Improving Evaporation Rates of Mining Wastewaters

Report to the
WATER RESEARCH COMMISSION

by

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EXECUTIVE SUMMARY

Minimization of desalination waste brine through its re-use or safe re-entry into the hydrological cycle is a critical part of wastewater management. Desalination waste brines are complex liquid mixtures of various salts with a composition dependent on the feed water properties, the chemical additives used in the desalination operation and the water treatment from which the final brine effluent derives. The eMalahleni water reclamation plant uses an evaporation pond for disposal of its brine effluent, which is concentrated to a manageable sludge. One problem with the utilization of evaporation ponds for brine disposal is that securing effective evaporation rates is challenging and requires awareness of how evaporation can be enhanced. The literature reviewed indicated that addition of dyes such as cobalt blue, methylene blue and black napthylamine to industrial brines led to enhanced evaporation rates of the brines, the premise being that coloured brine solutions absorb more radiant energy compared to clear solutions. Dyes aid absorption of radiant energy thus increasing the temperature of the solution, lowering the surface tension, increasing vapour pressure and consequently enhancing the evaporation rate of brine.

Thus, this study investigated whether dyes, either from a chemical or a biological source, could be used to enhance brine evaporation rates. Each source of dye has its own set of advantages and disadvantages, which need to be weighed against the level of improvement, if any. While methylene blue has already been demonstrated to improve evaporation rates, the application of pigments produced by halophilic bacteria for the purpose of industrial brine evaporation has never been evaluated before. Therefore, this project focused on investigating three main aspects:

i. chemical approach involving addition of methylene blue (MB) dye to the brine and evaluating its effect on evaporation rate,
ii. biological approach involving the isolation, characterization and culturing of pigmented halophilic bacteria and evaluating their effect on the evaporation rate,
iii. genetic engineering approach aimed at developing an autochthonous halophilic bacterial host for the expression of dark pigment to further enhance evaporation rates.

Evaporation studies were conducted in an access-controlled laboratory to minimize fluctuations so as to correlate the outcome directly to the dye/pigments being assessed. Three different scales of evaporation were conducted: micro-scale (200 ml), pan scale (1 L), and a pilot scale (100 L). Infrared lamps generating 240 Watts were employed as a source of radiation, ensuring a controlled and replicable design to enable direct correlation between the different approaches being assessed. Two different sources of brine were assessed: a

synthetic brine formulated to replicate the eMalahleni reclamation plant brine, and brine provided by NuWater. The constitution of the brines was compared and the significant differences were found to influence the implementation of the biological approach. This presents a major challenge to the future application of the findings presented in this study.

The chemical approach utilizing methylene blue focused on determining the minimal concentration of dye that would affect a marked improvement on brine evaporation rates when added to the synthetic brine. Five MB concentrations were assessed and 200 ppm resulted in a 21.4% increase in the evaporation rate of the brine. The resulting dried salts were reconstituted to investigate whether the MB dye “trapped” in the salt crystals could continue to effect an increased evaporation. Two successive reconstitutions were conducted and in each a comparable evaporation rate was achieved, i.e. the evaporation rate affected by adding 200 ppm of MB and those following the first and second reconstitutions were the same. The ability to reconstitute the methylene blue from precipitated salts in successive evaporation experiments, instead of replenishing the MB, represents a major advantage and may have important environmental and economic implications. Firstly, it may aid in keeping the MB concentration at manageable levels to prevent negative environmental impacts. Secondly, this could constitute an economic saving.

The biological approach was undertaken as it could represent a more environmentally friendly and sustainable option to enhancing brine evaporation rates. Pigmented halophilic bacteria were isolated from 2 different sources. The pigments produced by these bacteria are carotenoids, and since they absorb in the wavelength range of 300-600 nm, they in principle could cause an increase in the temperature of brine and enhanced evaporation rates, and is the premise on which solar salterns operate for the commercial production of salt. This red/pink pigmentation increases light absorption by the brine and increases its temperature, thus enhancing the salt production process.

Three isolates, CP5-4, EP3 and EP1, showed high growth rates as well as high pigment production levels when cultured in synthetic brine. Micro-scale evaporation experiments confirmed that the growth and pigmentation of the bacterial isolates resulted in up to 51% enhanced evaporation rates when compared to an uninoculated brine control. The rate of improvement seemed to be correlated to the pigment produced by each bacterial isolate, where the pink EP3 isolate affected the highest evaporation rate followed by the orange CP5-4 and the peach-coloured EP1 isolate. Carotenoids absorb light only in the blue range of the electromagnetic spectrum and the degree of absorption is dependent on the number of conjugated double bonds present in the structure of the pigment. The more conjugated double

bonds present, the more radiant energy absorbed and released as heat for enhanced evaporation rates of brine. Consequently, the pink/red pigment produced by isolate EP3 may have had a large number of double bonds that aided in the absorption of more radiant energy compared to the pigments produced by isolates CP5-4 and EP1.

When conducted using the NuWater brine, the carotenoid production was significantly and negatively impacted. A range of optimisations, involving the supplementation of the brine with various components, could restore, to some extent, the expression of the carotenoid in only one of the isolates, CP5-4. This demonstrated one of the biggest limitations to employing a whole cell biocatalyst approach. The production of carotenoids, as secondary metabolites, are usually under the control of numerous regulators which respond to a wide range of environmental queues. The regulatory mechanisms involved are not yet well-understood, making it difficult to predict how a particular whole cell biocatalyst will respond to different brines with respect to its growth and pigment production. This suggests that the performance of a pigmented bacterium would have to be assessed for every brine, making this a less feasible approach to adopt, given that brines from various sources/processes are not uniform in their composition.

Nevertheless, the performance of CP5-4 was assessed using a pilot scale setup, introducing two additional factors which were not relevant in the previous experiments but which represent a more “real world” scenario, i.e. depth and the presence of a mixed microbial community. Even though the desired effect of enhancing brine evaporation rate by 40% through utilizing a pure culture of the CP5-4 isolate was achieved, some insights from the experiments were that:

i. aeration and agitation of the brine are necessary to promote the growth of heterotrophic pigmented halophilic bacteria instead of sulfate reducing bacteria, and

ii. natural halophilic bacterial communities in the brine may compete with the introduced strain and take over as the main microbial community.

Thus, in a real world application there is no control over the type of bacteria that may colonize after addition of a pure culture of pigmented bacteria, although the ultimate outcome would still be enhanced evaporation rate as long as the pond is aerated and supplied with some form of nutrient supplementation. Given the costs, expertise and infrastructure required to prepare an inoculum to seed an evaporation pond, a nutrient supplementation solution to promote the growth of pigmented organisms already resident in the pond offers a much more cost-effective solution.

As a way to overcome the requirement to assess the performance of a pigmented bacterium for every brine, the selection and development of a halophilic bacterial host for the expression

of dark pigment was devised. While some progress was made towards this objective, achieving important breakthroughs in the development of a recombinant halophilic host, we did not achieve expression of the recombinant pathway and were not able to obtain a culture that could stably maintain the introduced operon. We do not, therefore, present this component of the project in this report; however, numerous strategies are currently being considered to overcome these challenges.

This study has achieved its objective of isolating, characterizing and assessing the applicability of pigmented halophilic bacteria as an environmentally friendly and sustainable option to enhancing current brine evaporation rates. In addition comprehensive comparisons between the effect of adding methylene blue dye and using the biological approach were made in each evaporation experiment conducted. The study has been concluded successfully in terms of assessing the effect of adding methylene blue dye on brine evaporation rates as well as utilizing halophilic bacteria for the enhancement of brine evaporation rate. Furthermore, the applicability of the biological approach was tested in a real world scenario and provided valuable insights on how microbial communities in the brine would affect the efficiency of the introduced strain.

