BBDuk' add on ("https://sourceforge.net/projects/bbmap/" n.d.). Trimming options were set with the option to 'Discard Short Reads' set to a minimum length of 20. As a result, the reads were trimmed from 5' and 3' ends according to their quality scores. Trimmed reads were subsequently mapped to the *Planococcus* sp. CP5-4-WT draft genome sequence using Geneious as the mapper. Sensitivity of the operation was set on 'Medium-Sensitivity/Fast' and the 'Fine Tuning' option set on 'iterate' up to five times to improve the results by aligning reads to each other in addition to the reference sequence. Following mapping, regions of low coverage were annotated for exclusion during variant calling. Annotation options for low coverage regions were set to exclude regions with coverage below a standard deviation of two from the mean. Thus, regions with coverage of 0 to 60 were annotated and subsequently excluded during variant calling. Variants were detected in the mapped data using default settings.

The option to 'Analyze the Effect of Polymorphisms on Translation' was chosen, and the default genetic code changed to 'Bacterial'. The Bacterial genetic code was chosen because it uses the coding sequence (CDS) annotations on the bacterial reference genome to determine the CDS of mapped reads and calculate whether the detected variants cause a change in the amino acid sequence.

4.2.9 PCR verification of variants

Verification of the detected sequence variants from the mapping and variant calling operations done using the paired end sequence reads of the yellow and unpigmented mutant strains' DNA was done by PCR amplification. Primer pairs CrtDel 1F/ CtrDel 1R (5'-TATGCTGGAATGGACATAT-3'/ 5'-GTTAATTGCTTTCGTTATC-3'), and CrtDel 2F/ CrtDel 2R (5'-ATGGCAAAACGAATGATTAT -3'/ 5'- TCATACATATCCCTCCACTT -3') were used for the verification of the sequence variations in the yellow and unpigmented mutant strains' genomes respectively. The primer pairs were designed using the 'manual creation of new primers' tool in Geneious ver. 11.1.5 (https://www.geneious.com, no date). Regions on the wild type genome missing the sequence variants responsible for the expression of the yellow and unpigmented mutant phenotypes following the read mapping and variant calling operations were selected for primer design. A detailed description of these regions and selected primer annealing sites is given in section 4.3.7.

PCR amplifications were performed in 50 µL volumes containing 100 ng template DNA, 1.25 units of Dream Taq polymerase, 10x Dream Taq PCR buffer, 0.2 mM of deoxynucleoside triphosphates (dNTPs), and 0.1 µM of each primer. Both verification PCRs were subjected to an initial denaturation of 5 min at 95 °C for 1 cycle followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing for 30 sec, extension at 72 °C and a final extension at 72 °C for 5 min. The CrtDel 1 primer pair was annealed at 52.6 °C while the CrtDel 2 primer pair was annealed at 58 °C, and extension carried out for 3 min 50 sec for the CrtDel 1 primer pair and 1 min 35 sec for the CrtDel 2 primer pair.

4.3 Results

4.3.1 Genome sequencing and analysis

Genome sequencing of the three *Planococcus* sp. CP5-4 strains and the analysis of the genomes was conducted to locate the MMS-induced mutations on the genomes of the yellow and white mutant strains, which would lead to the identification of the genes and/or pathways that encode the pigments. The number of paired end reads generated for the three *Planococcus* sp. CP5-4 strains following WGS is given in Table 4.1. The reads had a minimum length of 35 bp and a maximum length of 251 bp prior to trimming, and a minimum length of 10 bp post trimming using BBDuk Geneious trimmer.

Table 4.1 Number of paired end reads generated from sequencing the *Planococcus* sp. CP5-4 genomes

***Planococcus* sp. CP5 – 4 strain**	**Paired end reads**	**Coverage**	**Paired end reads after trimming**	**Coverage after trimming**
WT	2 336 074	104.3 x	2 308 726	103.1 x
YE	2 593 058	115.8 x	2 509 100	112 x
UN	2 246 878	100.3 x	2 223 122	99.2 x

De novo assembly of the *Planococcus* sp. CP5-4-WT strains' trimmed reads produced 3 875 contigs, 34 of which had a length greater than 1000 bp with a minimum length of 1 014 bp and a maximum length of 574 799 bp. Of the 2 308 726 reads, 2 279 190 were assembled into contigs leaving 29 536 unassembled reads. The detailed Geneious assembly report with the N50 values is given in Table 4.2. All contigs with a minimum length less than 1000 bp were excluded from further analyses.

Table 4.2 *De novo* assembly report for *Planococcus* sp. CP5-4 wild type strain

Statistics	All contigs	Contigs ≥ 100 bp	Contigs ≥ 1000 bp
Number of	3 875	3 734	34
Min length (bp)	25	100	1 014
Median length (bp)	289	294	48 919
Mean length (bp)	1 207	1 250	102 618
Max length (bp)	574 799	574 799	574 799
N50 length (bp)	182 393	182 393	241 991
Number of contigs ≥ N50	7	7	4

4.3.2 Taxonomic classification and novelty of Planococcus sp. *CP5-4-WT*

The closest relatives found through the GTDB-Tk analysis for *Planococcus* sp. CP5-4-WT are shown in Table 4.3, while the GTDB taxonomic classification of *Planococcus* sp. CP5-4-WT is depicted in Figure 4.1. The genus *Planococcus* was first proposed for accommodating motile cocci more than a century ago (Kocur *et al.*, 1970; Nakagawa, Sakane and Yokota, 1996). The motile cocci of the *Planococcus* genus were subsequently divided into two groups on the basis of the guanine-cytosine (G+C) content of their DNA (Kocur *et al.*, 1970; Shivaji, 2015). Species with a low G+C content (39.6 to 42.2 mole %) were assigned to group 1 and were identified as *Planococcus kocurii* (Van Hao and Komagata, 1985), while those with high G+C content (48.0 to 52.1 mole%) were assigned to group 2 and identified as *Planococcus citreus* (Shivaji, Srinivas and Reddy, 2014).

Consequently, the genus *Planococcus* was comprised of the following species: *P. citreus*, *P. kocurii, P. alkanoclasticus, P. okeanokoites* (previously classified as *Flavobacterium ohìkeanokoites* (ZoBell and Upham, 1944; Nakagawa, Sakane and Yokota, 1996)), *P. mcmeekinii, P. antarcticus, P. psychrophilus,* and *Planococcus* sp. isolate SOS orange (Romano *et al.*, 2003).

Subsequently, Yoon *et al.* (2001), proposed a new genus related to *Planococcus*, named *Planomicrobium*, comprising *Planomicrobium koreense*, *P. okeanokoites* and *P. mcmeekinii*, the latter previously classified as a species in the genus *Planococcus*. In 2005 the genus *Planococcus* comprised eight recognized species, *Planococcus citreus* (Kocur *et al.*, 1970), *Planococcus kocurii* (Van Hao and Komagata, 1985), *Planococcus alkanoclasticus* (Engelhardt *et al.*, 2001), *Planococcus antarcticus* and *Planococcus psychrophilus* (Reddy *et al.*, 2002), *Planococcus maritimus* (Yoon *et al.*, 2003), *Planococcus rifietoensis* (Romano *et al.*, 2003) and *Planococcus maitriensis* (Atam *et al.*, 2003). However, the number of recognized *Planococcus* species now stands at 27 and an additional 414 unclassified species in the NCBI taxonomy database (*http://www.ncbi.nlm.nih.gov/taxonomy*, no date; Schoch *et al.*, 2020).

Use of the GTDB-Tk for classification of *Planococcus* sp. CP5-4-WT revealed that the genus *Planococcus* in the NCBI taxonomic database is polyphyletic, and has been dereplicated into three distinct genera, i.e *Planococcus*, *Planococcus*_A, and *Planococcus*_B, based on relative evolutionary divergence. An alphabetic suffix in the GTDB taxonomy (e.g. *Planococcus*_B) indicates that, within the GTDB tree, the genus does not belong to a monophyletic group with the type species of that genus, in this case, *Planococcus maritimus* Y42.

Table 4.3 Average nucleotide identity comparison between *Planococcus* sp. CP5-4-WT and closely related GDTB *Planococcus* species

GTDB related reference	Unfiltered NCBI Taxonomy	Genome id	ANI (%)[a]	AF[b]	Isolate Attributes	Geographilcal location and environment of origin	Reference
Planococcus_B sp. 002833405	*Planococcus* sp. MB-3u-03	GCF_002833405.1	89.49	0.83	Gram-positive, orange/yellow, aerobic, non-sporulating, psychrotrophic coccoid bacterium.	Isolated from cold surface coastal water of the Pacific Ocean near Santa Cruz, CA (+37°0′12.32″N, −122°11′9.51″W)	Pearson and Noller, 2011
Planococcus_B sp. CP5-4	ND[c]	ND[c]	ND[c]	ND[c]	Gram-positive, orange, mesophilic, coccoid bacterium.	Isolated from brine samples from Cerebos crystallizer salt ponds in Velddrif, Western Cape, South Africa (S 32°47′10,632, E 18°10′9,499).	This study
Planococcus_B rifietoensis	*Planococcus rifietoensis* strain M8	GCF_001465795.2	89.44	0.87	Gram-positive, brilliant orange, mesophilic, aerobic/microaerophilic, non-sporulating coccoid bacterium.	Isolated from an algal mat collected from a sulfurous spring in Campania (Italy) (41.22852” N, 15.179250000000025 E”).	Romano *et al.*, 2003

GTDB related reference	Unfiltered NCBI Taxonomy	Genome id	ANI (%)[a]	AF[b]	Isolate Attributes	Geographilcal location and environment of origin	Reference
Planococcus_B maitriensis	*Planococcus maitriensis* strain S1	GCA_003289925.1	87.57	0.82	Gram-positive, orange, aerobic, psychrophilic, motile, non-sporulating, coccoid bacterium.	Isolated from a cyanobacterial mat sample collected from Schirmacher Oasis in Antarctica.	Atam *et al.*, 2003
Planococcus_B plakortidis	*Planococcus plakortidis* DSM 23997	GCF_001687605.2	86.80	0.82	Gram-positive, yellow-orange, aerobic, non-spore-forming, mesophilic, motile, coccoid bacterium.	Isolated from a marine sponge [*Plakortis simplex* (Schulze)], collected at a depth of 30 m in the Bay of Bengal, off the coast of Gopalpur in the Indian state of Orissa (84° 43.907′ N and 18° 57.169′ E).	Kaur *et al.*, 2012
Planococcus_B maritimus	*Planococcus maritimus* DSM 17275	GCF_001687625.2	86.54	0.83	Gram-positive, strictly aerobic, motile, yellow-orange, psychrotrophic, coccoid bacterium.	Isolated from sea water of a tidal flat in Korea	Yoon *et al.*, 2003
Planococcus_B maritimus	*Planococcus maritimus* MKU009	GCF_001592825.1	86.29	0.84	Gram-positive, yellow, coccoid bacterium.	Isolated from surface marine waters of Pichavaram (11.45N, 79.79E), South East Coast, Tamil Nadu, India	Ganapathy, Jayavel and Natesan, 2016

[a] GTDB determined percentage average nucleotide identity to *Planococcus*_B sp. CP5-4 query genome
[b] GTDB determined genome alignment fraction values for the query genome and the closet relative's genome
[c] Not determined for the query genome

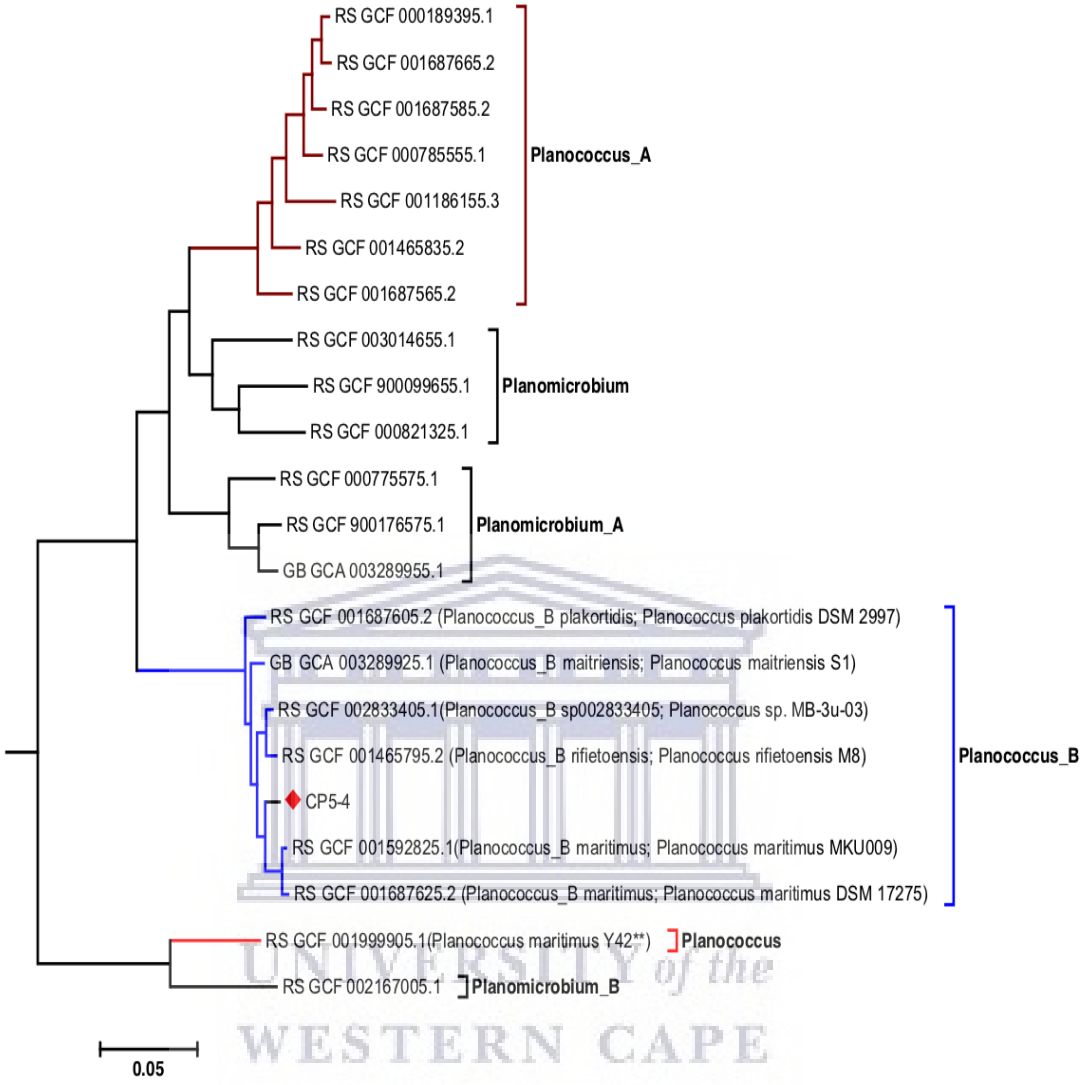

Figure 4.1 GTDB-Tk classification of *Planococcus* sp. CP5-4-WT based on the relative evolutionary distance of its genome to other members of the *Planococcus*_B genus. The genome identifiers of the organisms are shown on the tree branches, where appropriate, both the GTDB (Release 86.2). and unfiltered NCBI taxonomy names are given in parenthesis. The tree is based on the topology of the genomes and not on bootstrap resampling as this is computationally prohibitive and consequently may over-classify genomes relative to manual curation based on unsupported affiliations of user genomes to reference taxa. Part of the GTDB-Tk bac120 genome tree is shown.

All the closest related relatives to *Planococcus* sp CP5-4-WT belonged to the *Planococcus*_B genus. The species comprising the *Planococcus*_B genus were isolated from different saline environments except for *Planococcus*_B *rifietoensis* (Table 4.3).

Furthermore, members of the *Planococcus*_B genus are Gram-positive, yellow/orange, none-spore forming, coccoid motile bacteria with varying growth temperature and oxygen requirements (Atam *et al.*, 2003; Yoon *et al.*, 2003, 2010; Rajput *et al.*, 2013; Shivaji, Srinivas and Reddy, 2014; See-Too *et al.*, 2016) and genome sizes ranging from 3 251 644 bp to 3 614 431 bp with G+C contents ranging from 47.2 to 50.0 mole %.

The placement of *Planococcus* sp. CP5-4-WT in the concatenated bacterial protein genome tree (Figure 4.1) indicates that CP5-4 is a new species in the *Planococcus*_B genus. This designation is based on the assigned ANI of 89.49% to the closest related reference genome of *Planococcus*_B sp. 002833405 being less than the species-specific ANI circumscription radius of 95 % and the alignment fraction (AF) being greater than 0.65 (Table 4.3).

4.3.3 Gene prediction and annotation

Gene prediction and annotation were performed using B2G (Conesa *et al.*, 2005; Götz *et al.*, 2008). The draft genome of *Planococcus*_B sp. CP5-4-WT consisting of 34 contigs is 3 488 448 bp with a G+C content of 47.5 %. A total of 28 863 open reading frames (ORFs) were identified in the draft genome of which 3 469 were protein coding genes along with 59 tRNA and 17 rRNA genes (Table 4.4). Eighty three percent of the genes predicted by glimmer were annotated in B2G. Of the 17 % of genes without annotation, 11 % had Blast hits while 3 % had no Blast hits following the QBlast homology search operation during annotation. Furthermore, the remaining 2% of the genes without annotation were mapped to existing GO terms.

Table 4.4 Genome features of *Planococcus*_B sp. CP5-4-WT strain

Attributes	Value
Genome size*	3 488 448
Number of contigs	34
G+C content (%)	47.5
Number of ORFs	28 863
Number of genes	3 469
tRNA	59
rRNA	17
Genes per strand (+/-)	1 790 / 1 679
Length of genes (min/max) *	114 / 4 590
Average gene length*	844.73

*Length in base pairs

Eight genes associated with carotenoid biosynthesis were predicted on contigs 1 and 5 of the *de novo* assembled genome of *Planococcus*_B sp. CP5-4-WT by B2G. A query of the translated protein sequence of the B2G annotated carotenogenic genes of *Planococcus*_B sp. CP5-4-WT in the UniProtKB/Swiss-Prot database matched the query amino acid (aa) sequences to the homologs listed in Table 4.5, which also shows the assigned functions of the homologs as given in the UniProtKB/Swiss-Prot database.