Microbial pigmentation has been studied for centuries (Engelmann, 1882), and with the increased awareness in human safety and environmental conservation, a trend towards their application as eco-friendly and biodegradable commodities has followed from this emerging field of study (Venil and Lakshmanaperumalsamy, 2013). Pigments are mostly employed as colouring agents in the pharmaceutical, cosmetic, textile and food industries. In the crystallizer brines of saltern ponds for the production of salt from seawater, halophilic microorganisms are well-known for being responsible for the red colour of the brines at or approaching salt saturation. Although the influence on evaporation by pigmented microorganisms in salterns has been postulated, we present for the first time evidence that addition of pigmented isolates to industrial brine increases the evaporation rate.

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LIST OF ABBREVIATIONS

ARDRA	Amplified ribosomal restriction analysis
a_w	water activity
bp	base pair
CAF	Central analytical facility
CDS	Coding sequence
cfu/mL	Colony forming units per millilitre
Contig	Contiguous sequence
DDT	Dithriothreitol
DMAP	Dimethylallyl diphosphate
DNA	Deoxyribonucleic acid
DOXP/MEP	1-deoxy-D-xylulose-5-phosphate/2-C-methyl-D-erythritol-4-phosphate
dsDNA	Double stranded deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EWRP	eMalahleni water reclamation plant
$FeCl_3$	Ferric chloride
GGDP	Geranyl geranyl diphosphate
HiPRO	High recovery precipitating reverse osmosis
Hr	Hour
IPP	Isopentenyl pyrophosphate
IR	Infrared
Kb	Kilo base pair
KCl	Potassium chloride
KO	KeGG Orthology
mg/L	milligram per litre
MIC	Minimal inhibitory concentration
MMS	Methylmethane sulphonate
MVA	Mevalonate pathway
N^3-MeA	N^3-Methyladenine
N^7-MeG	N^7-Methylguanine
NaCl	Sodium chloride
NEB	New England Biolabs
NER	Nucleotide excision repair
ng	Nanogram

nm	Nanometre
OD	optical density
PDR	Phytoene desaturase
ppm	parts per million
PSY	Phytoene synthase
RAST	Rapid annotation using subsystems
RNA	Ribonucleic acid
rSAP	Recombinant shrimp alkaline phosphatase
sdH_2O	Sterile distilled water
ssDNA	Single stranded deoxyribonucleic acid
TE	Trisaminomethane ethylenediaminetetraacetic acid
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TVC	Total viable count
U	Units
v/v	Volume per volume
w/v	Weight per volume
xg	Gravitational force
µL	Microlitre

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CHAPTER 1: BACKGROUND

1.1. INTRODUCTION

Mine wastewater has historically been considered to be a lost resource. However, in recent years a scarcity of fresh water resources has resulted in mine wastewater being used as a viable source of potable water. The eMalahleni Water Reclamation Plant (EWRP), established as a joint initiative between Anglo Coal South Africa and BHP Billiton Coal Energy South Africa, makes use of a Hi recovery Precipitating Reverse Osmosis (HiPRO) system to recover potable water from mine wastewater generated from Greenside, Kleinkopje, South Witbank and Navigation collieries in the Witbank area (Kabuzire *et al.*, 2010). The process has recovery rates in excess of 99% and generates a concentrated reject brine stream that has to be disposed of.

In South Africa, the use of evaporation ponds is considered to be an effective and economical method for reject brine disposal due to ease of construction, low maintenance and minor operator attention (Mickley, 2007; El-naas, 2011). An evaporation pond is merely an excavated depression in the ground that serves as a reservoir for desalination wastewater. Often evaporation ponds are the final destination of the brine and work by allowing the evaporation of the water fraction of the brine. Evaporation of the water fraction results in the precipitation of residual solids (in the form of salt crystals) which may be landfilled *in situ* or collected and disposed of elsewhere (El-naas, 2011).

One problem with the use of evaporation ponds for brine disposal is that much of the industrial brine production is located in less arid parts of the country, especially Gauteng and Mpumalanga provinces where securing effective evaporation is more challenging (Petrik *et al.*, 2015). A number of researchers have looked at methods to enhance evaporation rates of sodium chloride brines (Kingdon, 1963; 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 water. The author suggested that this would result in an increase in 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 the EWRP pond is not feasible considering the cost and the potential environmental impacts associated with this method.

Other researchers looked at the possibility of adding synthetic dyes such as cobalt or methylene blue to maximize the absorption of solar energy (Bloch, Farkas and Spiegler, 1951; Winans, 1967). A coloured solution absorbs more solar energy than an uncoloured one 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.*, 2000).

Halophilic bacteria that grow in saltern evaporation ponds could offer a free and environmentally friendly option for enhancing the solar evaporation rates of the industrial reject brine produced at the EWRP. Not only are these microorganisms able to tolerate sodium chloride concentrations as high as 100 g/L they also produce pigments. Their pigments are responsible for the wide variety of orange to red colours seen in solar saltern ponds and absorb radiant energy in the wavelength range of 300-600 nm, which causes an increase of the brine temperature and enhanced evaporation rate (Vachali, Bhosale and Bernstein, 2012).

The present study was therefore commissioned to firstly, evaluate the effect of using different concentrations of methylene blue dye on the evaporation rate of desalination reject brine. Secondly, to assess the use of pigmented halophilic bacteria isolates as an environmentally friendly and sustainable option to enhancing current evaporation rates of the EWRP brine, and thirdly to develop a genetically modified halophilic bacterial host for the expression of a dark pigment to further improve evaporation rates as well as mining the genome of the selected host strain for potential biotechnological applications.

1.2. OBJECTIVES

The objectives of the project were:

- Determine the effect of methylene blue supplementation on the evaporation rate of laboratory-scale salt pans, i.e. the “chemical approach”.
- Isolate, identify, characterise and select pigment producing and/or metallotolerant halophilic bacteria.
- Assess the effect of inoculating pure cultures of pigmented halophilic bacterial isolates in eMalahleni brine on evaporation rate, i.e. the “biological approach”.
- Determine the feasibility of using pigmented halophilic bacteria for improving brine evaporation rates in a pilot plant.
- Select and genetically modify a halophilic bacterium to over-express pigment production.

- Screen developed host strain for biotechnologically or industrially applicable enzymes and biomolecules.

1.3. SCOPE AND LIMITATIONS

The study focused on improving evaporation rates of a reverse osmosis brine by-product disposed of in the eMalahleni water reclamation plant's evaporation pond. Use of evaporation ponds for brine disposal is an effective and economical method for reject brine disposal due to ease of construction, low maintenance and minor operator attention. However, securing effective evaporation is a challenge and requires awareness on how evaporation can be enhanced. Three approaches namely; the addition of different concentrations of methylene blue, inoculating the brine with pure cultures of pigmented halophilic bacteria and engineering a biocatalyst to produce a dark pigment for brine evaporation rate enhancement were proposed and investigated. Outcomes from these approaches will give insights into which of the three options is a more economical and feasible method for enhancing not only eMalahleni wastewater brine but other brine effluents produced by desalination plants in South Africa.

The major limitation to the study is the unpredictability of a biological system, with respect to the performance (growth and pigment production) of the bacteria in the brines. This makes it difficult to apply the findings of this study to other industrial brines. Furthermore, the scarcity of genetic tools and protocols for the genetic manipulation of halophilic bacteria, in addition to the many difficulties associated with heterologous expression, have made it impossible to complete the third component of the proposed project.

CHAPTER 2: LITERATURE REVIEW

2.1. REJECT BRINE

Brine is any water stream in a desalination process that has a higher salinity than the feed water while reject brine is the highly concentrated saline water in the last stage of the desalination process that is usually discharged as wastewater (El-naas, 2011).

Not only is reject brine highly saline, it also contains residual chemicals used during pre- and post-treatment cleaning processes of the desalination operations. As a result, the brine discharge 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. Acids such as sulfuric acid or hydrochloric acid to adjust the pH of the brine.

Not only are the reject brine characteristics dependent on the chemical additives used during the pre- and post-treatment processes but also on the type of feed water, the desalination technology used, as well as on the percentage recovery after desalination (Ahmed *et al.*, 2000; El-naas, 2011; Petrik *et al.*, 2015). Typical analyses of reject brines produced by different desalination plants in South Africa using reverse osmosis membrane technology are presented in Table 1.

Table 1. Characteristics of reject brine samples from South African desalination plants.

Parameter	Tutuka Brine[1]	Lethabo Brine[2]	Secunda Brine[3]	EWRP Brine[4]
Al, ppm	0.045 ± 0.1	2.8	0.519	62.5
Ca, ppm	106.99 ± 12	112	616.56	1340
K, ppm	106.2 ± 1	114.7	189.65	1810
Mg, ppm	163.36 ±1	198.4	22.31	75.0
Na, ppm	4804.88 ± 3	4719.1	1720.29	6720
Si, ppm	13.11 ± 0.1	42.6	-	-
Cl, ppm	2424 ± 17	-	1110.87	955.0
SO_4, ppm	8858 ± 86	-	3339.65	16000
pH	7.75 ± 0.0	7.8	8.48	7.67
EC (ms/cm)	16.69 ± 0.5	15.83	-	6.68
TDS	5400 ± 283	-	-	-

[1] Fatoba, 2011; [2] Sonqishe, 2009; [3] Muriithi, 2009; [4] Randall *et al.*, 2011

2.2. ENVIRONMENTAL IMPACTS OF REJECT BRINE

Numerous studies have evaluated the environmental impact of reject brine disposal on the soil, groundwater and marine environments. These studies showed that the surface discharge of reject brine from inland desalination plants could have negative impacts on soil and groundwater (Rao *et al.*, 1990; Areiqat and Mohamed, 2005; Al-Faifi *et al.*, 2010).

The composition and condition of reject brines also pose potentially negative impacts on marine life. The presence of the various concentrations of the chemicals used in pre- and post-treatment operations in reject brine discharged into the sea has the ability to change the salinity, alkalinity and the temperature averages of the sea water and cause changes in the marine environment (El-naas, 2011).

When reject brine is discharged into evaporation ponds, soil deterioration and/or ground water contamination become issues of concern. Evaporation ponds are a commonly used method for brine disposal by inland desalination plants such as the EWRP. Disposal of reject brine into unlined ponds has the potential to pollute ground water resources and the percolation of this brine into the subsurface may have profound effects on the properties of the soil (Areiqat and Mohamed, 2005). Reject brine disposal into unlined ponds increases the risk of saline brackish water intrusion into freshwater sources (El-naas, 2011).

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^{2+} ions in soil results in the reduction of the infiltration rate of water and soil aeration (El-naas, 2011). 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 reject brine can reduce plant productivity and increase the risk of soil salinization (El-naas, 2011). Proactive approaches such as the use of evaporation ponds, long term monitoring programs and field research to protect ground water from deterioration do help. However, 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 reject brine into the groundwater systems.

2.3. REJECT BRINE MANAGEMENT

Due to the large amounts of reject brine produced worldwide and the potentially harmful environmental impacts posed by uncontrolled brine disposal, several methods of disposal have been developed (El-naas, 2011). Adoption of a particular reject brine 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 disposal method for a particular reject brine stream (Masnoon and Glucina, 2011; El-naas, 2011).

Operating costs are an integral consideration when choosing a brine disposal option as these costs can contribute up to 33% of the total cost of the desalination operation (El-naas, 2011). The cost of brine disposal and minimization is dependent on the brine characteristics, the level of treatment of the reject brine before disposal, the volume of brine to be disposed of and the nature of the environment where disposal is intended (Ahmed *et al.*, 2002).

2.4. REJECT BRINE DISPOSAL OPTIONS

Although discharge into an open water body such as the ocean is a common way to dispose of reject brine from a reverse osmosis process, it is typically not cost-effective to transport the concentrate from inland regions to an ocean for disposal.

Because of the requirement that pipelines and tanker trucks be fitted with special liners for protection against corrosion by reject brine (El-naas, 2011), the cost of transportation for ocean disposal becomes more expensive the further the distance between the source and the final destination. In addition to its corrosive properties, reject brine contains significant amounts of environmentally harmful substances that cannot be released into inland surface waters or onto soil surfaces except under special circumstances. Many small desalination plants around the world add the reject brine effluent to their local municipality sewerage system. This practice lowers the biological oxygen demand (BOD) of the treated wastewater, which may be beneficial under some circumstances.

However, adding brine to the wastewater also increases the total dissolved solids (TDS) of the treated water. High TDS makes this water unsuitable for use in irrigation. 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).

Alternative options for inland brine disposal exist and include deep well injection, land application, zero-liquid discharge and the use of evaporation ponds.

2.4.1 Deep well injection

Deep well injection is an option for brine disposal from inland desalination plants where surface water discharge is not viable or is very costly. In this method reject 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 reject brine 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).

2.4.2 Land application

Land application is a reject brine reuse method in which reject brine is used for landscape and irrigation purposes. The reject brine is used in spray irrigation, rapid infiltration, percolation ponds and overland flow applications. Amongst the land applications of reject brine, spray irrigation is the most common (Masnoon and Glucina, 2011). However, spray irrigation is only be applicable when the resulting irrigation operation does not harm crops or groundwater (El-naas, 2011; 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 Zero liquid discharge

Zero liquid discharge involves the use of high recovery processes to dry out the reject brine to the extent that no liquid discharge leaves the desalination plant (Masnoon and Glucina, 2011). Thermal or mechanical evaporation is used to produce a solid end product from the liquid reject brine. 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 zero liquid

discharge system is the evaporation pond, which is a favoured brine disposal option for inland desalination plants.

2.4.4 Evaporation ponds

Evaporation ponds use solar energy to evaporate the water fraction of reject brine effluent. After a large portion of the water evaporates, the remaining concentrated sludge is either landfilled *in situ* or hauled offsite for disposal. Depending on the brine composition and other regulatory criteria, evaporation ponds maybe 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).

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 reject 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).

Ecologically, evaporation ponds are classified as extreme environments. According to Thomas D. Brock, extreme environments are those with low species diversity in which whole taxonomic groups are missing (Brock, 1979). In the case of reject brine evaporation ponds a combination of the high salinity, the low oxygen concentration, the temperature and the pH influence the biodiversity of individual ponds by promoting the growth of the limited number of species adapted to survive under the set of conditions (Ventosa, 2006). Other factors that may

influence the biodiversity of reject brine evaporation ponds include the nutrient availability, solar radiation levels and the presence of heavy metals and other toxic compounds (Rodriguez-Valera, 1988). With a few exceptions, most inhabitants of brine evaporation ponds are halophilic microorganisms (Ventosa, 2006).

2.5. METHYLENE BLUE

As part of the measures aimed at addressing the disadvantages associated with the disposal of brine using evaporation ponds, a number of studies have looked at the possibility of using dyes to maximise the absorption of radiant energy (Keyes and Gunaji, 1966; Blochi *et al*., 1966; Rajvanshi, 1981). The premise behind the use of dyes in this context is that a coloured solution absorbs more radiant energy compared to the clear brine solution. This increases the temperature of the solution, lowers surface tension, increases vapour pressure and consequently enhances the evaporation rate of brine (Ahmed *et al*., 2000; Kalidasa Murugavel *et al*., 2008; Hoque *et al*., 2010). Patel *et al*. (2010) evaluated the effect of different coloured dyes (black, blue and red) on distillation using a single slope active solar still coupled with an evacuated glass tube solar collector. The results showed that the addition of black dye increases the distillate output by 30.4% with the conclusion that black and blue dyes are the appropriate dyes to enhance the efficiency of evaporation. Similarly, Rajvanshi (1981) evaluated the effect of black napthylamine, red carmoisine and dark green dyes at various concentrations. Among the tested dyes, black napthylamine was found to increase the distillate output by 29% at the concentration of 172.5 ppm with no photochemical degradation. Other researchers observed an increased evaporation with the addition of absorbing material (ink and dye) when compared to the evaporation of water without any absorbing material (Priya and Mahadi, 2013). Considering the success and growing interest in the use of dyes for concentrating brine, this study seeks to evaluate the effect of various methylene blue (MB) concentrations on the evaporation rate of brine. Methylene blue was selected as per request by a commercial partner, specifically for the EWRP process.