Table 4.5 List of the genes associated with carotenoid biosynthesis identified on contigs 1 and 5 of the *de novo* assembled genome of *Planococcus*_B sp. CP5-4-WT

ORF	Size (bp)	B2G Annotation	UniprotKB/Swiss-Prot closest homolog	% Identity	Assigned Function
00648	678	Glycerol acyltransferase (*agpat*)	Acyl-phosphate glycerol 3-phosphate acyltransferase Protein (A0A2I0ESI3; *Planococcus sp*. Urea-3u-39) [a]	94.6	Mediates the transfer of an acyl group from one compound (donor) to another (acceptor).
00649	1104	Glucosyl transferase, family 2 (*gtf2*)	Glycosyl transferase family 2 protein (A0A0U2PCT2; *Planococcus rifietoensis*)[a].	94.6	Mediates the transfer of a glycosyl group from a donor compound to an acceptor.
00650	1506	Apo-4, 4'-lycopene oxygenase (*crtNb)*	4,4'-diapolycopene oxygenase (Q4VKU9*; Methylomonas* sp.)[b].	35.8	Mediates the oxidation of the terminal methyl side groups of 4,4'-diapolycopene to yield 4,4'-diapolycopen-4,4'-dial via the aldehyde intermediate 4,4'-diapolycopen-al. Also able to catalyze the oxidation of the terminal methyl side group of 4,4'-diaponeurosporene to form 4,4'-diaponeurosporen-4-al during C_{30} carotenoid biosynthesis
00653	1452	Apo-4, 4'-lycopene aldehyde oxidase (*crtNc*)	4,4'-diapolycopen-4-al dehydrogenase or alternatively 4,4'-diapolycopen-4-al oxidase (P0DPE9 (CRTNC_BACID); *Bacillus indicus*)[b].	53.5	Involved in the biosynthesis of C_{30} carotenoids. Mediates the oxidation of 4,4'-diapolycopen-4-al to yield 4,4'-diapolycopen-4-oic acid.

ORF	Size (bp)	B2G Annotation	UniprotKB/Swiss-Prot closest homolog	% Identity	Assigned Function
00652	831	Phytoene synthase (*crtM*)	Phytoene/squalene synthase family protein (A0A3M8P827; *Planococcus salinus*)[a].	77.9	Mediates the two steps reaction converting geranylgeranyl diphosphate to phytoene via prephytoene diphosphate, or conjugation of two prenylated farnesyl pyrophosphate (FPP) precursor molecules to form 4,4'-diapophytoene or apo-4,4'-phytoene during C_{50}, C_{40} or C_{30} carotenoid biosynthesis, respectively.
00654	1527	Apo-4, 4'-phytoene desaturase (*crtN*)	4,4'-diapophytoene desaturase (Q4VKV1 (CRTN_METSP); *Methylomonas* sp.)[b].	52.3	Involved in the biosynthesis of C_{30} carotenoids. Catalyzes four successive dehydrogenation reactions that lead to the introduction of four double bonds into 4,4'-diapophytoene (dehydrosqualene) to yield 4,4'-diapolycopene.
00174	1200	Cytochrome P450 hydroxylase	Cytochrome P450 107B1 (A0A098EMI8_9BACL; *Planococcus massiliensi*)[a].	77.3	Mediates the hydroxylation of carotenoids.
00178	1503	CrtNb-like apo-4, 4'- terminal methyl oxidase (*crtP*)	4,4'-diaponeurosporene oxygenase (Q4L978 (CRTP_STAHJ); *Staphylococcus haemolyticus* (strain JCSC1435))[b].	60.3	Involved in the biosynthesis of the yellow-orange carotenoid staphyloxanthin. Mediates the oxidation of the terminal methyl side group of 4,4'-diaponeurosporene to form 4,4'-diaponeurosporen-4-al.

[a] Unreviewed TrEMBLE top hit
[b] Reviewed Swiss-Prot top hit

4.3.4 Bioinformatic deduction of the type of pigment produced by Planococcus_B sp.CP5-4-WT

Guided by the results of the gene prediction and annotation processes, which pointed to the production of a carotenoid pigment by *Planococcus*_B sp. CP5-4-WT, a deduction of the type of carotenoid produced by this organism was conducted. A multiple sequence alignment and phylogenetic grouping of the phytoene synthase (CrtB) and the phytoene/squalene synthase family (CrtM) proteins from different bacterial families according to the method by Klassen, (2010) formed the basis of the analysis. Phytoene synthase (CrtB) mediates the formation of the C_{40} carotenoid phytoene by the head-to-head condensation of two molecules of C_{20} geranylgeranyl pyrophosphate (Dogbo *et al.*, 2006; Yatsunami *et al.*, 2014), while CrtM mediates the synthesis of the C_{30} carotenoid 4,4'-diapophytoene from two molecules of C_{15} farnesyl pyrophosphate (Klassen, 2010; Perez-Fons *et al.*, 2011; Sabine Steiger *et al.*, 2012; Yatsunami *et al.*, 2014; Mariutti and Mercadante, 2018).

The amino acid sequences of these homologous enzymes are conserved in all carotenogenic taxa and together represent the first dedicated step in carotenoid biosynthesis, making them highly informative to determine the overall phylogenetic topology of carotenoid biosynthesis (Klassen, 2010). The maximum likelihood phylogenetic tree produced from the multiple sequence alignment of all analyzed CrtB and CrtM is shown in Figure 4.2. Four major carotenoid evolutionary lineages are defined in Figure 4.2 based on the clustering of the CrtB/M sequences. The first lineage contains five sub-clades with CrtB sequences from the:

1. Bacteriodetes,
2. C_{50} carotenoid producing Actinobacteria,
3. C_{40} carotenoid producing Actinobacteria,
4. Beta carotene and linear C_{50} carotenoid producing Archaea, and the

5. Linear C_{50} carotenoid producing Archaea.

The second lineage contains CrtB sequences from the alphaproteobacteria, while the third and fourth lineages contain CrtM sequences from the gammaproteobacteria Methylomonas sp. DH1 and the Firmicutes, respectively.

The CrtM from *Planococcus*_B sp. CP5-4-WT and the other members of the *Planococcus*_B genus were clustered together with those of other C_{30} carotenoid producing Firmicutes. One major discrepancy, though, is the placement of the CrtB sequence from *Planococcus hypocryophilus* Ori 1, which is clustered with the CrtM sequences of the C_{30} carotenoid producing Firmicutes that include amongst others genera, the *Planococcus*_A and *Planococcus*_B genera in this analysis.

A probable reason for this is that the CrtB of *Planococcus hypocryophilus* was misannotated. According to Schnoes *et al.* (2009), misannotations of enzymes in superfamilies containing multiple enzyme families that catalyze different reactions (for example the phytoene/squalene/diapophytoene enzyme family) is a common problem in public unreviewed databases such as GenBank, TrEMBL, and the protein sequences in KEGG as a result of 'overprediction' of molecular function. Cases of overprediction include those proteins that have been annotated to functions that are more specific than the available evidence supports (Schnoes *et al.*, 2009). Thus, as the number of sequences deposited in these databases increases because of advancements in sequencing technologies and the drop in the price of sequencing, the problem of misannotation will continue to impact negatively on evolutionary studies as well as analyses of pathways, and metabolic processes if not addressed.

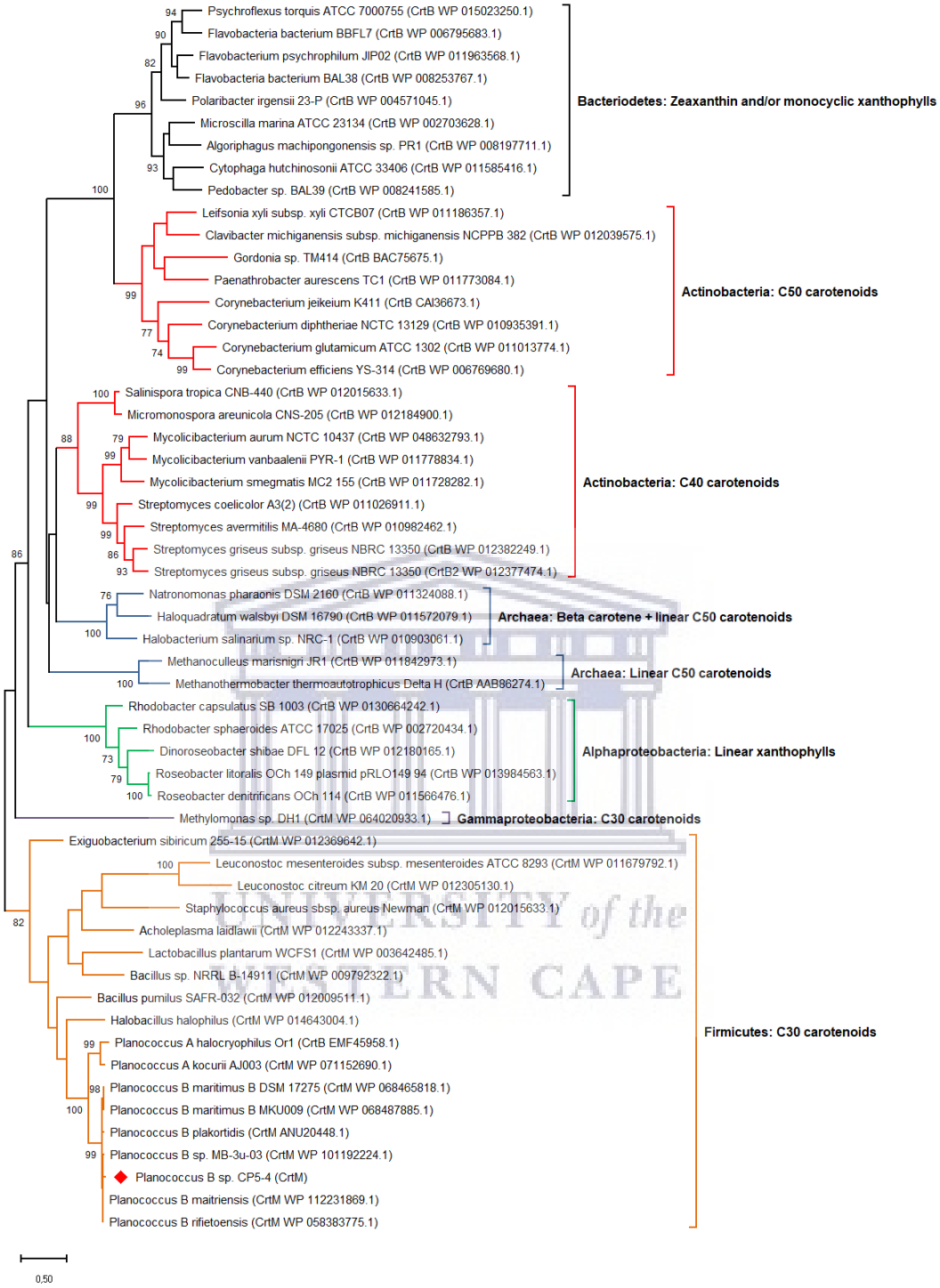

Figure 4.2 Maximum likelihood phylogenetic tree of bacterial CrtB and CrtM protein sequences constructed in MEGA 7 (Kumar, Stecher and Tamura, 2016). Bootstrap values greater than 70 % are shown on the tree. Carotenoid chain lengths of each bacterial family represented are indicated on the right of each clade. Note; not all carotenoid structures and bacterial lineages are represented on the tree. The analysis involved 38 CrtB and 17 CrtM sequences. The accession numbers of the sequences are included in parenthesis.

4.3.5 Pigment synthesis gene organization in Planococcus_B sp. CP5-4-WT

Following the tentative determination of the type of carotenoids produced by *Planococcus*_B sp. CP5-4-WT, the organization of the carotenogenesis genes, and how it differed from that of well-established C_{30} carotenoid producers, as well as from that of the other members of the *Planococcus*_B genus was investigated. Six of the eight carotenogenesis genes identified in this study were located on contig 1 as a 7876 bp biosynthetic cluster in which the *crtN*, *crtNc*, and *crtM* genes are separated by a 69 bp space from the *crtNb*, *gtf2*, *agpat*, and a hypotheical protein gene; both these sub-clusters are transcribed in opposite directions (Figure 4.3 A). The organization of the carotenogenic genes on contig 1 is similar to that of the gene clusters of other C_{30} carotenoid-synthesizing bacteria: *Halobacillus halophillus* (Köcher *et al.*, 2009) and *Planococcus*_A *kocurii* (NCBI unfiltered taxonomy name *Planococcus faecalis* AJ003T(Kim *et al.*, 2018)), albeit with little sequence similarity (Figure H1, Appendix H). The rest of the carotenogenesis genes, which include *crtP* and a putative cytochrome P450 hydrolyase gene were located on contig 5 (Figure 4.3 B). Cytochrome P450 hydroxylases mediating the hydroxylation of carotenoids in *Thermus thermophilus* HB27 and in *Deinococcus radiodurans* have been reported in the literature (Greule *et al.*, 2018).

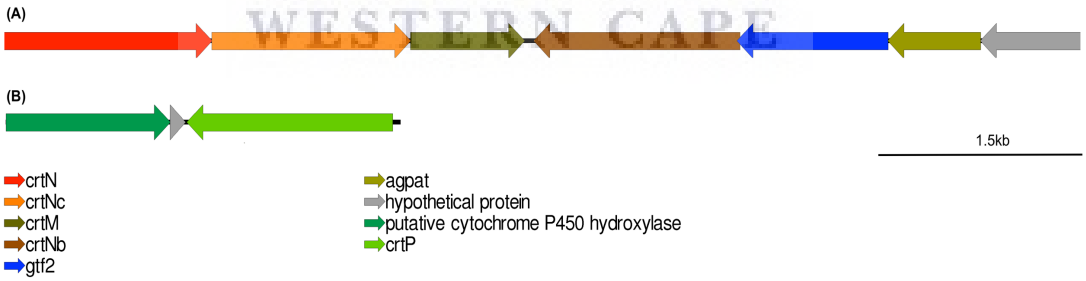

Figure 4.3 Organization of the carotenoid biosynthesis genes on (A) contig 1 and (B) contig 5 of the *Planococcus*_B sp. CP5-4-WT draft genome.

The location of the last 2 carotenoid biosynthesis genes away from the 7876 bp gene cluster on contig 1 is not unique to the *Planococcus*_B sp. CP5-4-WT strain because it is often reported that one or two carotenoid biosynthesis genes are located far away from a carotenoid gene cluster, for instance in the carotenoid pathway gene organization in *Staphylococcus aureus* (Kim and Lee, 2012), *Planococcus*_A *kocurii* (Kim *et al.*, 2018), *Halobacillus halophilus* (Köcher *et al.*, 2009), and *Methylomonas* sp. (Tao *et al.*, 2005). One or two carotenoid biosynthesis genes were also found to be located far away from the carotenoid biosynthetic gene cluster loci of the other members of the *Planococcus*_B genus, the number and locations of which are listed in Table 4.6. The names of the additional carotenogenic genes and their products are given in Table H 1 in Appendix H.

Table 4.6 Location of carotenoid biosynthesis genes in the genomes of members of the *Planococcus*_B genus

Organism	Genome id	Location of carotenoid BSGC[a] on genome	Number of additional Crt[b] synthesis genes	Location on genome
Planococcus_B sp. 002833405	GCF_002833405.1	2 877 287 – 2 885 162	2	487 280 – 488 110 745 224 – 746 726
Planococcus_B rifietoensis	GCF_001465795.2	2 695 415 – 2 703 298	2	194 975 – 195 805 462 371 – 463 873
Planococcus_B maitriensis	GCA_003289925.1	QLZQ01000001* 188 992 – 196 874	1	QLZQ01000003* 314 523 – 315 353
Planococcus_B plakortidis	GCF_001687605.2	2 815 485 – 2 823 385	2	521 501 – 522 331 730 457 – 731 959
Planococcus_B maritimus	GCF_001687625.2	2 866 001 – 2 873 867	2	500 800 – 501 630 740 416 – 741 924
Planococcus_B maritimus	GCF_001592825.1	LTZG01000001* 1 234 455 – 1 242 321	1	LTZG01000011* 183 819 – 185 321

[a] Biosynthetic gene cluster

[b] Carotenoid

* NCBI whole genome shotgun sequencing project accession number

An extra phytoene/squalene synthase gene was present in the genomes of *Planococcus*_B *maritimus* (GCF_001687625.2), *Planococcus*_B *maitriensis*, *Planococcus*_B *rifietoensis*, and *Planococcus*_B sp. 002833405 (Table H 1; Appendix H). Thus far, only one phytoene/squalene synthase gene has been identified to regulate either phytoene or 4, 4'-diapophytoene synthesis in carotenogenic bacteria, while in many plants it has been shown that they contain two or more phytoene synthase paralogs having overlapping roles in carotenogenesis. For example, in rice and maize, endosperm carotenoid accumulation requires the expression of one of the two phytoene synthase genes, while carotenogenesis in photosynthetic tissues requires the expression of both phytoene synthase genes (Gallagher *et al.*, 2004; Li *et al.*, 2008). However, because of the lack of research on the duplication of the phytoene/squalene synthase gene in the carotenoid biosynthetic pathway of bacteria and the effect of the duplication on carotenoid biosynthesis, it can only be surmised that this duplication allows for sophisticated cooperative control of carotenoid biosynthesis and accumulation than in bacteria with a single phytoene/squalene synthase gene. It is hoped that this finding will encourage the study of duplicated genes in the carotenoid biosynthetic pathways of bacteria and their impacts on the final carotenoid produced.

Notably, all the carotenoid biosynthetic gene clusters of the other members of the *Planococcus*_B genus are also comprised of two sub-clusters arranged in the same orientation as those on contig 1 of the *Planococcus*_B sp. CP5-4-WT strain, albeit with different lengths ranging from 7875 to 7900 bp. A BLASTx comparison of all the carotenoid biosynthetic gene clusters of the *Planococcus*_B species' listed in Table 4.6 with that of *Planococcus*_B sp. CP5-4-WT revealed that the carotenogenic protein products of the gene clusters shared a statistically significant similarity (70 to 100%; Figure 4.4) that reflects common ancestry. Thus, indicating that the proteins produced by the genes of the clusters compared may have similar structures and functions, and may mediate the synthesis of an identical C_{30} carotenoid chain by all the members of the *Planococcus*_B genus.

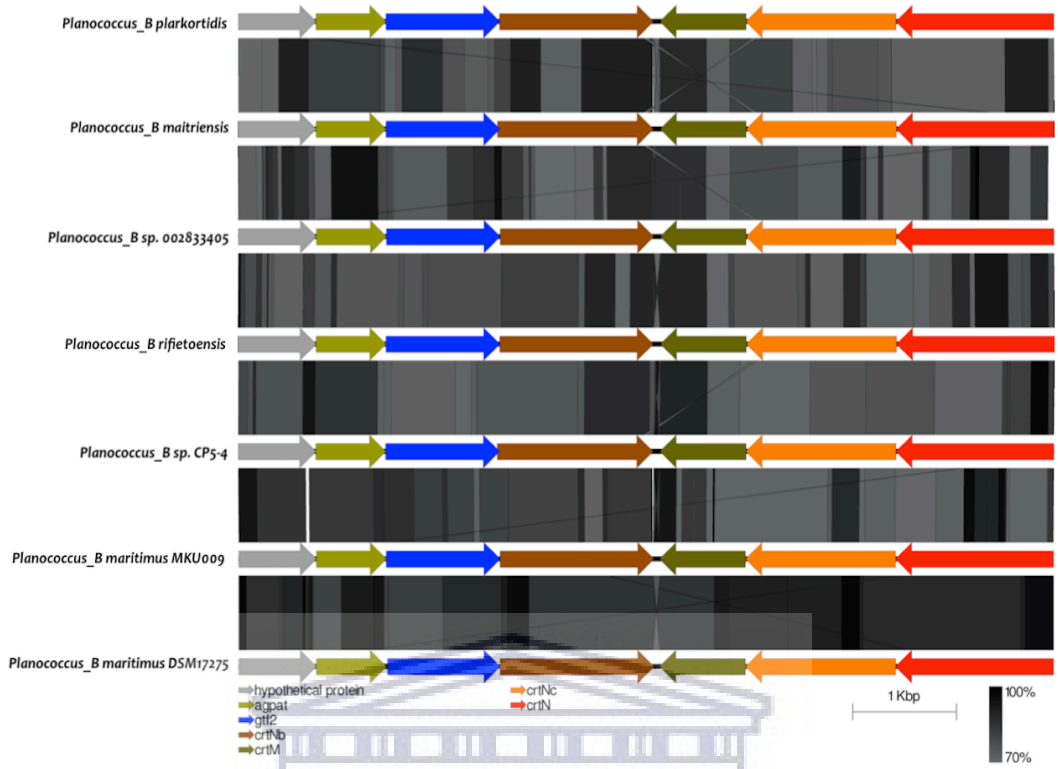

Figure 4.4 EasyFig BLASTx comparison of the carotenoid biosynthetic gene clusters of all the *Planococcus*_B species.