2.6. HALOPHILIC MICROORGANISMS

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 [equivalent to 0.85 M sodium chloride (NaCl)] or higher, and tolerate at least 100 g/l salt (equivalent to 1.7 M NaCl) (Oren, 2008). These extremophilic

microorganisms have the capacity to balance the osmotic pressure of the environment in which they reside, thereby resisting the denaturing effects of the salts (Ventosa *et al.*, 1998; 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).

2.6.1. 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 *et al.,* 1998). The most widely accepted and used of these schemes is that of Kushner (1978), depicted in a slightly modified form in Table 2.

Table 2. Classification of microorganisms according to response to salt

Category	Properties	Examples
Extreme halophiles	Grow best at salt concentrations of 2.5-5.2M	*Halobacterium salinarium, Hortaea werneckii*
Moderate halophiles	Grow best at salt concentrations of 0.5-2.5M	*Salinivibrio costicola, Flavobacterium gondwanense, Marinococcus halophilus* (formerly *Planococcus halophilus*)
Slight halophiles	Grow best at salt concentrations of 0.2-0.5M	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*

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 sulphate based brine.

2.6.2. Osmoadaptation of halophiles to saline environments

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 *et al.,* 1998; Grant, 2004).

a. Salt-in-cytoplasm strategy

A minority of halophiles, in particular members of the extremely halophilic Halobacteriaceae use this strategy to counterbalance levels of inorganic ions [usually potassium (K^+)] with the external environment to achieve osmotic stability (Grant, 2004). This adaptive approach requires a specific set of proteins that include:

i. Influx systems, such as the ATP-driven K+ pump Ktr, the multi-subunit ATP-driven K^+pump KdpFABCDE, the Na^+-dependent K^+ transporter Trk and the low affinity potassium uptake protein Kup systems, and
ii. Passive transport via K^+ channels and porins (OmpR and EnvZ) (Kempf and Bremer, 1998; Yaakop *et al.*, 2016).

Thus, halophiles using this strategy accumulate K^+ while exporting Na^+ via passive transport or via a Na^+/K^+ ATPase pump. In addition to acting as an osmotic solute, K^+ functions as an activator of intracellular enzymes, a regulator of cytoplasmic pH, and promotes the accumulation of other compatible solutes (Yaakop *et al.*, 2016). It is worth noting that almost all other halophiles accumulate some K^+ and Na^+ to varying amounts, K^+ usually being the dominant cation and the only one at levels above that in the medium. However, the intracellular concentrations that have been measured are insufficient to compensate for the osmotic potential of the growth medium (Grant, 2004).

b. Compatible solute accumulation strategy

Since the intracellular ion concentrations measured in the moderate halophiles are generally insufficient to provide osmotic balance with the external medium (Ventosa *et al*., 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 (trimethylammonium compounds) and thetines (dimethyl sulphonium 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 (Figure 1) (Welsh, 2000). 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).

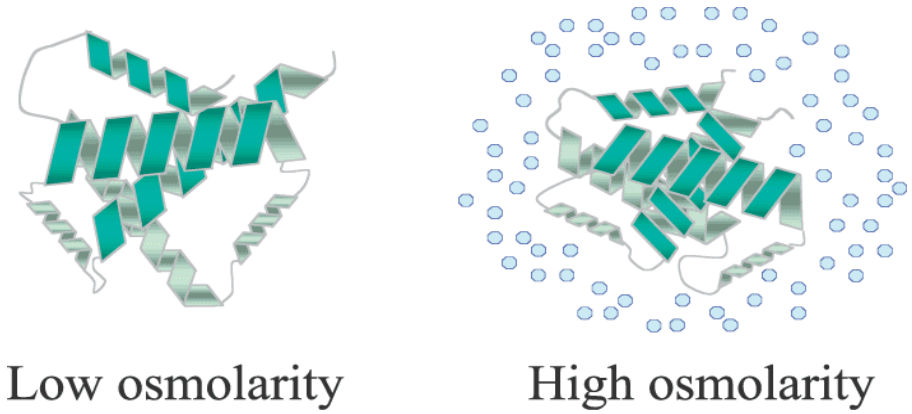

Figure 1. Structural stabilization of halotolerant enzymes at high osmolarity.

2.6.3. Adaptation to high levels of ultraviolet 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). These pigments are responsible for the wide variety of orange to red colours seen in solar saltern ponds and absorb light in the wavelength range of 300-600 nm (Vachali, Bhosale and Bernstein, 2012).

2.6.4. Pigments of halophilic microorganisms and their biosynthesis

Halophilic microorganisms mainly produce carotenoid pigments, through the expression of 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 such as β-carotene and α-carotene, 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). Figure 2 shows the structure of some carotenoids produced by halophiles.

The biosynthesis of carotenoids by halophilic microorganisms, especially bacteria, is well regulated and dependent on the biochemical make-up of the organism's environment and stress incurred during growth. Generally, carotenoid biosynthesis starts with the precursor isopentenyl pyrophosphate (IPP) produced in the mevalonate (MVA) or 1-deoxy-D-xylulose 5-phosphate / 2-C-methyl-D-erythritol 4-phosphate (DOXP/MEP) pathway (Figure 3). The distribution of the MEP and mevalonate pathways of isoprenoid biosynthesis is highly complex, with a clear bias towards the MEP pathway in bacteria and the classical mevalonate pathway in halophilic archaea (Lombard and Moreira, 2011).

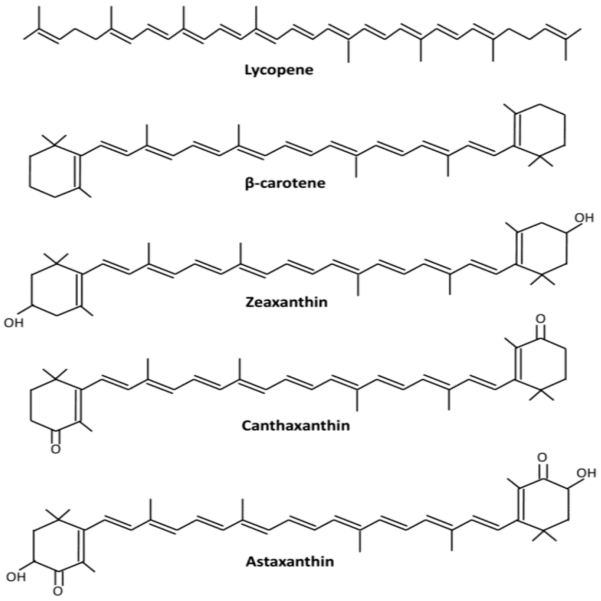

Figure 2. Structures of some carotenoid pigments produced by halophilic bacteria.

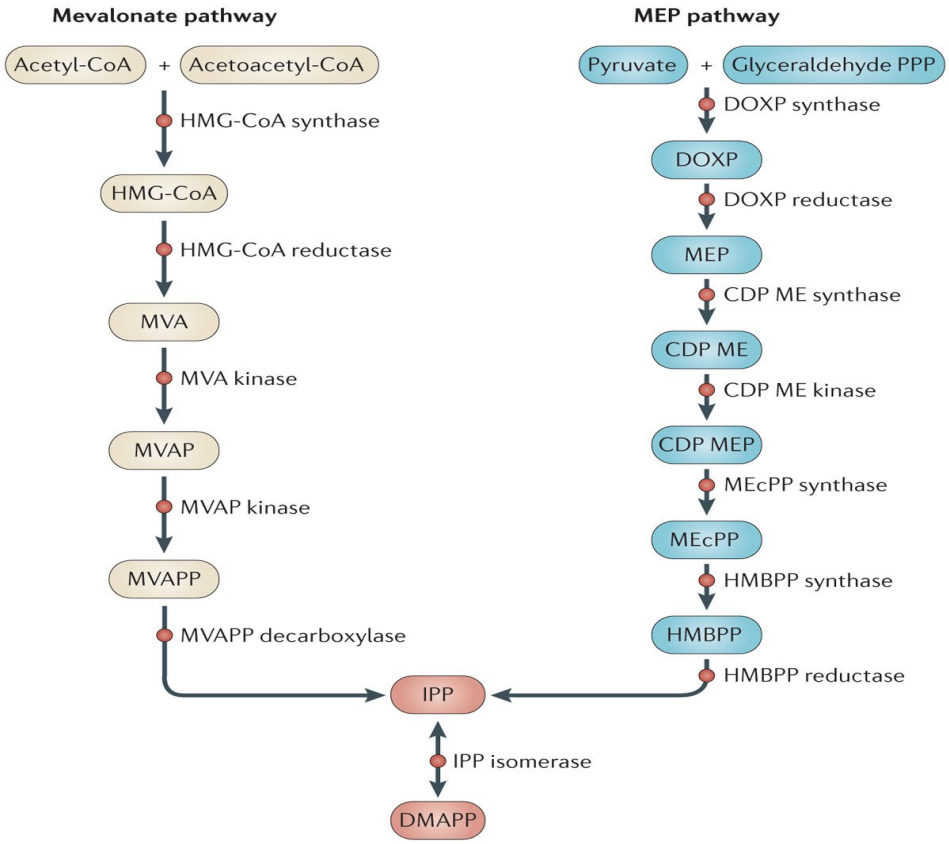

Figure 3. The mevalonate and 2-C-methyl-D-erythritol 4-phosphate (MEP) pathways for isopentenyl pyrophosphate (IPP) biosynthesis**.**

Three molecules of IPP are subsequently condensed with one molecule of dimethylallyl diphosphate (DMAP) by prenyltransferase to give a C20 diterpene geranylgeranyl diphosphate (GGDP) (Vachali *et al*., 2012). Two molecules of GGDP condense head to head to form the first carotenoid phytoene (Tian and Hua, 2010). Phytoene is then converted to lycopene a C40 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 catalysed by the bacterial type phytoene desaturase (CrtI) (Tian and Hua, 2010; Vachali, Bhosale and Bernstein, 2012). Lycopene is the precursor for all acyclic and cyclic carotenoids including the xanthophylls produced by microorganisms (Armstrong, 1997; Tian and Hua, 2010; Vachali, Bhosale and Bernstein, 2012).

The formation of carotene(s) by halophilic microorganisms commences by cyclization of desaturated lycopene at both ends to form either a β- or α-carotene molecule (Maresca *et al.*, 2007). These reactions are mediated by a lycopene β- or α-cyclase in bacteria. In the carotenoid biosynthetic pathway, phytoene synthase (CrtB), phytoene desaturase (CrtI), and lycopene cyclase (CrtY) are the three essential enzymes responsible for the biosynthesis of both acyclic and cyclic carotenoids (Mongodin *et al.*, 2005; Vachali, Bhosale and Bernstein, 2012). The cyclization of the linear compound lycopene to produce α- or β-carotene is a branch point in carotenoid biosynthetic pathways in many species of bacteria. Xanthophylls (oxygenated derivatives of carotenes) are formed by sequential oxidations of the post – carotene molecules yielding hydroxyl, epoxy, and oxo groups (Vachali, Bhosale and Bernstein, 2012). Introduction of a hydroxyl (-OH) group at the C3 position of the ionone ring leads to the formation of zeaxanthin, a C3-dihydroxy derivative of β-carotene (Vachali, Bhosale and Bernstein, 2012). Carotenoids with keto (-C=O) functional groups such as canthaxanthin and astaxanthin are formed by the introduction of -C=O groups at C4 and C4' with or without hydroxylation at C3 and C3' of the carotene backbone (Armstrong, 1997; Vachali, Bhosale and Bernstein, 2012).

2.6.5. Carotenoid biosynthesis in evaporation ponds and its effect on evaporation

Evaporation ponds present an environment with increasing salinity in which different halophilic organisms are found within the salinity gradient created as evaporation progresses. It has been long known that solar evaporation ponds and other hypersaline brines approaching halite saturation turn red as a result of carotenogenesis by dense communities of heterotrophic

archaea and bacteria (Baas-Becking, 1931; Oren, Stambler and Dubinsky, 1992). Without this natural colouration, evaporation proceeds only with great difficulty (Davis, 1974).

A field study by Saju (2011) to assess the impact of halophilic bacteria on the overall evaporation rate in a salt works primary concentrator pond in India revealed that the average evaporation percentage was higher than that for a control pond devoid of halophilic bacteria (Saju *et al.*, 2011). In fact, Saju (2011) found the average evaporation percent in the pond inoculated with halophilic bacteria to be 16.8 ± 1.33% during March 2004 whereas that of the control pond was 13.68% (Saju *et al.*, 2011).

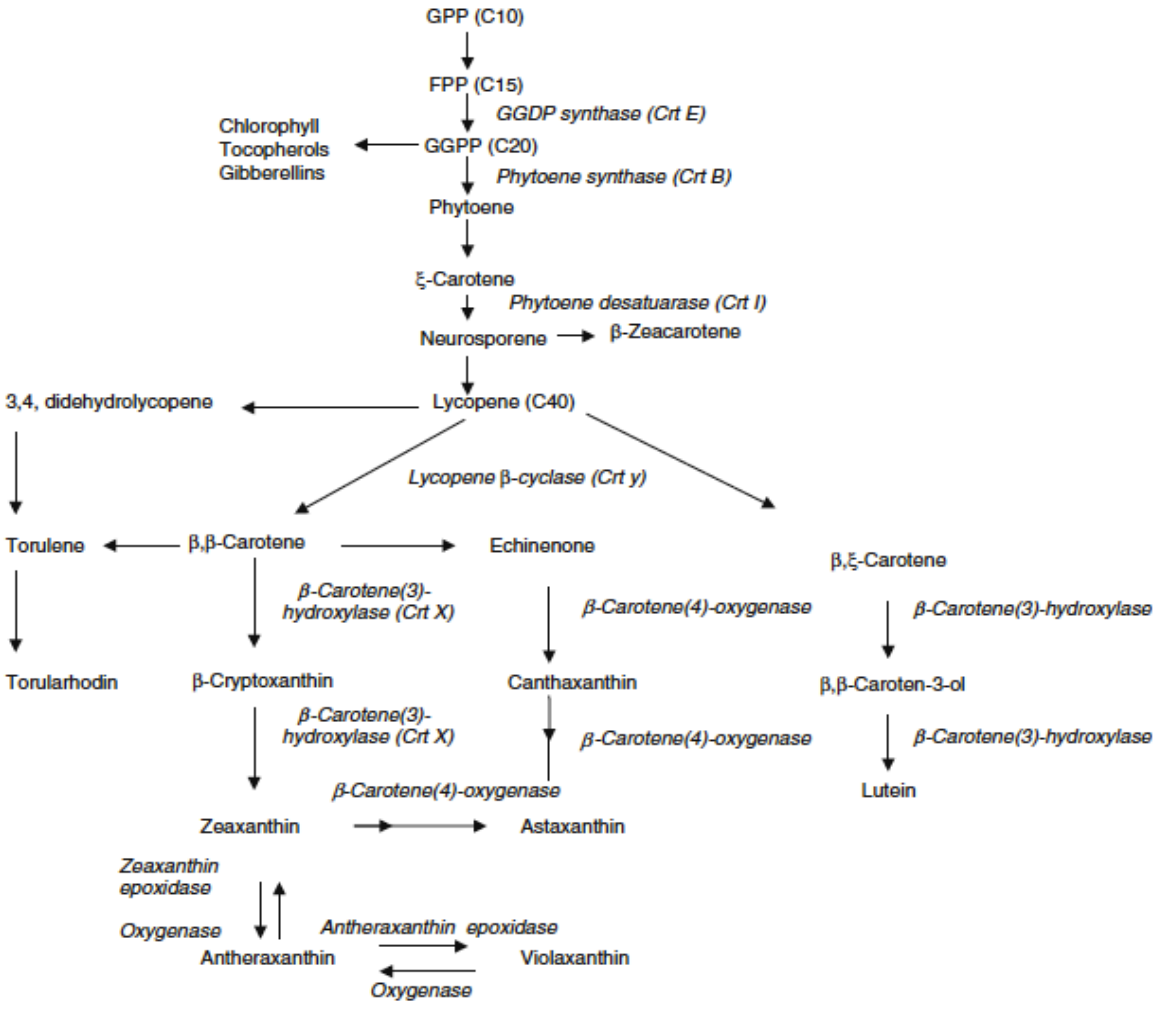

Figure 4. Carotenogenesis pathway compiled from several reports on microbial production.