4.3.6 Read mapping and variant calling for the identification of mutations in the Planococcus_B sp. CP5-4-YE and UN strains' genomes.

A major challenge with chemical mutagenesis using MMS stems from the large number of mutations induced per genome, which requires implementation of tedious genetic mapping techniques that make use of genetic markers and mapping strains to locate the causative mutation to a defined genomic region (Sarin *et al.*, 2008; Haelterman *et al.*, 2014; Li *et al.*, 2016). However, to save on time and to accurately locate the MMS induced mutations that resulted in the production of the *Planococcus*_B sp. CP5-4 yellow and unpigmented mutant strains, the genomes of these strains were sequenced and the sequenced reads mapped to the reference *Planococcus*_B sp. CP5-4-WT draft genome for variant calling.

Since *Planococcus*_B sp. 002833405 shared a large portion of its genome (89.49%) (Table 4.6) with *Planococcus*_B sp. CP5-4-WT, it was used as a reference for contig ordering using Mauve Contig Mover (MCM) (Darling *et al.*, 2004; Rissman *et al.*, 2009) to produce a draft genome, from the ordered and concatenated contigs, for use as a reference for the read mapping and variant calling operations to identify the mutations on the genomes of the yellow and unpigmented strains. Not all trimmed paired end reads generated following WGS of the *Planococcus*_B sp. CP5-4-YE and UN mutant strains were mapped to the *Planococcus*_B sp. CP5-4-WT draft genome. The numbers of both mapped, and unmapped sequence reads are given in Table 4.7.

Table 4.7 Number of paired end sequence reads from the yellow and unpigmented *Planococcus*_B sp. CP5-4 strains to the wild type strain draft genome.

***Planococcus* sp. CP5 – 4 strain**	**Number of trimmed paired end reads**	**Number of reads mapped to reference**	**Number of reads not mapped**
YE	2 509 100	2 426 677	82 423
UN	2 246 878	2 191 836	55 042

Variant calling following read mapping revealed the following types of variants: structural variations (SVs), single nucleotide polymorphisms (SNPs, transitions and transversions), substitutions, deletions, and truncations.

Structural variations, specifically long sequence deletions from 462 453 bp to 465 477 bp, and from 2 556 971 bp to 2 703 240 bp of the *Planococcus*_B sp. CP5-4 wild type strain draft genome were identified to be responsible for the yellow, and unpigmented phenotypes respectively. Until recently, SNPs were thought to be the predominant form of genomic variation induced by exposure to DNA alkylating agents such as MMS (Olivier, 2003). However, recent developments and applications of genome wide technologies have led to the discovery of thousands of copy number variants (CNVs; classified as SVs) in genomes (Peterson and Freeman, 2014). Copy number variants are defined as a duplication or deletion (i.e., a gain or loss of a genomic DNA segment relative to a reference sample) measuring greater than 1 Kb in size (Freeman *et al.*, 2006).

The mapped reads of the *Planococcus*_B sp. CP5-4 YE strain to the *Planococcus*_B sp. CP5-4 wild type draft genome revealed a truncation of the CrtNb-like apo-4, 4'-terminal methyl oxidase gene in the YE strains' genome, which was due to a 3 Kb sequence deletion encompassing part of the CrtNb-like apo-4, 4'-terminal methyl oxidase gene and three additional ORFs (Figure 4.5). The effect that this truncation had on the carotenoid structure will be discussed in section 4.3.8.

Likewise, the sequence reads of the *Planococcus*_B sp. CP5-4-UN strain mapped to the *Planococcus*_B sp. CP5-4 draft genome revealed a long sequence deletion, approximately 146 Kb, containing genes that included the *crtN, crtNb, crtNc, crtM,* glycosyl transferase, and glycerol acyl transferase genes (Figure 4.6). Deletion of the core carotenogenesis, and 'additional modifying' genes from the UN strain following MMS exposure resulted in the loss of pigment production by this strain.

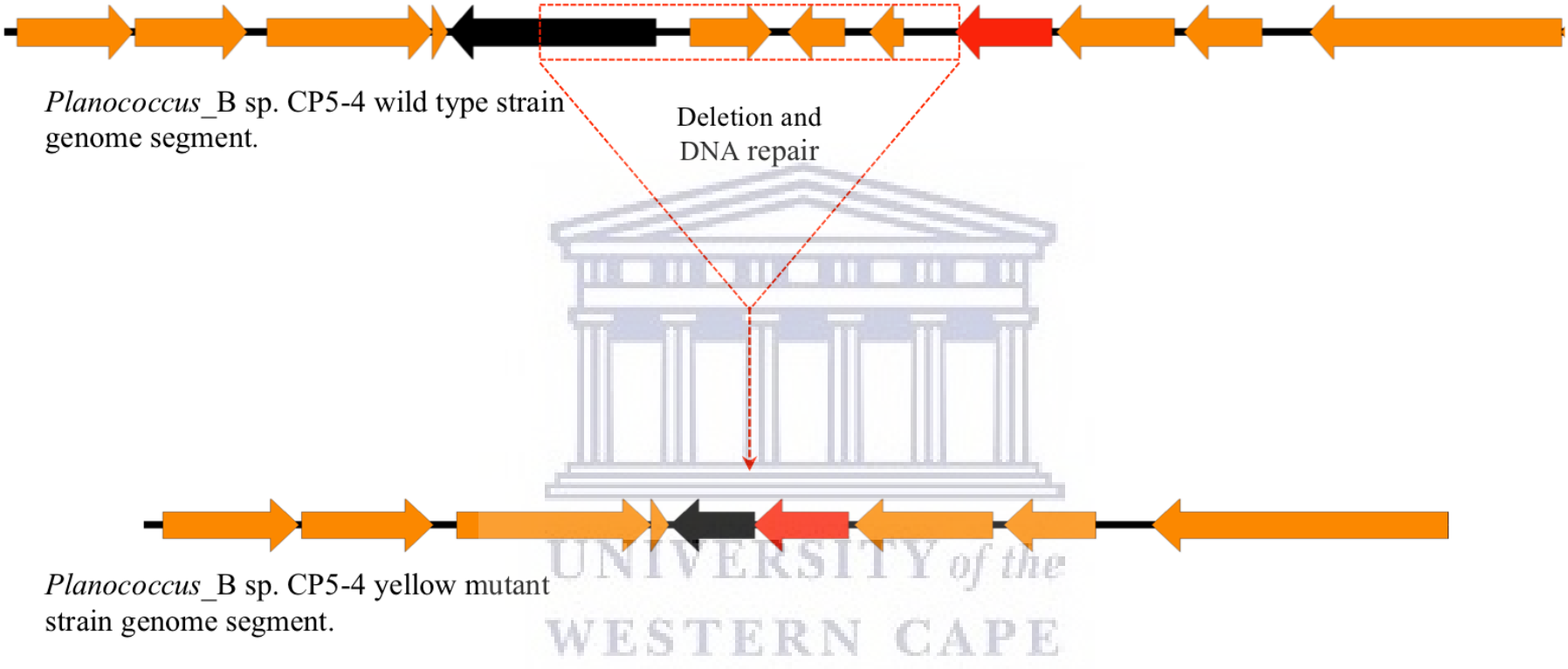

Figure 4.5 Deleted sequence region from the *Planococcus*_B sp. CP5-4-WT strain to produce the yellow mutant. The black coloured ORF represents the CrtNb-like apo-4, 4'-terminal methyl oxidase gene while the red ORF represents the flavodoxin reductase gene. Figure not drawn to scale.

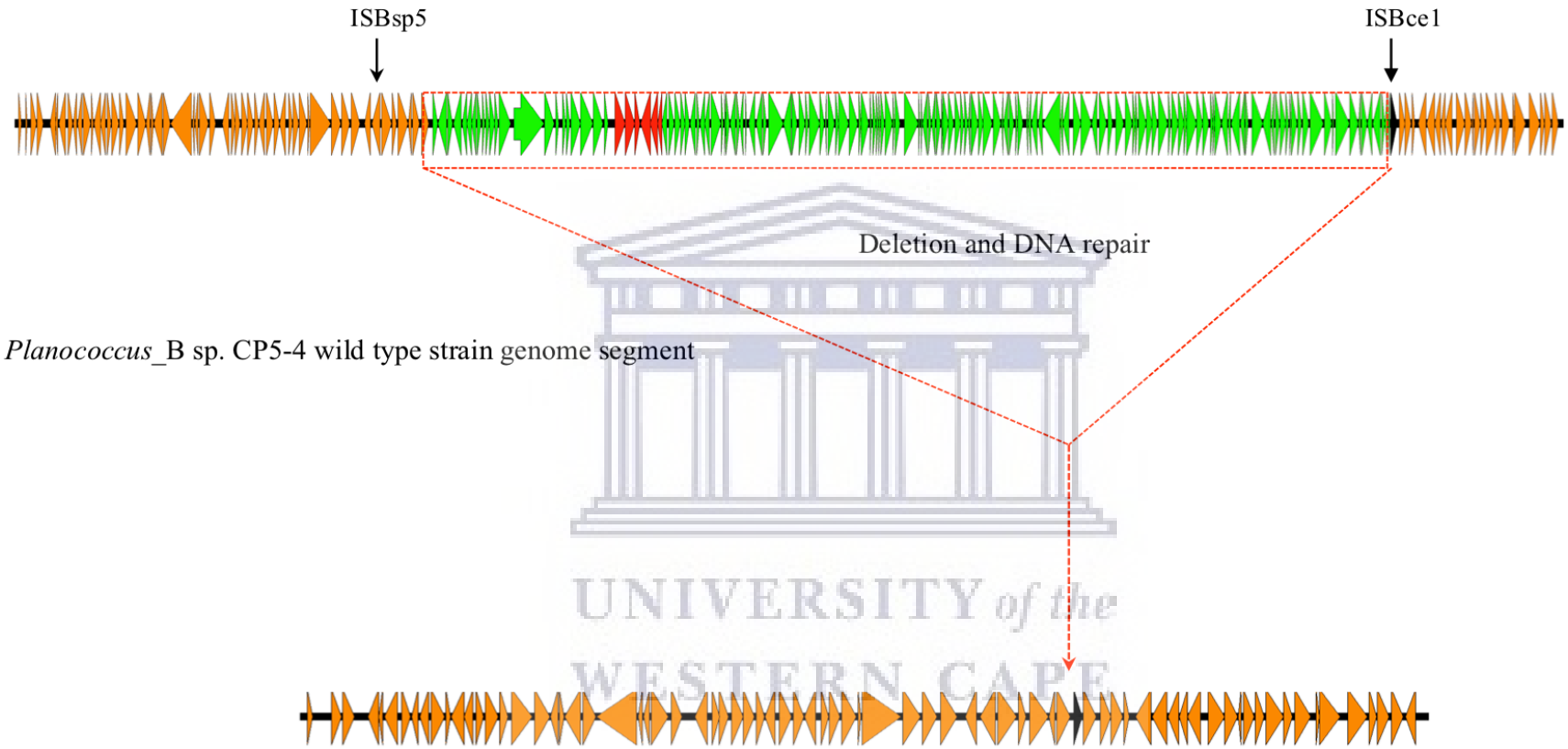

Figure 4.6 Deleted sequence region from the *Planococcus*_B sp. CP5-4-WT strain resulting in the loss of pigment production. The red ORFs represent the predicted carotenoid biosynthetic gene cluster while the green ORFs represent the additional genes that were also deleted as a result of DNA repair following MMS mutagenesis. The position of two insertion sequences (ISBsp5 / ISBce1, table 4.8) that may mediate the deletion event are indicated by arrows. Figure not drawn to scale.

According to Peterson, and Freeman (2014), copy number variant hotspots exist in microbial genomes where they mediate the frequent rearrangement of these genomes leading to a discontinuity in the DNA of strains of the same species. Thus, enabling the development of new phenotypes in response to environmental changes. Additionally, mobile genetic elements are frequently found near these hotspots, suggesting a mechanism for generating genomic instability following activation (Peterson and Freeman, 2014). Chemical mutagens such as MMS that cause little chromosome breakage but add bulky adducts to the DNA can induce the SOS response, which leads to the activation, and the transposition of the mobile genetic elements. Consequently, recombination following the generation of 2 copies of the same sequence in the same orientation during transposition can result in the deletion of the DNA sequence between them (Peterson and Freeman, 2014). This process may have mediated the deletion of the 146.691 Kb fragment from the *Planococcus*_B sp. CP5-4 wild type genome to give the unpigmented phenotype after MMS mutagenesis. Query of the *Planococcus*_B sp. CP5-4-WT draft genome in ISfinder (Zhang et al., 2000), revealed the presence of the insertion sequences listed in Table 4.8 which were near the deleted 146.691 Kb genome fragment from the *Planococcus*_B sp. CP5-4-WT strain to give the unpigmented mutant.

Table. 4.8 Detected genetic mobile elements bordering the deleted sequence fragment from the *Planococcus*_B sp. CP5-4-UN mutant strain.

Sequence producing significant alignment	IS family	% Identity	Origin	Start	End
ISI310	IS256	100	*Enterococcus hirae*	2306008	2306033
ISBsp5	IS1182	100	*Bacillus* sp.	2358859	2358880
ISEFm2	IS256	94	*Enterococcus faecium*	2306008	2306033
ISBce1	IS1182	91	*Bacillus cereus*	2878004	2878077
IS232	IS21	94	*Bacillus thuringesis*	2760538	2760569
IS1272	IS1182	93	*Staphylococcus haemolyticus*	2784056	2784099

Despite the deletion of such a large DNA fragment from its genome, the *Planococcus*_B sp. CP5-4-UN mutant strain displayed similar growth rate to that of the wild type and yellow mutant strains (Chapter 3, section 3.3.4). This finding suggested that the essential genes required for normal cell growth and metabolism by the *Planococcus*_B sp. CP5-4 unpigmented strain were retained following the deletion event. In fact, according to Luisi, Oberholzer, and Lazcano, (2002), as long as all the information for the cell to perform many essential (housekeeping) functions that maintain metabolic homeostasis is available, then bacteria with small genomes can reproduce and evolve in the presence of a full complement of essential nutrients and the absence of environmental stress. A reconstruction of the metabolic models of the three strains in KBase (http://kbase.us, no date; Arkin *et al.*, 2018), based on reactions, compounds, biomass, and proteins, revealed the key properties summarized in Table 4.9.

Table 4.9 Strain specific Kbase metabolic model statistics comparisons

	CP5-4-WT	**CP5-4-YE**	**CP5-4-UN**
Total reactions	1 118	1 115	1 095
Reactions with ORF assignment	840	840	817
Reactions without ORF assignment	278	275	255
Compounds produced	1 096	1 086	1 069

According to the metabolic models generated for the three strains in KBase (http://kbase.us, no date; Arkin *et al.*, 2018), the three *Planococcus*_B sp. CP5-4 strains models shared a common set of 982 compounds, 1094 reactions, and 100 biomass compounds (see Table I 1; Appendix I). Thus, providing evidence that all the information for the *Planococcus*_B sp. CP5-4-UN strain to perform the essential (housekeeping) functions that maintain metabolic homeostasis was retained in its genome following the deletion event. Furthermore, the generated metabolic models revealed the reactions and compounds that were not conserved in the *Planococcus* sp. CP5-4 YE and UN strains following mutation (see Table I 2; Appendix I).

4.3.7 PCR verification of variants

Verification of the sequence deletions detected during variant calling was through PCR amplification of the regions surrounding the SVs. Primer pair CrtDel 1 F/CrtDel 1 R was designed to amplify a 3 827 bp fragment from 462 289 bp to 466 116 bp in the absence of a deletion and a 522 bp fragment if the deletion was present in the yellow mutant genome sequence (Figure 4.7 A). The 3 827 bp amplicon included the entire CrtNb-like methyl oxidase, two hypothetical protein and phosphatidyl ethanolamine genes bordered by the flavodoxin reductase gene and another hypothetical protein gene.

Conversely, the 522 bp amplicon was that of the undeleted CrtNb-like terminal methyl oxidase gene bordered by the flavodoxin reductase and hypothetical protein genes. For verification of the deleted sequence from the *Planococcus*_B sp. CP5-4-UN mutant strain genome primer pair CrtDel 2 F/CrtDel 2 R was designed to amplify 1400 bp of the *crtN* gene from 2 657 933 bp to 2 659 333 bp in the absence of the deletion and to give no amplification if the 146 691 bp sequence deletion was present (Figure 4.7 B).

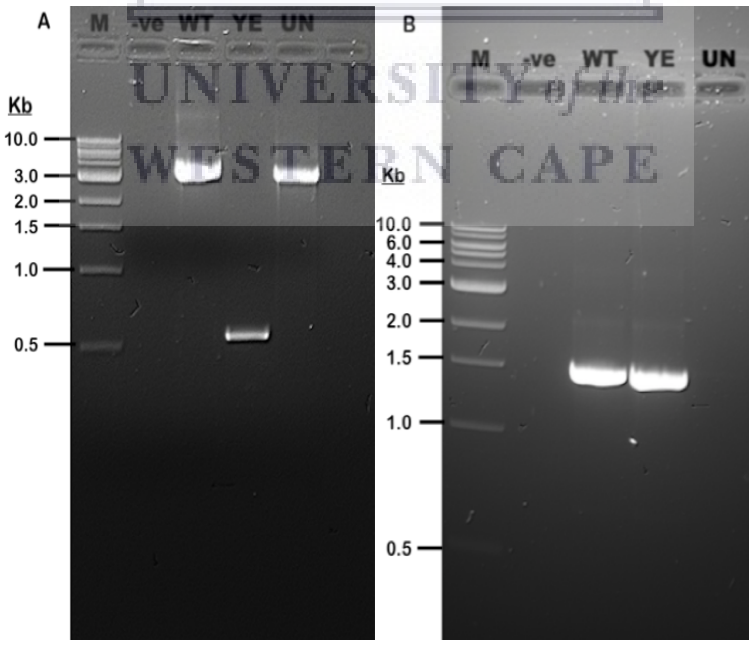

Figure 4.7 PCR verification of deleted sequence from *Planococcus*_B sp. CP5-4 yellow (A) and unpigmented (B) mutant strains. M – NEB 1Kb DNA marker, -ve – nuclease free water negative control, WT – wild type, YE – yellow mutant, and UN – unpigmented mutant strain,

4.3.8 Characterization of the Planococcus_B sp.CP5-4 pigments

The bioinformatics guided determination of the type of carotenoid produced by *Planococcus*_B sp. CP5-4-WT (Figure 4.2 in section 4.3.5) led to the prediction that *Planococcus_B sp.* CP5-4-WT likely produces a C_{30} carotenoid. Therefore, a mass spectrometry-guided characterization of the pigment extracts of the *Planococcus*_B sp. CP5-4 wild type and the yellow mutant strains was conducted to confirm the structure of the carotenoid pigment produced.