CHAPTER 3: EXPERIMENTAL PROCEDURES

3.1. ASSESSMENT OF METHYLENE BLUE ON REJECT BRINE EVAPORATION RATES

3.1.1. Brine Samples

Brine samples used in this study were collected from three different evaporation ponds; the eMalahleni Water Reclamation pond (EWRP) situated in the Mpumalanga province, South Africa (S 25°56'41.4, E 29°11'67.0); the Cerebos crystallizer salt ponds in Velddrift, Western Cape, South Africa (S 32°47'10,632, E 18°10'9,499); and NuWater Global (courtesy of Dr Jeeten Nathoo), Epping, South Africa, and stored at 4°C. For analysis of the eMalahleni and NuWater brines, debris was removed from a 2 L brine sample by filtration through 0.45 µm nitrocellulose Millipore™ membranes. The filtered brine sample was divided into two portions, one for the detection of anions and the other for cation detection. The brine portion for cation determination was first acidified with concentrated nitric acid prior to chemical analyses. The partitioned brine was analysed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) to determine its chemical composition and heavy metals, at the University of Stellenbosch analytical unit. Physical qualities such as pH, electrical conductivity and organic compounds (nitrate, nitrite, ammonia and total carbon) were analysed by Bemlab (Pty) Ltd water analytical services. Total carbon was analysed using the LECO CR-421 carbon analyser while nitrate, nitrite and ammonia were analysed using flow analyser SANS 13395 and SANS 5217 (model AA3). The results from the eMalahleni analyses (Table 3) were used to formulate a synthetic version of the brine for use in successive experiments. Synthetic brine was used to mitigate the difficulty faced in obtaining and transporting new brine from the EWRP, and to ensure consistency in the various assessments. The synthetic brine composition was as follows (w/v): 3497 mg/L Na^{+}; 758 mg/L K^{+}; 223 mg/L Mg^{2+}; 964 mg/L Ca^{2+}; 762 mg/L Cl^{-} and 10255 mg/L SO_4^{2-}.

3.1.2. Preparation of Methylene Blue solutions

Methylene blue (MB) (Figure 5) was purchased from Meric Millipore™, Cape Town. Stock solutions were prepared by dissolving 2 g of MB in 2000 mL of synthetic brine. The desired concentrations for the experiments were achieved by successive dilutions. Methylene blue solutions were prepared using synthetic brine to ensure that the concentration and chemical composition of synthetic brine was not altered.

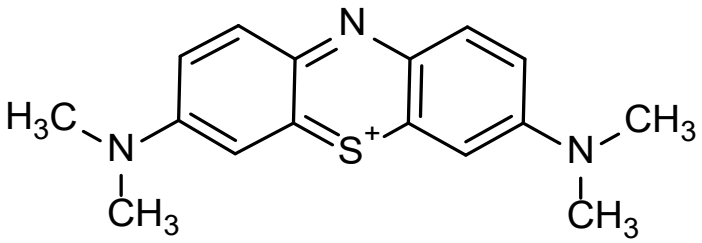

Figure 5. Chemical structure of methylene blue dye.

3.1.3. Evaluation of the effect of different concentrations of methylene blue on brine evaporation rate

The evaluation of the effect of different concentrations of methylene blue on the brine evaporation rate was done on 1 L volumes of synthetic brine in laboratory scale pans, which measured 17.8*27.9*2.4 cm^3. Five MB concentrations in the brine were tested. The final concentrations of MB in 1 L of the brine ranged from 100 to 300 ppm, with 50 ppm increments. The evaporation studies were conducted in a closed laboratory with controlled access to avoid constant temperature fluctuations due to free access. Access to the laboratory was only during the times when measurements of evaporation rate were conducted. The experiment was set up as shown in Figure 6.

Figure 6. Experimental set up for methylene blue evaporation studies.

Infrared lamps generating 240 watts of power were placed 36 cm above the pans to supply energy to facilitate brine evaporation. The lamps were distributed equidistant from each pan to ensure even heat distribution to each. Each experiment, together with its control were conducted simultaneously, therefore the comparative evaporation rates are relative. The control was 1 L synthetic brine without MB. All experiments were conducted in triplicate and measurements for brine loss taken at 6 h intervals for a period of 54 hours. The amount of brine loss was determined by weighing the saltpans on a Bright Led Display Economy Weight Measurement Scale (KETE 10), until the pans were completely dry. The rate of evaporation of the brine was calculated as in section according to the following equation (Ladewing and Asquilth, 2012):

$$Evaporation\ rate\left(\frac{cm}{h}\right)=\frac{volume\ of\ brine\ lost\ over\ time\left(\frac{cm^3}{h}\right)}{surface\ area\left(cm^2\right)}$$

Various equations are available for the calculation of evaporation rate. However, each equation is valid for a specific system. In this study laboratory scale pans were used and thus the above equation was chosen because it took into account the surface area of the pan as well as the volume of brine lost over time.

3.1.4. Reconstitution of Methylene blue

Reconstitution of MB was investigated following suggestions made during the WRC committee meeting held on the 22nd of October 2015 at the University of the Western Cape. The consensus was, if this approach worked it would drastically cut costs that would be involved with adding new batches of the dye to the pond as opposed to filling up the pond with fresh brine and having the rate of evaporation increased by the reconstituted dye. It was further agreed that this approach would keep the amount of the dye used at manageable levels as well, thus curbing detrimental environmental effects of the continuous incorporation of methylene blue with in the pond.

Methylene blue dye crystals trapped within the synthetic brine matrix at the end of the first evaporation rate experimental runs were reconstituted by adding 800 mL of freshly prepared synthetic brine in each pan. Methylene blue was reconstituted twice in this manner and all the experiments were done in triplicate. The reconstitution process was done in order to assess whether the remaining methylene blue would still be effective in increasing the rate of evaporation of the brine if the fresh brine is added.

3.2. ISOLATION OF HALOPHILIC BACTERIA

Halophilic bacteria were isolated from reject brine samples obtained from the eMalahleni water reclamation plant evaporation pond as well as from brine slurry from 5 salt concentrator ponds at Cerebos salt works in Velddrift along the West Coast, South Africa as follows.

One litre of eMalahleni reject brine was vacuum filtered through a 0.22 µm nitrocellulose Millipore™ membrane, which was crushed and suspended in 10 ml of sterile distilled water (sdH_2O). The suspension was vortexed for 2 minutes using a vortex 2 genie bench top vortexer from Scientific Industries™ USA, to dislodge bacterial cells bound to the membrane. The bacterial suspension was serially diluted using sdH_2O up to a factor of 10^{-6}. One hundred micro-litres of each dilution factor from 10^{0}-10^{-6} were spread plated onto R2A agar (Sigma Aldrich™, USA) made using sterile eMalahleni reject brine and incubated for 5 days at room temperature.

Isolation from samples obtained from the Cerebos salt works concentrator ponds was done by resuspending 1 g of the slurry in 10 ml of sterile seawater and then serially diluting the suspensions to a dilution factor of 10^{-6}. Each of the dilutions were inoculated onto agar plates as described above, the only difference being that 3 types of agar media were used namely, R2A, Zobel (ZBA) and Trypic soy agar (TSA). The plates were incubated at room temperature for a period of 5 days. Individual colonies were picked from plates that had growth based on pigmentation and subcultured on fresh R2A agar plates. Subculturing was repeated until pure cultures of differently pigmented isolates were obtained.

3.2.1. NaCl and $NaSO_4$ tolerance

Tolerance to salts was assessed by growing the isolates in R2A broth supplemented with a range (0-30%) of NaCl or $NaSO_4$ concentrations. The cultures were incubated at 30°C, 120 rpm for 48 hours and growth was noted by measuring the turbidity at 660 nm.

3.2.2. Molecular identification and phylogenetic analysis

3.2.2.1. DNA extraction

One loop full of actively growing culture was used to inoculate 50 ml McCartney bottles containing 10 ml of tryptic soy broth (TSB) supplemented with 5% (w/v) NaCl. Cultures were grown at 30°C for 18-24 hrs with constant shaking. Up to 5 ml of culture was harvested by centrifugation at 10 000 rpm for 5 minutes in a Labnet prismR™ (Woodbridge, New Jersey,

USA) refrigerated bench top centrifuge to obtain a pellet of approximately 500 µl. Genomic DNA for 16S rRNA gene amplification was extracted using a modified version of the Wang et al. (1996) DNA extraction protocol. The following modifications were made: the concentration of lysozyme was increased to 25 mg/ml, 0.2 mg/ml proteinase K was added to the lysozyme buffer and the harvested cells were incubated overnight in the lysozyme buffer. Extracted DNA was dissolved in 50 µl Tris EDTA (TE) buffer, quantified using the ND-1000 uv/vis Nanodrop™ (USA) spectrophotometer and stored at 4°C.

3.2.2.2. 16S rRNA gene amplification and sequence BLASTn analysis

The polymerase chain reaction (PCR) was used for amplifying the 16S rRNA gene from the extracted DNA. The universal primers E9F (5'-GAGTTTGATCCTGGCTCAG-3') and UR1510R (5'-GGTTACCTTGTTACGACTT-3') were used for amplification of the 16S rRNA gene. A Bio Rad T100™ thermocycler was used to conduct the amplification reaction. All PCR reactions were carried out in 25 µL volumes. Each reaction contained: 2.5 µL of 10X DreamTaq Buffer (Thermo Fisher Scientific, South Africa), 2.5 µL of 2 mM dNTPs, 1.2 µL of a 10 µM stock solution of each primer, 50 ng of template DNA, 0.125 µL of 5U/µL DreamTaq polymerase (Thermo Fisher Scientific, South Africa) and nuclease free water to make up to 25 µL.

The following cycling conditions were used for the amplification: 95°C for 5 mins, then 35 cycles of 95°C for 4 s, 55°C for 30 s and 72°C for 1 min 30 s, and a final extension at 72°C for 10 mins before stopping the reaction by cooling to 4°C. The PCR products were loaded onto a 1.2% (w/v) agarose gel, which was run at 100 volts for 1 hour in Tris-acetic acid-EDTA (TAE) buffer, and the PCR products excised from the gel. The excised products were purified using the Nucleospin™ gel purification kit as per the manufacturer's instructions, and sequenced by the central DNA sequencing facility (CAF) at Stellenbosch University.

Sequence chromatograph files in .ABI format from CAF were opened and viewed using SnapGene™ (version 4.0.2). The forward and reverse sequences of all the isolates were checked for quality before reverse complimenting the reverse sequences and merging them to their respective forward sequences. The merged sequences for each isolate were queried against the curated 16S rRNA bacterial gene database on the NCBI server for matches.

3.2.3. Growth and pigment production kinetics of EP1, EP3 and CP5-4 isolates in NuWater brine

Growth of and pigment production by EP1, EP3 and CP5-3 isolates in the NuWater brine (see section 3.3) was evaluated in 100 ml Erlenmeyer flasks. Tryptic soy broth-brine medium was prepared with variations in the amount of TBS added, pH, KCl concentration and iron source. These variables were chosen to assess their influence on the growth and pigment production capacity of the isolates in the NuWater brine as well as to determine the optimum values and/or concentrations that result in good growth and pigment production. The weight per volume percentages of TBS added in preparing the TSB-brine medium varied from 25 to 100%, while the pH of the medium was varied from 6 to 8. Potassium chloride concentration was varied by adding 1.2 and 1.8 g/L of the salt to the medium. Iron chloride salts were added in different experiments and their concentrations varied from 20 to 60 mg/L with 20 mg increments. Overnight cultures of the isolates were inoculated into the different media and incubated at room temperature with shaking at 150 rpm for 4 days.

Aliquots of the cultures were used to measure growth in terms of the change in turbidity at 660 nm using a spectrophotometer. Increase in cell biomass was measured by obtaining 1 ml samples of the cultures, placing them in pre-weighed eppendorff tubes which were then centrifuged to pellet the cells. Centrifugation was done for 10 minutes at 10 000 rpm. The supernatant was removed and the pellet dried before determining the cell biomass.

Pigment was extracted by harvesting the cells from culture by centrifugation, the cell pellet washed and resuspended in methanol. The resuspended cell pellet in methanol was incubated for 15 minutes at 60ºC after which the cell suspension was centrifuged and the methanol extract used for spectrophotometric analyses. The absorbance of the methanol extract was recorded at 200-700 nm using a uv/vis spectrophotometer. The yield of pigments was calculated from total absorbance of acetone extract divided by the volume of culture broth and expressed as Absorbance*/mL broth. The efficiency of pigment production was calculated by dividing the yield of pigments by the turbidity (OD_{660nm}) of culture broth at the time of extraction and expressed as A* nm/A660 nm (Fang *et al.*, 2010).* refers to a range of wavelengths that were measured.

3.3. ASSESSMENT OF PIGMENTED ISOLATES FOR IMPROVEMENT OF REJECT BRINE EVAPORATION RATES

The purpose of these experiments was to evaluate the effect of culturing the pigmented isolates in the brine on brine evaporation rate compared to a brine control. Two types of brines were assessed in this study: i) synthetic brine and ii) brine supplied by NuWater (see section 3.1.1). Experiments were conducted in 3 phases namely micro (200 ml), laboratory pan (1 L) and pilot scale (100L). The details of each experimental phase are described below.

3.3.1. Micro-scale experiments

To screen the isolates for their ability to increase evaporation of synthetic brine, twenty millilitres of overnight cultures grown in TSB supplemented with 5% (w/v) NaCl were used to inoculate 180 ml of filter sterilized synthetic brine in 9 cm diameter deep well petri dishes. A control (180 ml synthetic brine + 20 ml TSB/5% NaCl media) was included. The petri dishes were incubated under infrared (IR) lamps generating 240 watts inside a laminar airflow cabinet (Figure 7). Incubation was conducted for 72 hours during which the evaporation rate in each dish was determined by measuring the amount of brine lost over time in cm^3/hr as described in section 3.1.3. Micro-scale experiments conducted with NuWater brine were supplemented with TSB, KCl, and $FeCl_3$ as determined in section 3.2.3.

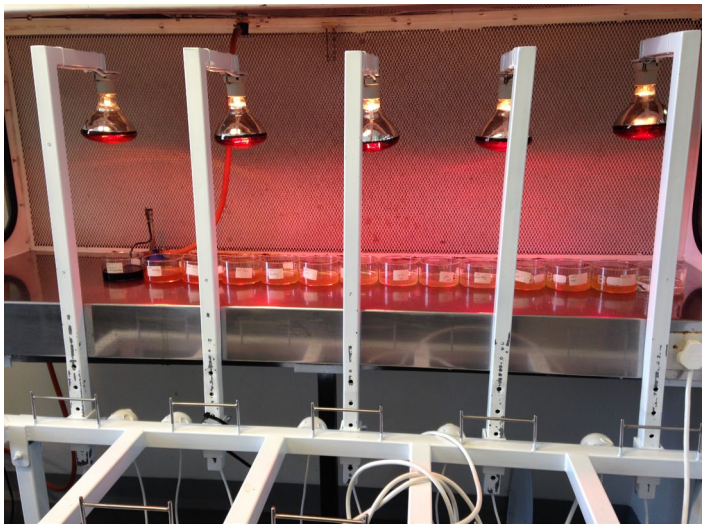

Figure 7. Set up of the micro scale evaporation rate experiment.