Ultra-performance liquid chromatography coupled to an electrospray ionization quadrupole time-of-flight mass spectrometer operating in MSE mode (UPLC-qTOF-MSE) was used for the sensitive, fast and effective characterization of the pigment extracts from the *Planococcus*_B sp. CP5-4 wild type and yellow mutant strain. Both saponified, and unsaponified extracts were analyzed. Saponification involves treatment of the pigment extracts with KOH to remove lipids that may interfere with chromatographic separation.

However, the process of saponification also removes those fatty acid (FA) moieties which are part of the structure of the carotenoid pigments (Armstrong, 1997). The carotenoids extracted from the *Planococcus*_B sp. CP5-4-WT strain were primarily identified based on the combined information obtained from UPLC-qTOF-MSE spectra together with data from published references. The main compounds identified from the saponified pigment extract are presented in Table 4.10.

Table 4.10 UPLC-diode array detector-mass spectrometry analysis of saponified pigment extract from *Planococcus*_B sp. CP5-4-WT

*Peak	t_R (mins)	Formula	λ_{max} (nm)	Measured Mass (m/z)	Calculated Mass (m/z)	Error (ppm)
1	1.74	ND	271.1	ND	ND	ND
2	2.10	ND	236.1		ND	
3	3.04	$C_{37}H_{53}O_8$	447, 467.12, 492.12	625.3732	625.3737	-1.3
4	3.46	$C_{26}H_{37}O_6$	268.12, 277.12, 283.12	445.2589	445.2590	-0.2
5	6.06	ND	230	ND	ND	ND
6	7.62	$C_{30}H_{43}O_2$	451.12	435.3277	435.3263	1.4

ND – not determined

*Peak numbers refer to Figure 4.5

Chromatograms of the saponified pigment extract monitored at 280 nm and at 450 nm are presented in Figure 4.8. The pigment extracts were monitored at 280 nm and 450 nm to detect both the shorter polyene chain colourless carotenoids, which absorb maximally in the 280 – 320 nm region (Meléndez-Martínez *et al.*, 2015), and the long polyene chain coloured carotenoids that absorb in the visible region of the spectrum between 400 – 500 nm (Machmudah and Goto, 2013).

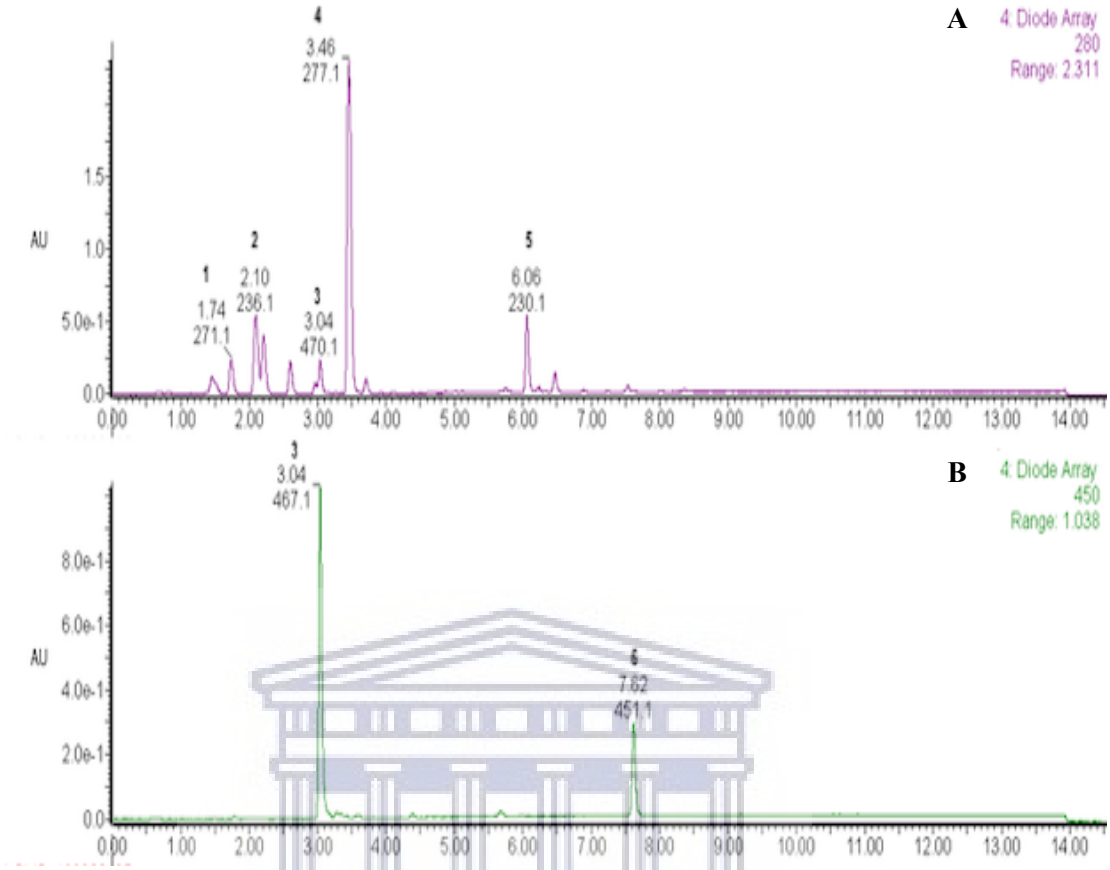

Figure 4.8 UPLC chromatograms of saponified pigment extract from *Planococcus*_B sp. CP5-4-WT recorded at (A) 280 nm and (B) 450 nm, respectively.

The molecular formulae deduced for the compounds present in the saponified pigment extract from *Planococcus*_B sp. CP5-4-WT were mostly for C_{30} compounds (Table 4.5). Amongst these, the predominant peak (3) in the UPLC chromatogram obtained at 450 nm in Figure 4.8 B was taken to represent the most abundant C_{30}-carotenoid compound in the saponified extract.

The C_{30}-carotenoid compound was deduced to be methyl 5-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate from comparison with the MS data of the C_{30} carotenoid discovered by Shindo *et al.*, (2014), and from the predicted molecular formula ($C_{37}H_{53}O_8$), molecular ion at m/z 625.3737 (error: -1.3), and the three finger peak carotenoid uv-vis spectrum shown in Figure 4.9 B.

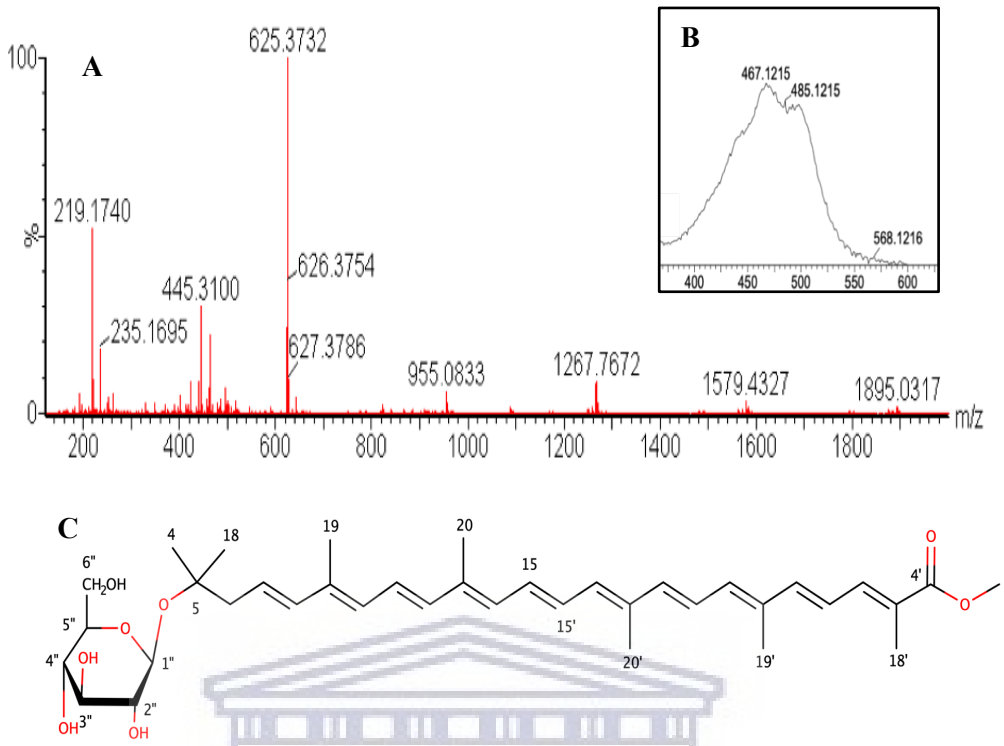

Figure 4.9 Characteristic MS spectrum (A), UV-Vis spectrum (B), and structure (C) of main C_{30} carotenoid pigment in saponified pigment extract from *Planococcus*_B sp. CP5-4-WT

The presence of a hexose sugar (Δm/z of 162) on the structure of methyl 5-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate was revealed by the presence of peak 4 (Figure 4.8 A) with a measured m/z of 445.2589 $[M+H]^+$ (error: -0.2) and a characteristic apo-4, 4'-lycopene uv-vis spectrum (Figure 4. 10 A).). Hence, the 445.2589 parent mass of peak 4 can be accounted for as a difference of 180 (hexose + H_2O) in the mass of methyl 5-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate detected upon collisionally induced fragmentation (Figure 4. 10 C).

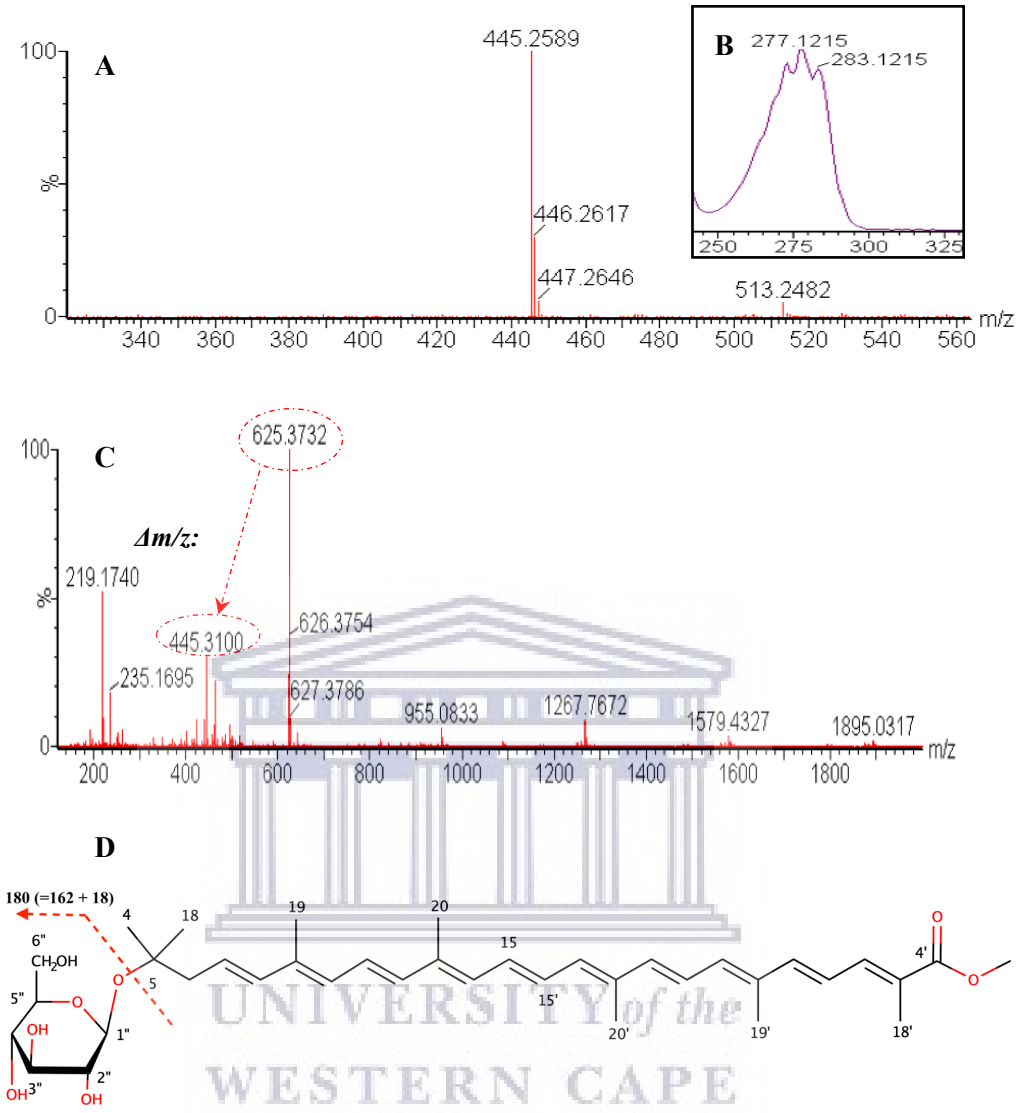

Figure 4.10 Characteristic MS spectrum (A), UV-Vis spectrum (B) of dihydro methyl-apo-4, 4'-lycopenoate, and collisionally induced fragmentation pattern of methyl 5-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate (C and D) to give dihydromethyl-apo-4, 4'-lycopenoate.

Figure 4.11 details the chromatographic profile obtained for the unsaponified carotenoid extract at 450 nm. Peak 3 eluting at 8 minutes 37 seconds was predominant with a UV-Vis spectral absorption maximum of 466.1215 nm (Figure 4.12) and an absorption spectrum typical of a methyl-glucosyl-dihydro-apo-4, 4'-lycopenoate fatty acid ester (Perez-Fons *et al*., 2011; Sabine Steiger *et al*., 2012).

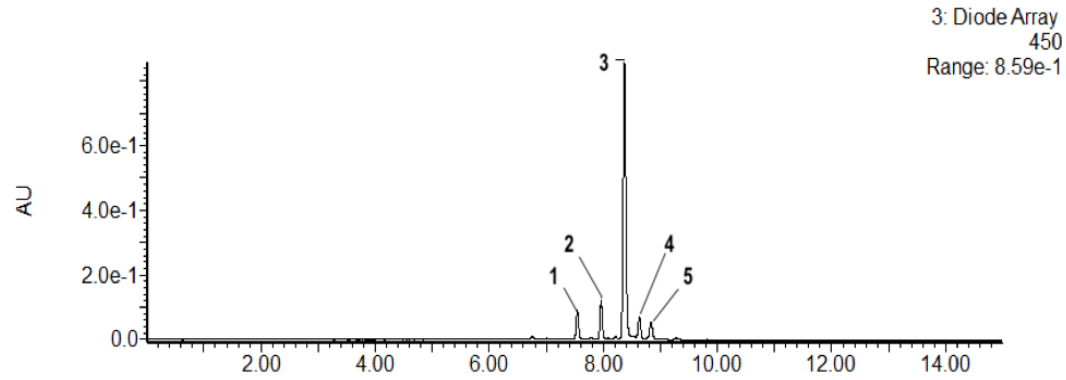

Figure 4.11 UPLC chromatogram of unsaponified pigment extract of *Planococcus*_B sp. CP5-4-WT recorded at 450 nm.

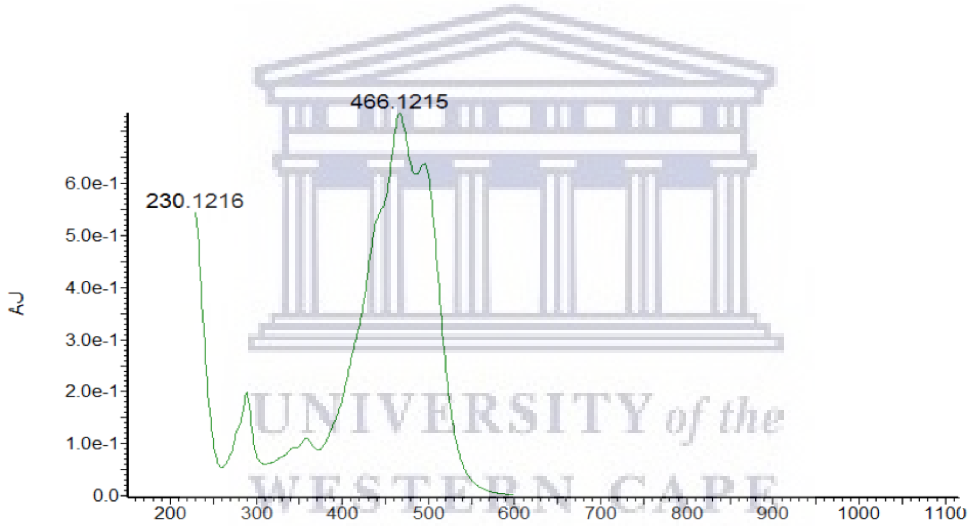

Figure 4.12 UV-Vis spectrum of unsaponifed pigment extract of *Planococcus*_B sp. CP5-4-WT

A parent ion mass of m/z 871.5715 [M+H]+ was obtained for peak 3 (Figure 4.11) and the presence of a fatty acid moiety revealed after subtracting the parent mass of the saponified methyl 5-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate (predominant peak (3) at 450 nm of saponified extract) from m/z 871.5715 [M+H]+ to give a Δm/z of 246.1993. Considering that a H_2O molecule was eliminated during the esterification process the mass of the fatty acid moiety then becomes 264.1993, which represents that of (8Z, 11Z, 14Z) - heptadecatrienoic acid.

Thus, peak (3) in Figure 4.8 was designated as methyl 5-(6-$C_{17:3}$)-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate derived from the parent mass of 871.5715 $[M+H]^+$ comprised of a hexose sugar (Δm/z 162) esterified to a (8Z, 11Z, 14Z) -heptadecatrienoic acid (Δm/z 264) and attached to 445.31 $[M+H]^+$ dihydro-methyl-apo-4, 4'-lycopenoate.

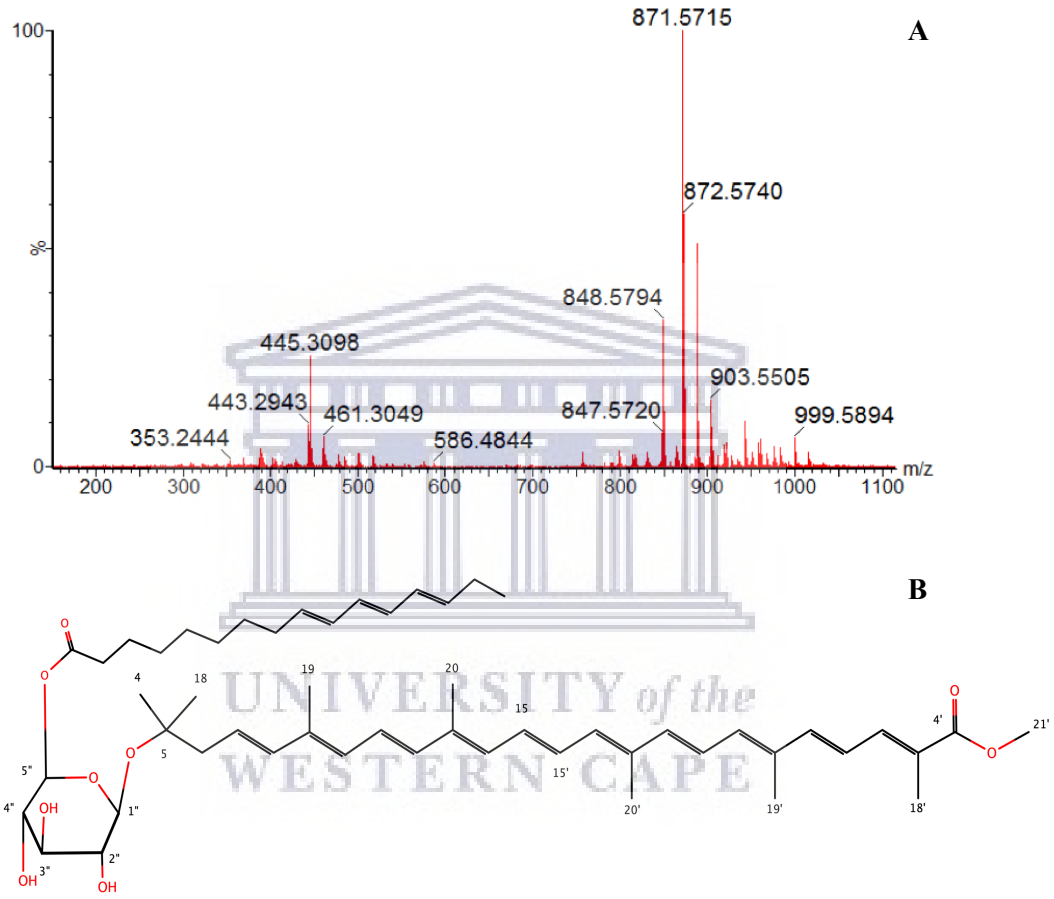

Figure 4.13 Characteristic MS spectrum (A) and structure of the unsaponified methyl 5-(6-$C_{17:3}$)-glucosyl-5, 6'-dihydro-apo-4, 4'-lycopenoate produced by *Planococcus*_B sp. CP5-4-WT (B).

Sequencing of the *Planococcus*_B sp. CP5-4-WT strain genome, the identification and characterization of the carotenogenic genes in the genome, and the UPLC-PDA-qTOF MS analysis of the pigment extract of *Planococcus*_B sp. CP5-4-WT enabled the prediction of the putative carotenoid biosynthesis pathway depicted in Figure 4.14. The structure of the carotenoids and the putative carotenoid biosynthesis pathway of *Planococcus*_B sp. CP5-4-WT were established using obtained MS spectra together with fragment characteristics from published data (Shindo *et al.*, 2008, 2014; Osawa *et al.*, 2010a, 2010b; Perez-Fons *et al.*, 2011; Sy *et al.*, 2013). Thus, the resulting structural suggestions for the *Planococcus*_B sp. CP5-4-WT carotenoids were mainly based on product ion spectra from collisionally induced fragmentation. As a result, the structural fittings conducted in this study were intended to find as many fragments in the spectra as possible that matched previously characterized structures.

Figure 4.14 Proposed biosynthetic cascade for methyl 5-(6-$C_{17:3}$)-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate in *Planococcus*_B sp. CP5-4-WT. CYP - cytochrome P450 hydroxylase, CrtNb-like-CH_3OH - CrtNb-like terminal methyl oxidase, and FPP - farnesyl pyrophosphate.

According to the UPLC chromatogram shown in Figure 4.15 of the saponified carotenoid extract from the *Planococcus*_B sp. CP5-4-YE mutant, peak 6 previously observed eluting as a minor peak from the chromatogram of the wild type strains' saponified extract in Figure 4.5 B, was detected as the predominant peak eluting at 7 minutes 59 seconds.

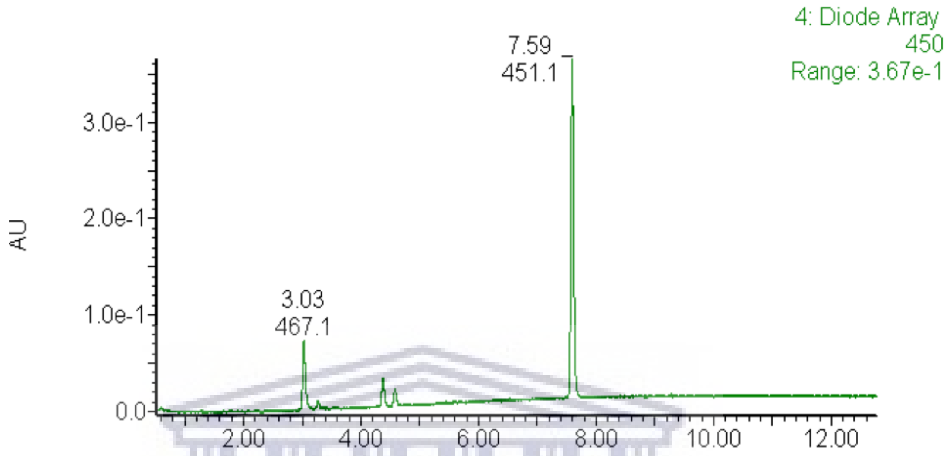

Figure 4.15 UPLC chromatogram of saponified pigment extract from *Planococcus*_B sp. CP5-4-YE strain recorded at 450 nm.

The aforementioned peak gave a visible spectral maximum at 451.1215 nm and a $[M+H]^+$ ion peak at m/z 435.3269 $[M+H]^+$ corresponding to a predicted formula of $C_{30}H_{43}O_2$ (error: 1.4). These data suggest that the yellow pigment may be a putative glucosylated diapolycopene. This deduction of the putative structure of the saponified yellow pigment is supported by Pérez-Fons and Fraser, (2012), who reported structures of yellow glucosylated diapolycopene and glucosylated diapolycopene fatty acid esters absorbing maximally between 449 to 454 nm from saponified and unsaponified carotenoid extracts from yellow *Bacillus* spore formers. The truncation of CrtNb-like apo-4, 4'-terminal methyl oxidase gene in the yellow mutant's genome is theorized to have resulted in the translation of a non functional enzyme that could not mediate the addition of a terminal O-methyl ester group to glucosylated diapolycopene during the biosynthesis of the orange methyl 5-(6-$C_{17:3}$)-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate, thus resulting in the production of the yellow pigment. According to Sy *et al.*, (2013), the main structural difference between the glucosylated orange and yellow C_{30} pigments is the absence of the terminal O-methyl ester group in the yellow pigment.

4.4 Discussion

In this chapter, whole-genome sequencing and sequence analysis were used for (i) the taxonomic classification of the *Planococcus* sp. CP5-4-WT strain, (ii) the identification and characterization of pigment biosynthesis genes in the *Planococcus* sp. CP5-4 strains, and (iii) for the identification of the type(s) of DNA mutations caused by MMS to produce the yellow, and unpigmented mutant phenotypes. Furthermore, UPLC-PDA-qTOF mass spectrometry was used for identifying the pigments produced by the pigmented *Planococcus* sp. CP5-4 strains.

Genome based taxonomic classification of the *Planococcus* sp. CP5-4-WT using the GTDB-Tk placed it into one of 3 dereplicated *Planococcus* genera on the bac120 tree (Figure 4.1) distinguished from each other by alphabetical suffixes to resolve polyphyly. This approach of splitting/merging only taxa that are outliers in terms of diversity within their rank is appealing for the taxonomic ranks from phylum to genus because these are relatively arbitrary and have no meaningful cutoffs in terms of diversity (Wittouck *et al.*, 2019). However, at the species level it is different because, in contrast to the other taxonomic ranks (e.g., the genus rank), relatively fixed similarity cutoffs to separate species are generally accepted and largely seem to correspond to the historically defined species (Konstantinidis and Tiedje, 2005; Jain *et al.*, 2018). According to Chaumeil *et al.*, (2019) the cutoff used by the GTDB to separate bacterial species based on their genome sequences is 95% average nucleotide identity (ANI). Thus, a query genome is classified as a new species in a particular genus if, and only if, the ANI cutoff is less than 95%, and the alignment fraction is ≥0.65 (Chaumeil *et al.*, 2019; Coil *et al.*, 2019). Following the above rules, *Planococcus*_B sp. CP5-4-WT was classified as a new species in the *Planococcus*_B genus (Figure 4.1) based on the calculated ANI scores of the reference genomes in the GTDB to that of *Planococcus*_B sp. CP5-4-WT and the respective alignment fractions (Table 4.3).

Eight genes, six of which organised into two adjacent sub-clusters that are transcribed in opposite directions on contig 1 (Figure 4.3 A) and the remaining two located on contig 5 (Figure 4.3 B), were predicted to be responsible for the production of a carotenoid pigment by the *Planococcus*_B sp. CP5-4-WT strain. A BLASTp search of the translated amino acid (aa) sequences of the predicted carotenogenic genes in the UniProtKB/Swiss-Prot database revealed that several of the sequences had similarities to 4,4'-diapolycopene oxygenase, 4,4'-diapolycopen-4-al dehydrogenase, 4,4'-diapophytoene desaturase, and 4,4'-diaponeurosporene oxygenase enzymes responsible for the biosynthesis of C_{30}-carotenoids in *Bacillus indicus*, *Methylomonas* sp., and *Staphylococcus haemolyticus* (strain JCSC1435) (Table 4.5). Thus, indicating the possibility of the *Planococcus*_B sp. CP5-4-WT strain producing a putative C_{30}-carotenoid pigment.

The clustering of the predicted *Planococcus*_B sp. CP5-4-WT CrtM sequence, and that of the other members of the *Planococcus*_B genus, with 4,4'-diapophytoene synthases from C_{30}-carotenoid producing Firmicutes (Figure 4.2) provided the first piece of evidence that a C_{30}-carotenoid pigment was produced by the *Planococcus*_B sp. CP5-4-WT strain, and possibly by the other members in this genus. This prediction is based on what is understood about phytoene and 4,4'-diapophytoene synthases dictating the formation of C_{50}, C_{40}, or C_{30}-carotenoids, respectively, by mediating the synthesis of either phytoene or 4,4'-diapophytoene in the first committed step of carotenoid biosynthesis (Dogbo *et al.*, 2006; Klassen, 2010). Phytoene undergoes successive modifications by other enzymes in the biosynthetic pathway to produce either C_{40} or C_{50}-carotenoids, while 4,4'-diapophytoene is modified to produce C_{30}-carotenoids (Mercadante *et al.*, 2004; Dogbo *et al.*, 2006; Klassen, 2010; Perez-Fons *et al.*, 2011; Yatsunami *et al.*, 2014; Mariutti and Mercadante, 2018). Compared to C_{50} and C_{40}-carotenoids, C_{30}-carotenoids are relatively rare in nature, and there is little information in the literature regarding the organization and regulation of the genes responsible for their biosynthesis in bacteria (Kim *et al.*, 2016).

In this study, not only were the carotenogenic genes of *Planococcus*_B sp. CP5-4 found to be arranged similarly to those in the carotenoid biosynthetic gene clusters of *H. halophilus* and *P. faecalis* AJ003T (Figure H1; Appendix H), the arrangement of the carotenogenic genes was also found to be similar in all the members of the *Planococcus*_B genus (Figure 4.4). The high aa sequence similarities of 70-100% shared by the translated carotenogenic genes forming the carotenoid biosynthetic gene clusters of the organisms of the *Planococcus*_B genus (Figure 4.4) suggests similarities in the biosynthetic function of the translated proteins meaning that these proteins may mediate the synthesis of an identical C_{30}-carotenoid structure by the *Planococcus*_B species (Watson, Laskowski and Thornton, 2005; Addou *et al.*, 2009; Higdon, Louie and Kolker, 2010).

A glucosylated C_{30}-carotenoid, methyl 5-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate (m/z 625.3732 (calc: 625.3737)), was identified to be the main pigment compound in the saponified extract obtained from *Planococcus*_B sp. CP5-4-WT (Table 4.10 and Figure 4.8), in this study using UPLC-PDA-qTOF mass spectrometry. Notably, Shindo *et al.*, (2008, 2014), and Shindo and Misawa, (2014) also identified methyl 5-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate (m/z 625.37330 (calc: 625.37404)) in a saponified pigment extract from *Planococcus maritimus* isolate iso-3, a close relative of the type strain *Planococcus*_B *maritimus* (GCF_001687625.2) (Unfiltered NCBI Taxonomic name *Planococcus maritimus* DSM17275).

Thus, providing strong evidence to the possibility that the enzymes produced by the genes in the two carotenoid sub-clusters identified to be present in all the members of the *Planococcus*_B genus may mediate the formation of a similar C_{30}-carotenoid backbone structure. The taxonomical characterization of *Planococcus maritimus* isolate iso-3 was done through DNA-DNA hybridization analysis, chemotaxonomic marker analysis, and 16S rRNA sequence similarity search in RDP as described by Katsuta *et al.*, (2005) revealing 99.5 % similarity to *Planococcus*_B *maritimus* (GCF_001687625.2).

Glucosylated carotenoid acid esters, in which a glucose molecule is esterified to both a triterpenoid carotenoid carboxylic acid and fatty acid (FA) (Pelz *et al.*, 2005), have been reported to be the end products of carotenoid biosynthesis in heterotrophic bacteria that include but are not limited to the C_{30}-carotenoid producing *Staphylococcus aureus* (Marshal and Wilmoth, 1981; Marshall and Wilmoth, 1981; Kim and Lee, 2012), *Planococcus maritimus* (Shindo *et al*., 2008, 2014), *Planococcus faecalis* AJ003T (Kim *et al.*, 2015, 2018), *Methylomonas* sp. (Tao *et al.*, 2005), *Halobacillus halophilus* (Köcher *et al.*, 2009), *Bacillus indicus* (Perez-Fons *et al.*, 2011; Steiger *et al.*, 2015), and *Bacillus firmus* (S. Steiger *et al.*, 2012; Steiger *et al.*, 2015).

However, the occurrence of glucosylated carotenoid esters in bacteria is often overlooked because of the routine use of saponification to hydrolyze the esters and remove the fatty acids before subsequent characterization of the carotenoid compounds. This practice results in the generation of data for glucosylated carotenoid compounds devoid of the esterified fatty acid chain (Amorim-Carrilho *et al.*, 2014; Mariutti and Mercadante, 2018; Manzoni Maroneze *et al.*, 2019). In this study, UPLC-PDA-qTOF MS analysis of the unsaponified extract from *Planococcus*_B sp. CP5-4-WT and determining the difference in the m/z of the parent ions of the main C_{30}-carotenoid compounds in both the saponified and unsaponified extracts of the *Planococcus* sp. CP5-4-WT strain (Figures 4.9 and 4.13) revealed the hydrolyzed fatty acid moiety to be (8Z, 11Z, 14Z)-heptadecatrienoic acid, a $C_{17:3}$ polyunsaturated fatty acid.

Similar results have been found by other researchers where if saponification was not used in the extraction procedure, complex carotenoids structurally compatible with membrane integration were found (Davies and Taylor, 1982; Taylor, 1984; Perez-Fons *et al.*, 2011). In these studies, membrane integration of the glucosylated carotenoid compounds was shown to be made possible by the esterified the fatty acid chain, which mediates the localization of the carotenoid compound in the hydrophobic fatty acid tail region of the lipid bilayer of the bacterial membrane (Taylor, 1984; Mariutti and Mercadante, 2018).

Notably, the final structure of the glucosylated carotenoid fatty acid ester produced by a bacterium is determined by the esterification of the predominantly produced fatty acid. In bacteria, fatty acid synthesis is achieved by a highly conserved set of genes that each encodes an individual step in the type II fatty acid biosynthetic pathway (De Mendoza and Schujman, 2019), which plays a fundamental role in the synthesis of the phospholipid bilayer forming the cellular membrane (de Carvalho and Caramujo, 2018). However, according to Herbert, (1961); O'Leary, (1962); Dembitsky, (2004); and de Carvalho and Caramujo,(2018) the fatty acid composition of bacteria is markedly affected by the nature of the medium and by the conditions under which the culture is grown. For example, de Carvalho *et al.*, (2014) reported a change in the total lipid fatty acid composition of *Rhodococcus erythropolis* cells to a high percentage of the polyunsaturated eicosapentaenoic ($C_{20:5\omega3}$), arachidonic acid ($C_{20:4\omega6}$), and docosapentaenoic ($C_{22:5\omega3}$) fatty acids after exposure to osmotic stress caused by the addition of 7.5% NaCl to the growth medium.

Thus, based on the fact that in this study a modified TSB salt-sorbitol medium with 5% (w/v) NaCl added to it was used to culture the *Planococcus*_B sp. CP5-4-WT strain, it is probable that the increased NaCl concentration could have resulted in the production of the $C_{17:3}$ polyunsaturated fatty acid (8Z, 11Z, 14Z)-heptadecatrionic acid deduced to be esterified to the unsaponified methyl 5-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate compound (section 4.3.8, Figure 4.13).

Although several polyunsaturated fatty acids have been reported to be produced by bacteria (Schujman *et al.*, 1998, 2006; de Mendoza, Schujman and Aguilar, 2014; De Mendoza and Schujman, 2019), prior to this study there was no report of the production of the $C_{17:3}$ polyunsaturated fatty acid (8Z, 11Z, 14Z)-heptadecatrionic acid by bacteria including by species in the *Planococcus* genera.

The proposed carotenoid biosynthesis pathway of *Planococcu*s_B sp. CP5-4-WT is outlined in Figure 4.14. Biosynthesis of methyl 5-(6-$C_{17:3}$)-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate in the *Planococcus*_B sp. CP5-4-WT strain begins with the conjugation of two prenylated farnesyl pyrophosphate (FPP) precursor

molecules to form apo-4, 4-phytoene (4, 4'-diapophytoene) by CrtM. This contradicts early reports on the structure of this carotenoid which was deduced from NMR data to be an 8'-apocarotenoid, methyl-hydroxy-3, 4-dihydro-apo-8'-lycopenoate (Shindo *et al.*, 2008; Perez-Fons *et al.*, 2011). This led to the hypothesis that the original prenylated precursors might be the C_{20} geranylgeranyl pyrophosphate and the C_{10} geranyl pyrophosphate molecules instead of two C_{15} farnesyl molecules (Perez-Fons *et al.*, 2011).

However, revision of the NMR data generated for the carotenoid extracted from *P. maritimus* iso 3 revealed its structure to be that of the 4, 4'-apocarotenoid, methyl 5-glucosly-5, 6-dihydro-apo-4, 4'-lycopenoate (Shindo *et al.*, 2014), supporting the fact that its synthesis begins with the conjugation of two C_{15} FPP molecules. This initial reaction is identical in all of the bacterial species that produce C_{30}-carotenoids because CrtM is highly conserved functionally in the C_{30}-carotenoid producers (Klassen, 2010). However, carotenoid biosynthetic gene presence and function in different taxa diverge following CrtM, leading to a myriad of biosynthetic "branches" collectively denoting the various downstream modification steps seen in different C_{30}-carotenoid producing bacteria to produce a vast catalogue of glucosylated C_{30}-carotenoid fatty acid structures (Klassen, 2010).

For example, in *Methylobacterium rhodium* (formally *Pseudomonas rhodos*) 4, 4'-diapophytoene is desaturated to the conjugated undecaene, 4, 4'-diapolycopene before oxygenation and subsequent glucosylation with two glucose moieties to form 4, 4'-D-β-D-glucopyranosyl-4, 4'-diapolycopen-4, 4'-dioic acid diester (Taylor, 1984), while in 4, 4'-diapophytoene is desaturated to diaponeurosporene in *Staphylococcus aureus* and *Streptococcus faecium* before oxygenation and subsequent glucosylation to form 4'-D-glucopyranosyloxy-4, 4'-diaponeurosporen-4oic acid ester and 4'-D-glucopyranosyloxy-4, 4'-diaponeurosporene, respectively (Kleinig *et al.*, 1979; Marshal and Wilmoth, 1981; Marshall and Wilmoth, 1981; Davies and Taylor, 1982; Kleinig and Schmitt, 1982; Taylor, 1984; Tao *et al.*, 2005).

In *Planococcus*_B sp. CP5-4-WT, after the formation of apo-4, 4'-phytoene is a series of four desaturation reactions in which apo-4, 4'-phytoene is converted to apo-4, 4'-lycopene via apo-4, 4'-phytofluene, apo-4, 4'-ζ-carotene and apo-4, 4'-neurosporene by CrtN. At this stage, the pathway proceeds towards the biosynthesis of 5-hydroxy-5, 6-dihydro-apo-4, 4'-lycopene mediated by cytochrome P450 hydroxylase. Cytochrome P450 hydroxylases that mediate the hydroxylation of carotenoids in *Thermus thermophilus* HB27 and the radiation-resistant bacterium *Deinococcus radiodurans* have been reported (Greule *et al.*, 2018). The P450 hydroxylase CYP175A1 was shown to hydroxylate two symmetry-related methylenes at either end of the carotene skeleton in *T. thermophilus* HB27 (Nishida and de Montellano, 2005), while CYP287A1 was shown to be a C_2 methylene hydroxylase of the monocyclic carotenoid, deinoxanthin produced by *D. radiodurans* (Zhou *et al.*, 2015).

Following this hydroxylation reaction is the oxidation of C4′ by CrtNb to give 5-hydroxy-5, 6-dihydro-apo-4, 4'-lycopenal, which is further oxidized by CrtNc and the CrtNb-like terminal methyl oxidase to produce methyl 5-hydroxy-5, 6-dihydro-apo-4, 4'-lycopenoate. The desaturase gene *crtN* and the two following genes in the biosynthesis pathway, *crtNb*, and *crtNc* although encoding proteins with oxidase function, belong to the same C_{40} phytoene desaturase (crtI)-related gene family (Raisig and Sandmann, 2001; Köcher *et al.*, 2009). The pathway culminates with the glucosylation of the P450 hydroxylated terminal methylene group by a glycosyltransferase to give methyl 5-glucosly-5, 6-dihydro-apo-4, 4'-lycopenoate, which is further acetylated with the polyunsaturated (8Z, 11Z, 14Z)-heptadecatrionic fatty acid by a glycerol acyltransferase to give the final glucosylated C_{30}-carotenoid fatty acid ester product methyl 5-(6-$C_{17:3}$)-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate.

As already discussed in section 4.3.6 exposing *Planococcus*_B sp. CP5-4-WT to MMS resulted the truncation of the CrtNb-like apo-4, 4'-terminal methyl oxidase gene due to a 3 kb sequence deletion encompassing part of the CrtNb-like apo-4, 4'-terminal methyl oxidase gene and three additional ORFs (Figure 4.5). The CrtNb-like terminal methyl oxidase mediates the addition of a terminal O-methyl

ester group to glucosylated diapolycopene (Tao *et al.*, 2005; Osawa *et al.*, 2010a) during the biosynthesis of the orange methyl 5-(6-$C_{17:3}$)-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate. Thus, the inability of this enzyme to mediate the addition of the terminal O-methyl ester group to glucosylated diapolycopene resulted in the production of the yellow pigment in the *Planococcus*_B sp. CP5-4-YE strain.

In conclusion the main results of this chapter revealed that:

(i) *Planococcus* sp. CP5-4-WT classified, as a tentatively novel species based on a 16S rRNA sequence identity threshold of 97% in chapter 3, is indeed a novel species belonging to the *Planococcus*_B genus. According to the GTDB phylogeny inferred from the concatenation of 120 ubiquitous single-copy bacterial proteins, many taxa such as the genus *Planococcus* circumscribe polyphyletic groupings because of historical phenotype-based classification and the uneven application of taxonomic ranks across the 16S rRNA tree. To resolve polyphyly at the lower taxonomic ranks such as at the genus level, the GTDB retains existing names of the genera with alphabetical suffixing in its taxonomic ranks. Only the group(s) containing type material (if known) keep(s) the original unsuffixed name to indicate the validity of the name assignment. As such, 4 *Planococcus* genera were proposed. Based on the GTDB classification of the *Planococcus*_B sp. CP5-4 as a proposed novel species in the *Planococcus*_B genus, it is recommended that the number of characterized species in this genus be updated to 7.

(ii) Eight genes are responsible for carotenogenesis in *Planococcus*_B sp. CP5-4 and the existence of the carotenoid biosynthetic gene cluster on contig 1 of the *de novo* assembled genome of *Planococcus*_B sp. CP5-4 plus the organization of the carotenogenesis genes on the said cluster is an evolutionary trait shared by all the members of the *Planococcus*_B genus. Furthermore, the high sequence similarity (70-100%) of the translated aa sequences of the genes in the carotenogenesis clusters of all the *Planococcus*_B species suggests functional similarity and the probable synthesis of the same carotenoid structure, and

(iii) The C_{30}-glucosylated carotenoid acid ester methyl 5-(6-$C_{17:3}$)-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate was produced by *Planococcus*_B sp. CP5-4 and that the structure of the orange methyl 5-(6-$C_{17:3}$)-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate was altered to that of a putative glucosylated diapolycopene fatty acid ester as a result of MMS mutagenesis and truncation of the crtNb-like terminal methyl oxidase gene in the yellow mutant. The resulting yellow C_{30}-glucosylated carotenoid fatty acid ester had a high IR reflectance, which caused it to absorb less IR radiation during the brine evaporation experiment in chapter 3, and to consequently affect a lower increase in the brine evaporation rate compared to the orange methyl 5-(6-$C_{17:3}$)-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate.

That being said, to improve the efficiency of the *Planococcus*_B sp. CP5-4-WT strain in increasing brine evaporation rates, the strain would have to produce a pigment with a lower IR-reflectance than that of the orange methyl 5-(6-$C_{17:3}$)-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate, and this may be accomplished through engineering the expression of such a pigment in the *Planococcus*_B sp. CP5-4 strains. The prospect of this approach is elaborated on in the recommendations section of the conclusions chapter.

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Chapter 5

Conclusions and recommendations

5.1 Introduction

Currently, the evaporation of desalination brine effluent in ponds is the most economical method available for its management by inland desalination plants. However, the effectiveness of this method is dependent on several factors that include temperature, relative humidity, solar radiation, wind speed, and the composition of salts dissolved in the brine. As part of efforts to improving the effectiveness of brine evaporation ponds, Bloch, Farkas, and Spiegler, (1951); Keyes, and Gunaji, (1966); and Rajvanshi, (1981), looked at the possibility of using chemical dyes to maximize the absorption of radiant energy by the brine to increase the evaporation rate. However, according to Hassaan and Nemr (2017), the use of chemical dyes to enhance brine evaporation rates poses a threat to both the environment and human health because of their non-biodegradable and potentially carcinogenic nature. Thus, the focus of this thesis was to assess the use of an alternative environmentally friendly biological approach to enhancing the evaporation of desalination brine contained in evaporation ponds; the aims of which were:

I. To assess the effect of the reflected light spectrum (colour) of pigment produced by halophilic bacteria on desalination brine evaporation, and
II. To identify the type of pigment(s) produced by the halophilic bacterial strain(s) affecting the highest increase on brine evaporation rate.

As such, the main findings regarding the use of the biological method for improving mine wastewater desalination brine evaporation rates will be discussed in this chapter. Furthermore, the findings relating to the effect of the colour of pigment produced by halophilic bacteria on the brine evaporation rate will be discussed and the general conclusions of the thesis presented.

The limitations of the study will be discussed, and the chapter closed by presenting recommendations for future studies.

5.2 General discussion and conclusions

Bacterial pigments have long been implicated in aiding the evaporation of NaCl brines during salt production. According to Davis (1974), without this colouration, the process of evaporation proceeds with some difficulty. However, this link has not been established for pigmented bacteria and industrial wastewater desalination brine effluents with differing salt compositions to those of saltern pond brines. Thus, the focus of this study was to assess the effects of pigmented halophilic bacteria on a Na_2SO_4 based desalination brine effluent produced at the EWRP and to investigate the effects of the reflected light spectrum (colour) of the pigment on brine evaporation.

In this study, the mutagenesis of the *Planococcus*_B sp. CP5-4-WT strain to produce pigment mutants made it possible to investigate the effects of the reflected light spectra of pigments produced by a single halophilic bacterial species on brine evaporation. Moreover, the production of the mutant strains ensured the elimination of possible factors associated with the use of different genera and/or species, which Silva-Castro *et al.*, (2019) highlighted as limitations in their study. The *Planococcus*_B sp. CP5-4 strains used in this study had comparable growth rates in the brine (Chapter 3, section 3.3.4) and the wild type and yellow mutant strains exhibited similar pigment production capacity, except for the colour of the pigment produced.

UPLC-PDA-qTOF-mass spectrometry provided sufficient data to show that the pigments produced by the *Planococcus*_B sp. CP5-4-WT and YE strains were a glucosylated C_{30}-carotenoid fatty acid ester methyl 5-(6-$C_{17:3}$)-glucosyl-5, 6-dihydro-apo-4, 4'-lycopenoate (Me-5-(6-$C_{17:3}$)-DL) and a putative glucosylated diapolycopene fatty acid ester (5-(6-$C_{17:3}$)-DL) derivative devoid of a O-methyl ester group (HCO_2) at the terminal end of the chromophore molecule 4, 4' diapolycopene, respectively. Similar structural differences between an orange methyl 1-(6-$C_{11:0}$)-glucosyl-3, 4-dehydro-apo-8'-lycopenoate produced by

Bacillus indicus HU36 and a yellow intermediate, 1-(6-$C_{11:0}$)-glucosyl-3, 4-dehydro-apo-8'-lycopene, have been reported by Perez-Fons *et al.*, (2011). Thus, indicating that a direct change to the highly conjugated polyene backbone can alter the reflected light spectrum of a carotenoid compound unlike the acetylation of fatty acid chains to the hydroxyl groups at the terminal ends of the carotenoid compound, which does not change the UV/VIS and the reflected light spectrum of the carotenoid compound (Hornero-Méndez and Mínguez-Mosquera, 2000; Bunea, Socaciu and Pintea, 2014; Mariutti and Mercadante, 2018).

Increases of 18.56% and 10.31% in the evaporation rate were observed in the brine inoculated with the WT and YE *Planococcus*_B sp. C5-4 strains, respectively. How these increases were achieved can be explained by the localization of the glucosylated carotenoid fatty acid esters within the phospholipid bilayer of the bacterial membrane where they play a large role in light-harvesting and photoprotection under excessive light conditions (Davies and Taylor, 1982; Taylor, 1984; Christensen, 1999; Gradinaru *et al.*, 2001; Valery M. Dembitsky, 2004; Perez-Fons *et al.*, 2011). Carotenoids can carry out their light-harvesting function thanks to their highly conjugated polyene backbone, which allows for the absorption of radiant energy and attainment of an excited state by the electrons within these double bonds, the excited state electrons, in turn, transfer the absorbed energy to nearby molecules when they return to the ground state (Christensen, 1999; Gradinaru *et al.*, 2001; Mariutti and Mercadante, 2018). It is thus, this energy transfer from the glucosylated carotenoid fatty acid ester compounds embedded in the phospholipid bilayer of the bacterial cell membrane to the surrounding that contributed to raising the temperature of the brine temperature and ultimately the evaporation rate of the brine.

The differences in the increment of the brine evaporation rates can be attributed to the degree by which Me-5-(6-$C_{17:3}$)-DL and 5-(6-$C_{17:3}$)-DL reflected the incident infrared radiation used to drive the evaporation process. According to Deshmukh (2005), infrared radiation flowing through the air heats any object or pigment it comes into contact with; and the amount of heat gain is dependent on the IR-reflectance of that particular object or pigment.

Thus, the YE strain which had the enhanced IR-reflectance due to the altered reflected light spectrum of 5-(6-$C_{17:3}$)-DL, absorbed less heat compared to the Me-5-(6-$C_{17:3}$)-DL, which ultimately reduced the amount of heat transfer from the YE strain to its surroundings (the brine). Notably, the 6.19% increase in the evaporation rate of the brine inoculated with the *Planococcus*_B sp. CP5-4-UN strain was also as a result the ability of this strain to reflect the incident infrared radiation used to drive the evaporation process. However, because of the lack of pigment, the *Planococcus*_B sp. CP5-4-UN strain had a more enhanced IR-reflectance compared to the YE and WT strains, which resulted in the absorption and transference of less heat to the surroundings. According to Canlas (2016), pigments or objects with enhanced IR-reflectances are cooler than those with less IR-reflectances and as such pigments or objects with low IR-reflectances absorb the most heat energy and transfer it to surrounding surfaces.

It is important to note that although the increases in the evaporation rate of the brine was mainly because of the low infrared reflectance of Me-5-(6-C17:3)-DL and 5-(6-C17:3)-DL produced by the WT and YE *Planococcus*_B sp. CP5-4 strains, the increments in the evaporation rate of the brine in this study cannot be extrapolated as direct estimates of evaporation for open water bodies. The reasons being the differences in the depth, thermal conduction, and brine capacity of the evaporation pans compared to the open water bodies, as well as, the exclusion of studying the effects of environmental factors such as air pressure, humidity, temperature, and solar radiation, together with inoculating the brine with the *Planococcus*_B sp. C5-4 strains in this study. As such, the achieved increments in the evaporation rates of the EWRP brine inoculated by the *Planococcus*_B sp. C5-4 strains represent an over-estimate compared to what might be achieved in the brine evaporation pond at the EWRP. Moreover, because the evaporation experiments in this study were conducted under very controlled laboratory conditions that excluded changing climatic conditions experienced by brine in evaporation ponds and radiant energy was supplied 24 hours a day, the attained evaporation rate increments represent a best-case scenario. Nonetheless, the main results of this thesis have demonstrated the effectiveness of the biological approach to increasing the evaporation rate of the Na_2SO_4 based brine from the

EWRP pond, and how the reflected light spectrum of carotenoid pigment(s) produced by halophilic bacteria affect brine evaporation rate. Also demonstrated is the fact that pigmentation is not a prerequisite for a bacterial strain to affect an increment in the brine evaporation rate, but an important factor to attaining even higher evaporation rate increments.

That being said, the application of this approach to real-world industrial desalination brine evaporation pond systems, either using the *Planococcus*_B sp. CP5-4-WT strain or any other pigmented halophilic bacterial species would require the consideration of the following:

- The effect of brine composition on pigment production. Bacterial growth and carotenoid production is governed by external factors such as culture conditions and environmental parameters, which directs their biosynthesis pathways (Ram *et al.*, 2020). It has been shown that the variations in the concentrations of ions such as potassium, magnesium, sodium, calcium, sulphate and other transition metal ions such as the ferric or ferrous ions in desalination brines from different feed water sources cause significant changes in pigment production, in some cases even inhibiting pigment production in some bacteria (Silva-Castro *et al.*, 2019). Thus, such an organism-specific response to the brine composition makes the application of the biological approach to real-world situations difficult and the outcome unpredictable.
- The brine ecology, which is comprised of several genera of bacteria that include, but are not limited to pigmented halophilic eubacteria, archaea, sulphur-oxidizing bacteria, etc. Thus, the non-sterile nature of the brine ponds may result in the autochthonous microorganisms outcompeting the CP5-4 strain and also result in an unpredictable outcome. This was observed to be the case when CP5-4 was used to inoculate 100 L of non-sterile desalination brine from NuWater in a pilot evaporation study conducted by Moyo *et al.* (2018). Consequently, the increase in the evaporation rate of the brine could not be confidently attributed to the effect of CP5-4.

- The need for aeration and agitation of the brine to promote the growth of heterotrophic pigmented halophilic bacteria instead of sulphate-reducing bacteria.

However, with proper planning, and strain improvement for adaptability and constitutive carotenoid production in different types of desalination brines, the biological approach to improving industrial wastewater brine evaporation rates may offer a cheap and environmentally friendly solution to RO desalination brine management with the potential for generating products of value such as food colouring, halophilic enzymes, compatible solutes, etc., from pigmented halophilic bacteria isolated from the brine.

Compared to other carotenoid production methods in South Africa such as the use of the halotolerant algae *Dunaliella salina* to produce astaxanthin and beta-carotene (Lalloo, 2007), bacterial carotenoids provide a compelling forte to be exploited as a promising alternative. This is largely because of the potential for higher biomass from bacterial cultures, shorter cultivation periods, lack of seasonal restrictions, production of a wide variety of novel carotenoids with higher antioxidant activities and larger colour palette, ease of extraction, and the potential of converting low-cost substrate such as industrial wastewater into value added products (Numan *et al.*, 2018; Ram *et al.*, 2020). If the use of pigmented halophilic bacteria in enhancing brine evaporation rates can be successful then the benefits of having a natural bioreactor for the production of the carotenoids may help offset costs of brine storage and disposal. The global market for carotenoids is anticipated to reach US$ 2.0 billion by 2022, at a compound annual growth rate of (CAGR) of 5.7% from a 2017 US$ 1.5 billion global carotenoid market price (McWilliams, 2018; Ram *et al.*, 2020).

5.3 Limitations of the study

A major limitation of the study was the inability to assess the combined effect of the pigmented *Planococcus*_B sp. CP5-4 strains, as well as those of fluctuating temperatures, wind speed, pond depth, and humidity on the brine evaporation rate. Furthermore, the unpredictability of the adaptation (i.e., growth and pigment production) of the bacteria in different evaporation pond systems and brine compositions makes it difficult to apply the findings of this study to other industrial brines.

5.4 Recommendations for future research

After completing the study and in view of its limitations, the following recommendations can be made for future research:

- A similar study is conducted in an environmental chamber allowing for the study of prevailing meteorological conditions, brine depth, and the effect of the pigmented strains on the evaporation rate simultaneously, so the attained evaporation rates can be extrapolated to real-world evaporation ponds.
- Moreover, a variety of industrial brines from the desalination of different feed water sources would have to be assessed for their ability to support the growth and pigment production by the halophilic bacterial strain to be used for enhancing the evaporation rate. The reason for this recommendation is that the composition of brine was shown to be influenced by the feed water quality, pre-treatment method, and the cleaning procedures and chemicals used during the desalination process by Yoon *et al.* (2004); Ersever, Ravindran and Pirbazari (2007); Gomes *et al.* (2007); Lee *et al.* (2009); and Pramanik, Shu and Jegatheesan, (2017). Because of all the factors mentioned above, the growth and pigment production capacity of the halophilic bacterial strain may be impacted as observed by Silva-Castro *et al.* (2019). In their study, Silva-Castro *et al.* (2019) reported the loss of constitutive pigment production from *Arthrobacter* EP3 and *Planococcus* CP5-4 isolates when grown in brine

from NuWater instead of in a synthetic brine medium formulated from the chemical composition of the EWRP brine.

- The pigment producing strain is engineered to constitutively express the carotenoid pathway under changing conditions as would be experienced in an evaporation pond, by either promoter refactoring for constitutive carotenoid production (Silva-Castro *et al.*, 2019) or by identifying and knocking-out non-essential biosynthetic genes to divert more carbon flux into carotenoid synthesis, and thus further enhancing carotenoid biosynthesis (Zuo *et al.*, 2018).

Last but not least, because of the biotechnological potential of moderately halophilic bacteria and several attractive features that make them ideal candidates for genetic engineering such as (1) their high salt tolerance, which decreases to a minimum the necessity for aseptic conditions; (2) their simple nutritional requirements that make them easy to grow and maintain in the laboratory; (3) the majority can use a large range of compounds as a sole carbon and energy source (Argandoña *et al.*, 2012) and; (4) their accumulation of compatible solutes that enhance solubility and proper folding of recombinant proteins (Yin *et al.*, 2015), a case is made for the heterologous expression of pigment compounds such as the violet pigment violecien in the *Planococcus*_B sp. CP5-4-UN strain for the further improvement of brine evaporation rates. Violacein [3-(1,2-dihydro-5-(5-hydroxy-1 *H*-indol-3-yl)-2-*oxo*-3*H*-pyrrol-3-ilydene)-1,3-dihydro-2*H*-indol-2-one], is an indole derivative compound with a deep purple hue that is produced by various bacteria such as *Pseudoalteromonous luteoviolacea*, *Alteromonas luteoviolacea*, *Chromobacterium violaceum*, *Janthinobacterium lividum, Janthinobacterium* sp. HH01 (Laatsch, Thomson and Cox, 1984; Yada *et al.*, 2008; Zhang, 2010; Rodrigues *et al.*, 2012; Hornung *et al.*, 2013).

The deep violet chromophore violacein is of particular interest because it has an absorbance spectrum peaking around 590 nm with substantial absorbance above 650 nm (Jiang *et al.*, 2015), unlike carotenoids which absorb maximally between 400 and 500 nm and produce light reflected spectra corresponding to yellow, orange, and red (Zigmantas *et al.*, 2002, 2004; Flores-Hidalgo *et al.*, 2017;

Takaya, Anan and Iwata, 2018; Ram *et al.*, 2020). Also, violacein is of interest because the genes required for its production are known to exist within a single operon, i.e., *vioABCDE*, which several researchers have had success in cloning and expressing in other bacterial hosts like *E. coli* (Sarovich and Pemberton, 2007; Ahmetagic and Pemberton, 2010; Harrison and Ronczka, 2013; Hornung *et al.*, 2013; André Luis Rodrigues, 2014).

However, compared to the genetically tractable bacterial hosts like *E. coli*, the genetic manipulation of the non-model microorganism *Planococcus*_B sp. CP5-4-UN will be challenging because as of the time of conducting the experimental work and writing this thesis, there were no known genetic tools and protocols for the *Planococcus* species.

Thus, for this approach to be feasible appropriate cloning and expression vectors have to be developed. Broad-host range (BHR) plasmids are the obvious choice for the development of both cloning and expression vectors for non-model organisms like the *Planococcus*_B sp. CP5-4-UN strain because of their ability to be transferred and maintained among bacteria belonging to different subgroups, which is made possible by the ability of the BHR plasmids to replicate independently of the host's replication factors. The cloning and expression vectors would have to have an appropriate selection marker for the identification of transformants on the growth media containing high salt concentrations, as halophilic bacteria often show a natural antibiotic-resistant phenotype in the presence of high salt concentration (Nieto *et al.*, 1993; Coronado *et al.*, 1995; Lv *et al.*, 2015). As such, a non-antibiotic resistance selection marker such as an auxotrophic maker would have to be developed and included on the cloning and expression vectors; auxotrophic markers have been widely used to construct genetic systems in bacteria, eukaryotes and some halophilic archaeal species (Lv *et al.*, 2015). Additionally, the expression vector would have to have a tightly regulated expression system such as the inclusion of an inducible promoter for the controlled expression of the genes of interest following successful transformation.

Most importantly, there needs to be a high efficiency transformation system that allows for DNA transfer, transposon mutagenesis, and expression of specific

genes. Different methods that include conjugal transfer (Vargas *et al.*, 1997; Ventosa, Nieto and Oren, 1998; Argandoña *et al.*, 2012) and electroporation (Mellado, Nieto and Ventosa, 1995; Harris *et al.*, 2016) have been used for transforming halophilic bacteria.

However, compared to conjugal transfer, electroporation has a broad range of microorganisms on which it can be used, involves fewer steps, is quicker, and more efficient (Aune and Aachmann, 2010). Thus, a detailed electroporation method would have to be developed through the systemic examination of the factors involved in the entire electroporation process, which include the addition of osmoticums in both the electroporation wash buffer and growth medium to balance the high internal turgor pressure of the halophilic bacterial cells (Trevors, Dower and Blaschek, 1992) and the optimization of the field strength used for electroporation to ensure high transformation efficiencies.

In closing, this study has contributed to the body of knowledge available on the type, structure and the biosynthesis of the pigment(s) produced by the *Planococcus* species, specifically for those belonging in the *Planococcus*_B genus. The knowledge on the biosynthesis pathway in *Planococcus*_B sp. CP5-4-WT, the identification of the genes for C_{30}-carotenoid synthesis, and production of the pigment mutants have paved the way for future studies to elucidate the developmental regulation of the pathway as well as determining the potential biotechnological importance of these molecules in the health, food and feed sectors.

Most importantly, from an industrial application point of view, the current study has shown the potential of pigmented halophilic bacteria as an environmentally friendly and sustainable option to the use of chemical dye for improving evaporation rates of desalination brine disposed of in evaporation pond systems.

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APPENDICES

Appendix A

Properties of the eMalahleni water reclamation plant desalination brine.

Table A 1 Chemical and physical properties of eMalahleni water reclamation plant desalination brine

Parameter	Value or concentration[a] in eMalahleni brine
pH – Value at 25 °C	7.76
Electrical Conductivity in mS/m at 25 °C	2190
Chloride	1072.78[a]
Sulphate	14520[a]
Fluoride	ND
Nitrate	122.12[a]
Nitrite	0.01[a]
Sodium	3973[a]
Potassium	710[a]
Calcium	1200[a]
Magnesium	459[a]
Aluminium	0.03[a]
Barium	0.21[a]
Copper	0.004[a]
Iron	0.07[a]
Manganese	0.01[a]
Phosphorus	0.03[a]
Zinc	0.008[a]
Salinity	2.12[b]

a. Concentration of the brine components is expressed in parts per million (ppm).
b. Salinity in mg/L

Appendix B

Average evaporation rates of synthetic brine with various concentrations of methylene blue dye in 200 mL synthetic brine.

Table B 1 Average evaporation rates of synthetic brine with various concentrations of methylene blue dye in 200 mL synthetic brine as determined by Silva-Castro *et al.*, 2019.

Methylene blue Concentration [mg·L^{-1}]	**Evaporation rate [cm/ h]**
0	0.027 ± 0.002
100	0.034 ± 0.003
150	0.037 ± 0.005
200	0.039 ± 0.004
250	0.037 ± 0.003
300	0.043 ± 0.003

Mean ± standard deviation

Appendix C

Evaporation rate experiment pan set up

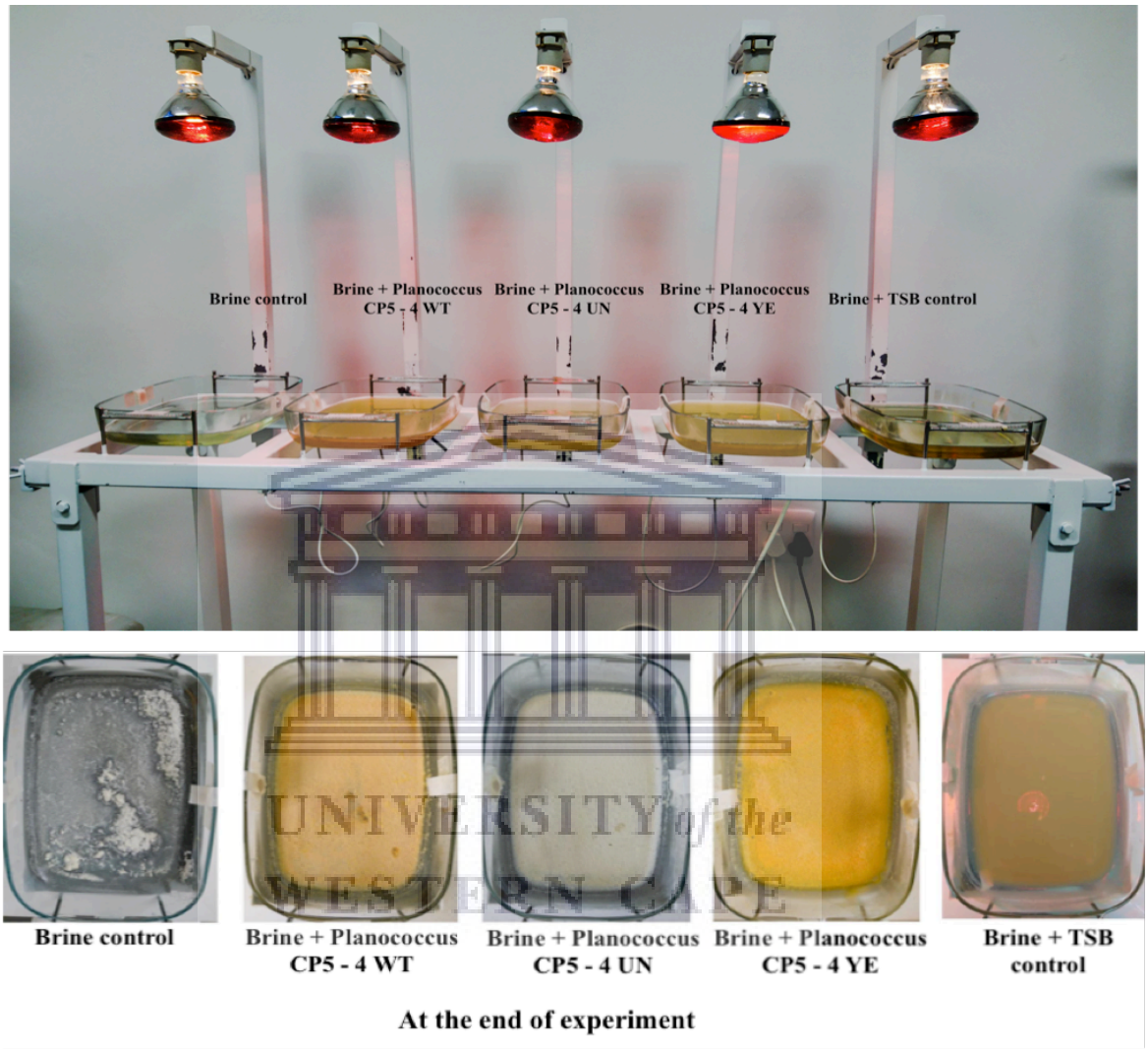

Figure 6.1 Evaporation rate experiment set up. The top panel shows the position of the pans under the infrared light heat sources while the bottom panel shows the pan contents at the end of the experiment.

Appendix D

Study variables, normality and homogeneity of variance tests on dependent variables.

A one-way MANOVA was used to determine whether there were differences in the evaporation rate of the brine during the 12-hour intervals as influenced by the different additives used. The additives used were: none, TSB-salt-sorbitol medium, and cultures of *Planococcus* sp. CP5-4 WT, YE and UN strains in TSB-salt-sorbitol medium. As such, the evaporation rates of the brine with the different additives were assessed. Therefore, the dependent variables of the study were the brine evaporation rates per 12-hour period of the total duration of the experiment, whilst the independent variable was the additive used, which consisted of the five categories: none, TSB, *Planococcus* sp. CP5-4 WT, *Planococcus* sp. CP5-4 YE, and *Planococcus* sp. CP5-4 UN.

The data for the dependent variables were checked for normal distribution and homogeneity of variance in SPSS®. Normality of the data was confirmed by the Kolmogorov-Smirnov/Shapiro-Wilk tests. Since the sample size was less than 50 (N=15, three replicates per additive), the outcome of the Shapiro-Wilk test was taken into consideration (Field, 2009; Mayers, 2013). Table D1 indicates that data for the dependent variables (duration of brine evaporation and brine evaporation rate per 12-hour period) were normally distributed for all the additives.

The Kolmogorov-Smirnov and Shapiro-Wilk tests investigate whether data are significantly different to a normal distribution, thus for the data to be similar to a normal distribution the significance (Sig.) must be greater than 0.05 (Mayers, 2013). Since the sample sizes were equal for each group in the evaporation rate experiments (N = 3, three replicates for each additive) the homogeneity of variance assumption was ignored and the variances taken to be equal in all cases.

Table D 1 Kolmogorov - Smirnov/Shapiro-Wilk test for normality of dependent variables data.

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
Dependent variable	**Statistic**	**df**	**Sig.**	**Statistic**	**df**	**Sig.**
Evaporation rate for 0 to 12 hour period	.266	15	.005	.822	15	.007
Evaporation rate for 12 to 24 hour period	.136	15	.200*	.933	15	.302
Evaporation rate for 24 to 36 hour period	.177	15	.200*	.921	15	.200
Evaporation rate for 36 to 48 hour period	.271	15	.004	.849	15	.017
Evaporation rate for 48 to 60 hour period	.207	15	.085	.913	15	.150
Evaporation rate for 60 to 72 hour period	.157	15	.200*	.940	15	.379
Evaporation rate for 72 to 84 hour period	.189	15	.156	.885	15	.056

*. This is a lower bound of the true significance.
a. Lilliefors Significance Correction

Appendix E

Means, standard error of means and percentages of total brine loss per 12-hour period

Additive		Volume of brine loss (mL) per 12-hour interval						
		0 to 12 hours	12 to 24 hours	24 to 36 hours	36 to 48 hours	48 to 60 hours	60 to 72 hours	72 to 84 hours
None	Mean	120.00	343.33	530.00	650.00	830.00	916.00	1000.00
	SEM	5.77	8.82	5.77	11.55	10.00	8.33	0.00
	% of Total Sum	17.2%	21.8%	20.5%	19.0%	18.8%	19.0%	20.0%
TSB	Mean	123.33	230.00	406.67	596.67	790.00	942.67	1000.00
	SEM	3.33	10.00	8.82	8.82	5.77	2.67	0.00
	% of Total Sum	17.7%	14.6%	15.7%	17.5%	17.8%	19.6%	20.0%
Planococcus sp. CP5-4 WT	Mean	190.00	370.00	590.00	790.00	993.33	1000.00	1000.00
	SEM	5.77	10.00	11.55	10.00	3.33	0.00	0.00
	% of Total Sum	27.3%	23.5%	22.8%	23.1%	22.4%	20.8%	20.0%
Planococcus sp. CP5-4 YE	Mean	140.00	343.33	553.33	713.33	923.33	990.00	1000.00
	SEM	5.77	6.67	14.53	3.33	3.33	5.77	0.00
	% of Total Sum	20.1%	21.8%	21.4%	20.9%	20.9%	20.5%	20.0%
Planococcus sp. CP5-4 UN	Mean	123.33	290.00	503.33	663.33	890.00	970.00	1000.00
	SEM	3.33	5.77	6.67	27.28	11.55	15.28	0.00
	% of Total Sum	17.7%	18.4%	19.5%	19.4%	20.1%	20.1%	20.0%

Appendix F

Analysis of variance (ANOVA) for duration of brine evaporation

Table F 1 Descriptive statistics for duration of brine evaporation

				95% Confidence Interval for Mean	
Additive	**N**	**Mean**	**Std. Error**	**Lower Bound**	**Upper Bound**
None	3	3 06:36:00	00:21:39.4	3 05:02:49	3 08:09:10
P. maritimus CP5 - 4 WT	3	2 23:38:20	00:10:10.2	2 22:54:34	3 00:22:05
P. maritimus CP5 - 4 YE	3	3 02:10:40	00:18:53.3	3 00:49:23	3 03:31:56
P. maritimus CP5 - 4 UN	3	3 04:27:40	00:32:32.3	3 02:07:39	3 06:47:40

Table F 2 One-way analysis of variance (ANOVA) output.

	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	1044913200.000	3	348304400.000	64.890	.000
Within Groups	42940800.000	8	5367600.000		
Total	1087854000.000	11			

Table F 3 Fisher's LSD post hoc test on duration of brine evaporation

(I) Additive	(J) Additive	Sig.	95% Confidence Interval	
			Lower Bound	Upper Bound
None	*P. maritimus* CP5 - 4 WT	.000	05:44:57	08:10:22
	P. maritimus CP5 - 4 YE	.000	03:12:37	05:38:02
	P. maritimus CP5 - 4 UN	.004	00:55:37	03:21:02
P. maritimus CP5 - 4 WT	None	.000	- 08:10:22	- 05:44:57
	P. maritimus CP5 - 4 YE	.001	- 03:45:02	- 01:19:37
	P. maritimus CP5 - 4 UN	.000	- 06:02:02	- 03:36:37
P. maritimus CP5 - 4 YE	None	.000	- 05:38:02	- 03:12:37
	P. maritimus CP5 - 4 WT	.001	01:19:37	03:45:02
	P. maritimus CP5 - 4 UN	.002	- 03:29:42	- 01:04:17
P. maritimus CP5 - 4 UN	None	.004	- 03:21:02	- 00:55:37
	P. maritimus CP5 - 4 WT	.000	03:36:37	06:02:02
	P. maritimus CP5 - 4 YE	.002	01:04:17	03:29:42

*. The mean difference is significant at the 0.05 level

Appendix G

Multivariate and univariate analysis of variance and Fisher's LSD post hoc test

Table G 1 Multivariate test (MANOVA) on effect of bacterial pigments on brine evaporation

Effect		Value	F	Hypothesis df	Error df	Sig.	Partial Eta Squared
Intercept	Pillai's Trace	1.000	8507.617[b]	6.000	3.000	.000	1.000
	Wilks' Lambda	.0001	8507.617[b]	6.000	3.000	.000	1.000
	Hotelling's Trace	17015.234	8507.617[b]	6.000	3.000	.000	1.000
	Roy's Largest Root	17015.234	8507.617[b]	6.000	3.000	.000	1.000
Additive	Pillai's Trace	2.302	2.746	18.000	15.000	.027	.767
	Wilks' Lambda	.0005	6.910	18.000	8.971	.003	.922
	Hotelling's Trace	138.063	12.784	18.000	5.000	.005	.979
	Roy's Largest Root	129.083	107.569[c]	6.000	5.000	.000	.992

a. Design: Intercept + Additive

b. Exact statistic

c. The statistic is an upper bound on F that yields a lower bound on the significance level.

Table G 2 Univariate tests of between-subjects effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	0 to 12 hours	.000[a]	3	4.197E-5	37.600	.000
	12 to 24 hours	7.591E-5[b]	3	2.530E-5	4.722	.035
	24 to 36 hours	2.534E-5[c]	3	8.447E-6	1.513	.284
	36 to 48 hours	.000[d]	3	3.617E-5	2.817	.107
	48 to 60 hours	5.984E-5[e]	3	1.995E-5	1.418	.307
	60 to 72 hours	.000[f]	3	5.311E-5	15.328	.001
	72 to 84 hours	.000[g]	3	5.642E-5	16.714	.001
Intercept	0 to 12 hours	.003	1	.003	2958.400	.000
	12 to 24 hours	.006	1	.006	1121.333	.000
	24 to 36 hours	.007	1	.007	1240.020	.000
	36 to 48 hours	.004	1	.004	327.270	.000
	48 to 60 hours	.007	1	.007	472.508	.000
	60 to 72 hours	.001	1	.001	166.084	.000
	72 to 84 hours	.000	1	.000	45.762	.000
Additive	0 to 12 hours	.000	3	4.197E-5	37.600	.000
	12 to 24 hours	7.591E-5	3	2.530E-5	4.722	.035
	24 to 36 hours	2.534E-5	3	8.447E-6	1.513	.284
	36 to 48 hours	.000	3	3.617E-5	2.817	.107
	48 to 60 hours	5.984E-5	3	1.995E-5	1.418	.307
	60 to 72 hours	.000	3	5.311E-5	15.328	.001
	72 to 84 hours	.000	3	5.642E-5	16.714	.001

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Error	0 to 12 hours	8.931E-6	8	1.116E-6		Error
	12 to 24 hours	4.287E-5	8	5.358E-6		.035
	24 to 36 hours	4.465E-5	8	5.582E-6		.284
	36 to 48 hours	.000	8	1.284E-5		.107
	48 to 60 hours	.000	8	1.407E-5		.307
	60 to 72 hours	2.772E-5	8	3.465E-6		.001
	72 to 84 hours	2.701E-5	8	3.376E-6		.001
Total	0 to 12 hours	.003	12			
	12 to 24 hours	.006	12			
	24 to 36 hours	.007	12			
	36 to 48 hours	.004	12			
	48 to 60 hours	.007	12			
	60 to 72 hours	.001	12			
	72 to 84 hours	.000	12			
Corrected Total	0 to 12 hours	.000	11			
	12 to 24 hours	.000	11			
	24 to 36 hours	6.999E-5	11			
	36 to 48 hours	.000	11			
	48 to 60 hours	.000	11			
	60 to 72 hours	.000	11			
	72 to 84 hours	.000	11			

a. R Squared = .934 (Adjusted R Squared = .909)
b. R Squared = .639 (Adjusted R Squared = .504)
c. R Squared = .362 (Adjusted R Squared = .123)
d. R Squared = .514 (Adjusted R Squared = .331)
e. R Squared = .347 (Adjusted R Squared = .102)
f. R Squared = .852 (Adjusted R Squared = .796)
g. R Squared = .862 (Adjusted R Squared = .811)
h. Computed using alpha = .05

Appendix H

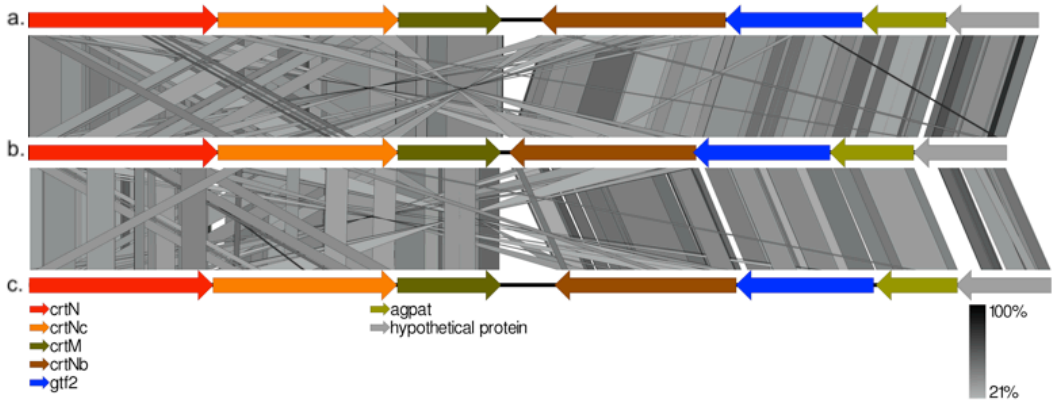

Figure H 1 EasyFig tBLASTx comparison of the carotenogenic gene clusters of *Planococcus*_A *kocurii* (a.), *Planococcus*_B sp. CP5-4-WT (b.), and *Halobacillus halophilus* (c.). The carotenogenic gene organization between the organisms is similar, though with little sequence similarity.

Table H 1 Names and locations of the additional carotenogenic genes in the genomes of the other *Planococcus*_B genus

Organism	Genome id	Name of additional Crt synthesis genes	Location on genome
Planococcus_B sp. 002833405	GCF_002833405.1	1. Phytoene/squalene synthase family protein	487 280 – 488 110
		2. Diapolycopene oxygenase	745 224 – 746 726
Planococcus_B rifietoensis	GCF_001465795.2	1. Phytoene synthase	194 975 – 195 805
		2. Diapolycopene oxygenase	462 371 – 463 873
Planococcus_B maitriensis	GCA_003289925.1	1. Phytoene/squalene synthase family protein	QLZQ01000003* 314 523 – 315 353

Table H 1 Continued…

Organism	Genome id	Name of additional Crt synthesis genes	Location on genome
Planococcus_B plakortidis	GCF_001687605.2	1. Phytoene/squalene synthase family protein	521 501 – 522 331
		2. Diapolycopene oxygenase	730 457 – 731 959
Planococcus_B maritimus	GCF_001687625.2	1. Phytoene/squalene synthase family protein	500 800 – 501 630
		2. Diapolycopene oxygenase	740 416 – 741 924
Planococcus_B maritimus	GCF_001592825.1	1. Diapolycopene oxygenase	LTZG01000011* 183 819 – 185 321

Appendix I

Metabolic model comparisons of *Planococcus*_B sp. CP5-4 WT, YE, and UN strains.

Table I 1 Conserved biomass compounds in the three *Planococcus*_B sp. CP5-4 strains

Compound	Name	Number of Models	Conserved	WT	YE	UN
cpd00001	H_2O	3	1	present	present	present
cpd00002	ATP	3	1	present	present	present
cpd00003	NAD	3	1	present	present	present
cpd00006	NADP	3	1	present	present	present
cpd00008	ADP	3	1	present	present	present
cpd00009	Phosphate	3	1	present	present	present
cpd00010	CoA	3	1	present	present	present
cpd00012	PPi	3	1	present	present	present
cpd00015	FAD	3	1	present	present	present
cpd00016	Pyridoxal phosphate	3	1	present	present	present
cpd00017	S-Adenosyl-L-methionine	3	1	present	present	present
cpd00023	L-Glutamate	3	1	present	present	present
cpd00028	Heme	3	1	present	present	present
cpd00030	Mn^{2+}	3	1	present	present	present
cpd00033	Glycine	3	1	present	present	present
cpd00034	Zn^{2+}	3	1	present	present	present
cpd00035	L-Alanine	3	1	present	present	present
cpd00038	GTP	3	1	present	present	present
cpd00039	L-Lysine	3	1	present	present	present
cpd00041	L-Aspartate	3	1	present	present	present
cpd00042	GSH	3	1	present	present	present
cpd00048	Sulfate	3	1	present	present	present
cpd00051	L-Arginine	3	1	present	present	present
cpd00052	CTP	3	1	present	present	present
cpd00053	L-Glutamine	3	1	present	present	present
cpd00054	L-Serine	3	1	present	present	present
cpd00056	TPP	3	1	present	present	present
cpd00058	Cu2+	3	1	present	present	present
cpd00060	L-Methionine	3	1	present	present	present
cpd00062	UTP	3	1	present	present	present
cpd00063	Ca^{2+}	3	1	present	present	present
cpd00065	L-Tryptophan	3	1	present	present	present
cpd00066	L-Phenylalanine	3	1	present	present	present
cpd00067	H^+	3	1	present	present	present
cpd00069	L-Tyrosine	3	1	present	present	present
cpd00084	L-Cysteine	3	1	present	present	present
cpd00087	Tetrahydrofolate	3	1	present	present	present
cpd00099	Cl^-	3	1	present	present	present
cpd00107	L-Leucine	3	1	present	present	present
cpd00115	dATP	3	1	present	present	present
cpd00118	Putrescine	3	1	present	present	present
cpd00119	L-Histidine	3	1	present	present	present
cpd00129	L-Proline	3	1	present	present	present

Table I 1 Continued…

Compound	Name	Number of Models	Conserved	WT	YE	UN
cpd00149	Co^{2+}	3	1	present	present	present
cpd00156	L-Valine	3	1	present	present	present
cpd00161	L-Threonine	3	1	present	present	present
cpd00166	Calomide	3	1	present	present	present
cpd00201	10-Formyltetrahydrofolate	3	1	present	present	present
cpd00205	K^{+}	3	1	present	present	present
cpd00220	Riboflavin	3	1	present	present	present
cpd00241	dGTP	3	1	present	present	present
cpd00254	Mg	3	1	present	present	present
cpd00264	Spermidine	3	1	present	present	present
cpd00322	L-Isoleucine	3	1	present	present	present
cpd00345	5-Methyltetrahydrofolate	3	1	present	present	present
cpd00356	dCTP	3	1	present	present	present
cpd00357	TTP	3	1	present	present	present
cpd00557	Siroheme	3	1	present	present	present
cpd01997	Dimethylbenzimidazole	3	1	present	present	present
cpd02229	Bactoprenyl diphosphate	3	1	present	present	present
cpd03422	Cobinamide	3	1	present	present	present
cpd10515	Fe^{2+}	3	1	present	present	present
cpd10516	Fe^{3+}	3	1	present	present	present
cpd11416	Biomass	3	1	present	present	present
cpd11459	Tcam	3	1	present	present	present
cpd11493	ACP	3	1	present	present	present
cpd12370	apo-ACP	3	1	present	present	present
cpd15352	2-Demethylmenaquinone 8	3	1	present	present	present
cpd15500	Menaquinone 8	3	1	present	present	present
cpd15533	phosphatidylethanolamine dioctadecanoyl	3	1	present	present	present
cpd15540	Phosphatidylglycerol dioctadecanoyl	3	1	present	present	present
cpd15560	Ubiquinone-8	3	1	present	present	present
cpd15665	Peptidoglycan polymer (n subunits)	3	1	present	present	present
cpd15666	Peptidoglycan polymer (n-1 subunits)	3	1	present	present	present
cpd15667	glycerol teichoic acid (n=45), linked, unsubstituted	3	1	present	present	present
cpd15668	glycerol teichoic acid (n=45), linked, D-ala substituted	3	1	present	present	present
cpd15669	glycerol teichoic acid (n=45), linked, glucose substituted	3	1	present	present	present
cpd15695	Diisoheptadecanoylphosphatidylethanolamine	3	1	present	present	present
cpd15696	Dianteisoheptadecanoylphosphatidylethanolamine	3	1	present	present	present

Table I 1 Continued…

Compound	Name	Number of Models	Conserved	WT	YE	UN
cpd15722	Diisoheptadecanoylphosphatidylglycerol	3	1	present	present	present
cpd15723	Dianteisoheptadecanoylphosphatidylglycerol	3	1	present	present	present
cpd15748	Stearoyllipoteichoic acid (n=24), linked, unsubstituted	3	1	present	present	present
cpd15749	Isoheptadecanoyllipoteichoic acid (n=24), linked, unsubstituted	3	1	present	present	present
cpd15750	Anteisoheptadecanoyllipoteichoic acid (n=24), linked, unsubstituted	3	1	present	present	present
cpd15757	Stearoyllipoteichoic acid (n=24), linked, glucose substituted	3	1	present	present	present
cpd15758	Isoheptadecanoyllipoteichoic acid (n=24), linked, glucose substituted	3	1	present	present	present
cpd15759	Anteisoheptadecanoyllipoteichoic acid (n=24), linked, glucose substituted	3	1	present	present	present
cpd15766	Stearoyllipoteichoic acid (n=24), linked, N-acetyl-D-glucosamine	3	1	present	present	present
cpd15767	Isoheptadecanoyllipoteichoic acid (n=24), linked, N-acetyl-D-glucosamine	3	1	present	present	present
cpd15768	Anteisoheptadecanoyllipoteichoic acid (n=24), linked, N-acetyl-D-glucosamine	3	1	present	present	present
cpd15775	Stearoyllipoteichoic acid (n=24), linked, D-alanine substituted	3	1	present	present	present
cpd15776	Isoheptadecanoyllipoteichoic acid (n=24), linked, D-alanine substituted	3	1	present	present	present
cpd15777	Anteisoheptadecanoyllipoteichoic acid (n=24), linked, D-alanine substituted	3	1	present	present	present
cpd15793	Stearoylcardiolipin (*B. subtilis*)	3	1	present	present	present
cpd15794	Isoheptadecanoylcardiolipin (*B. subtilis*)	3	1	present	present	present

Table I 1 Continued…

Compound	Name	Number of Models	Conserved	WT	YE	UN
cpd15795	Anteisoheptadecanoylcardiolipin (*B. subtilis*)	3	1	present	present	present
cpd17041	Protein biosynthesis	3	1	present	present	present
cpd17042	DNA replication	3	1	present	present	present
cpd17043	RNA transcription	3	1	present	present	present
cpd15795	Anteisoheptadecanoylcardiolipin (*B. subtilis*)	3	1	present	present	present
cpd17041	Protein biosynthesis	3	1	present	present	present
cpd17042	DNA replication	3	1	present	present	present
cpd17043	RNA transcription	3	1	present	present	present
cpd15795	Anteisoheptadecanoylcardiolipin (*B. subtilis*)	3	1	present	present	present

Table I 2 Reactions and coding sequences absent from the *Planococcus* sp. CP5-4-YE and UN strains' metabolic models

Reaction	WT	YE	UN	CDS Product Function
NADP + (2) L-Glutamate <=> NADPH + 2-Oxoglutarate + L-Glutamine + H	CDS.520 CDS.521	CDS.1511 CDS.1512	absent	Glutamate synthase [NADPH] small chain (EC 1.4.1.13), Glutamate synthase [NADPH] large chain (EC 1.4.1.13)
H_2O + L-Glutamine => NH_3 + L-Glutamate	CDS.392	CDS.1384	absent	Glutaminase (EC 3.5.1.2)
H_2O + Acetamide => NH_3 + Acetate	CDS.483	CDS.1474	absent	Aliphatic amidase AmiE (EC 3.5.1.4)
NADPH + O_2+ L-Arginine+ H => H_2O + NADP + N-(omega)-Hydroxyarginine	CDS.429	CDS.1420	absent	Nitric oxide synthase oxygenase (EC 1.-.-.-)

Table I 2 continued …

Reaction and compound	WT	YE	UN	CDS Product Function
H_2O+ H + 2-Phenylacetamide <=> NH_3 + PACT	CDS.483	CDS.1474	absent	Aliphatic amidase AmiE (EC 3.5.1.4)
ADP + H + deoxyribose-5-phosphate <=> ATP + Thyminose	CDS.416	CDS.1407	absent	Ribokinase (EC 2.7.1.15)
H_2O + Indole-3-acetamide => NH_3 + Indoleacetate	CDS.483	CDS.1474	absent	Aliphatic amidase AmiE (EC 3.5.1.4)
H_2O + 4-Guanidinobutanamide => NH_3 + 4-Guanidinobutanoate				
NH_3 + Acrylate <= H_2O + Acrylamide	CDS.483	CDS.1474	absent	Aliphatic amidase AmiE (EC 3.5.1.4)
H_2O + Benzamide => NH_3 + Benzoate				
NADH + $2O_2$ + 2NO <=> NAD + H + (2) Nitrate	CDS.382	CDS.1374	absent	Flavohemoprotein (EC 1.14.12.17)
NADPH + $2O_2$ + 2NO <=> NADP + H + (2) Nitrate				
L-Glutamate + 4-methylthio 2-oxobutyrate <=> 2-Oxoglutarate + L-Methionine	CDS.484	CDS.1475	absent	Glutamine-dependent 2-keto-4-methylthiobutyrate transaminase
H_2O + ATP + D-Ribose => ADP+ Phosphate + H + D-Ribose	CDS.412 CDS.415	CDS.1406 CDS.1403	absent	Ribose ABC transport system, periplasmic ribose-binding protein RbsB (TC 3.A.1.2.1),
H_2O + ATP + Thyminose => ADP + Phosphate + H + Thyminose	CDS.413 CDS.414	CDS.1405 CDS.1404		Ribose ABC transport system, high affinity permease RbsD (TC 3.A.1.2.1), Ribose ABC transport system, permease protein RbsC (TC 3.A.1.2.1), and Ribose ABC transport system, ATP-binding protein RbsA (TC 3.A.1.2.1)

Table I 2 continued …

Reaction and compound	WT	YE	UN	CDS Product Function
H+[e0] + Phenylpropanoate[e0] <=> H + Phenylpropanoate	CDS.2097	absent	CDS.1601	Probable 3-phenylpropionic acid transporter
Farnesyldiphosphate <= (2) PPi + (2) H + Dehydrosqualene	CDS.509	CDS.1500	absent	Dehydrosqualene synthase (EC 2.5.1.96)
H_2O + 2',3'-Cyclic AMP=> H+ 3'-AMP H_2O + 2',3'-Cyclic UMP => H + 3'-UMP H_2O + 2',3'-Cyclic CMP => H + 3'-CMP H_2O + 2',3'-Cyclic GMP => H + Gp	CDS.3616	absent	absent	2',3'-cyclic-nucleotide 2'-phosphodiesterase (EC 3.1.4.16)
UMP+ ATP + H => ADP + UDP	absent	present	absent	ND
H_2O + S-Adenosyl-homocysteine => Homocysteine + Adenosine	absent	present	absent	ND
NADPH + O_2 + (2) N-(omega)-Hydroxyarginine <=> NADP + (5) H+ (2) Citrulline + (2) NO	CDS.429	CDS.1420	absent	Nitric oxide synthase oxygenase (EC 1.-.-.-)

[e0] Extracellular

CDS Coding sequence

ND Not defined

*Unless specified, all reactions were predicted to occur in the cytosol.