3.3.2. Scaled up pan experiments

Isolates that gave a high evaporation rate in the micro-scale experiments were used in the 1 L pan experiments. The purpose of these experiments was to further assess the effect of culturing the pigmented isolates in a larger volume on brine evaporation, and also to assess the extracted pigment on brine evaporation. Synthetic bring and NuWater brine were assessed, and both were sterilised prior to the start of the experiment.

3.3.2.1 The effect of bacterial pigment on the evaporation rate of brine

EP3 was grown in 100 ml TSB in an Erlenmeyer flask and incubated at room temperature with shaking set at 120 rpm for 3 days. At the end of the incubation period pigmented cells were harvested by centrifuging 50 ml of the culture for 10 minutes at 7274 ×*g*. The supernatant was removed and the cell pellet washed twice with sterile distilled water. The pigment was extracted from the pelleted cells using methanol, as follows. The pellet was re-suspended in 2 ml of methanol and the mixture incubated at 65^{o}C for 10 minutes. At the end of the incubation, bleached cells were pelleted by centrifugation at 7274 ×*g* for five minutes and the coloured supernatant filtered through Whatman number 1 filter paper. The filtered supernatant was transferred to a new pre-weighed tube and a scanning spectrophotometer was used to determine the wavelength at which the pigment absorbed maximally. The absorption range scanned was from 300 to 800 nm. After determining the maximum wavelength, the remaining samples were incubated in oven at 60^{o}C until they were completely dry. Total carotenoid content was calculated as detailed in section 3.2.3. One gram of the extracted pigment was added to 1 L of synthetic brine, which was then allowed to evaporate. The experimental set up was as shown in Figure 8, and performed as described in section 3.1.3.

3.3.2.2 The effect of the bacterial culture on the evaporation rate of brine

Two hundred millilitres of overnight cultures of the isolates grown in TSB-synthetic brine broth were used to inoculate 800 ml of filter-sterilized synthetic brine in 1 L pans. For the experiments conducted with NuWater brine, 200 ml of overnight cultures of the isolates grown in TSB-NuWater brine supplemented with 20 mg/ml $FeCl_3$ were used to inoculate 800 ml of NuWater brine+ 20 mg/ml $FeCl_3$ in 1 L pans. Amount of liquid loss, growth rate of, and pigment production by the isolates were measured at 6-hour intervals until the pans were completely dry. Control experiments included: 1 L of un-inoculated i) synthetic brine, ii) synthetic brine+20% TSB or iii) NuWater brine+20% TSB+20 mg/ml $FeCl_3$. All experiments were carried out in triplicate. The experimental set up was as shown in Figure 8, and performed as described in section 3.1.3.

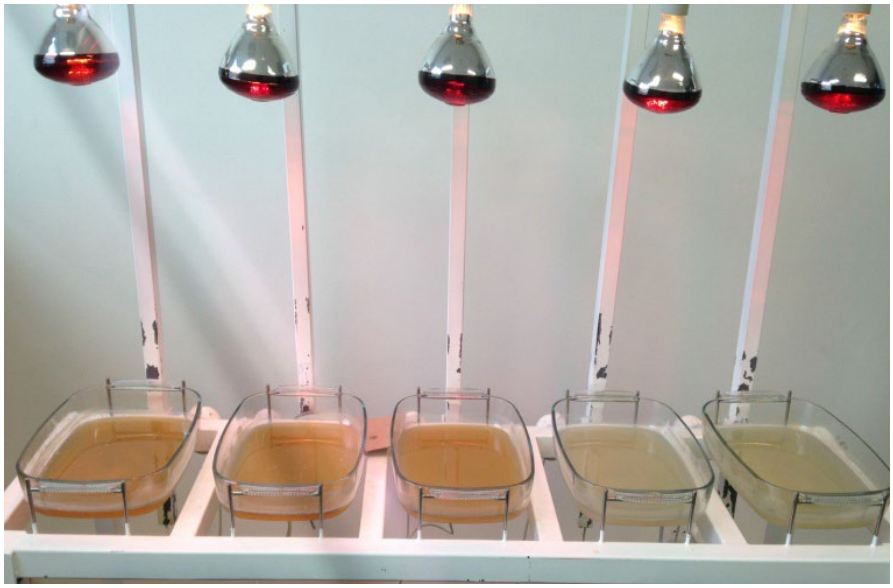

Figure 8. Set up of 1 L pan evaporation rate experiments.

3.3.3. Pilot plant experiments

Due to the large volume of synthetic brine, time spent preparing and the amount of chemical components needed to make 100 L for pilot plant studies as well as logistical difficulties in obtaining brine from eMalahleni, it was suggested by the WRC committee to carry out evaporation studies using brine supplied by NuWater. It was acknowledged that the NuWater brine would have different chemical and physical properties to those of eMalahleni brine (Table 3) and that the isolates might behave differently in the NuWater brine. One thousand liters of brine were received in May 2016 and used in all the experiments described here on.

The ability of the isolates to grow and produce pigment in the NuWater brine was evaluated, followed by an assessment of the effect that these strains had on the evaporation rate of the brine over time as described above. Strains that grew produced pigment and affected high evaporation rates of the NuWater brine in 1 L pans were used in a series of pilot plant experiments to demonstrate the feasibility of using this approach in a non-laboratory setting.

3.3.3.1. Seed culture production for application in pilot plant experiments

CP5-4 was chosen for seed culture production based on its ability to grow and produce its carotenoid pigment in TSB-NuWater brine broth supplemented with 20 mg/L $FeCl_3$. Optimised pigment production was achieved with 50 mg/ml $FeCl_3$. Seed culture production was conducted in a Minifors™ 2 L bioreactor. One hundred and fifty millilitres of a 48-hour-old

culture grown in TSB-NuWater brine broth supplemented with 50 mg/L $FeCl_3$ were inoculated into 1.5 L of the same broth inside the bioreactor. The culture was incubated at room temperature with stirring at 300 rpm and aeration for 72 hours.

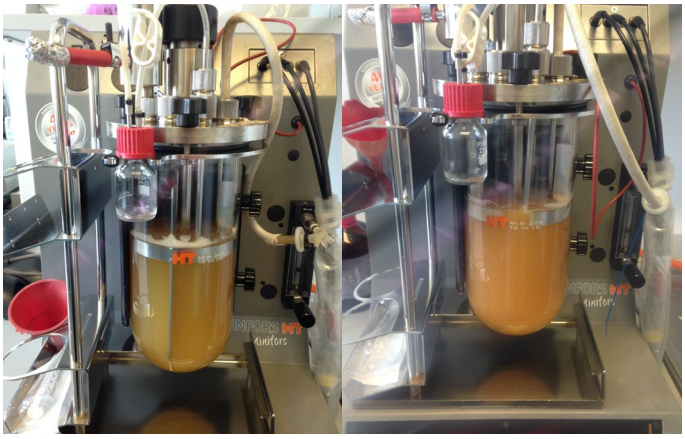

Figure 9. CP5-4 seed culture production in Minifors™ bioreactor.

3.3.3.2. Pilot plant set up and evaporation rate studies

Structurally the pilot plant consisted of a cubic Perspex container measuring 50*50*50 cm^3. The container was well insulated with reflective material to minimize the impact of external conditions on the experiment. Two 250 watt IR-bulbs were suspended above the pilot plant to supply the heat energy required for the experiment. Three temperature sensors were installed 6, 12 and 18 cm below the brine surface to record the heat energy transfer from the IR-bulbs to the brine (Figure 10).

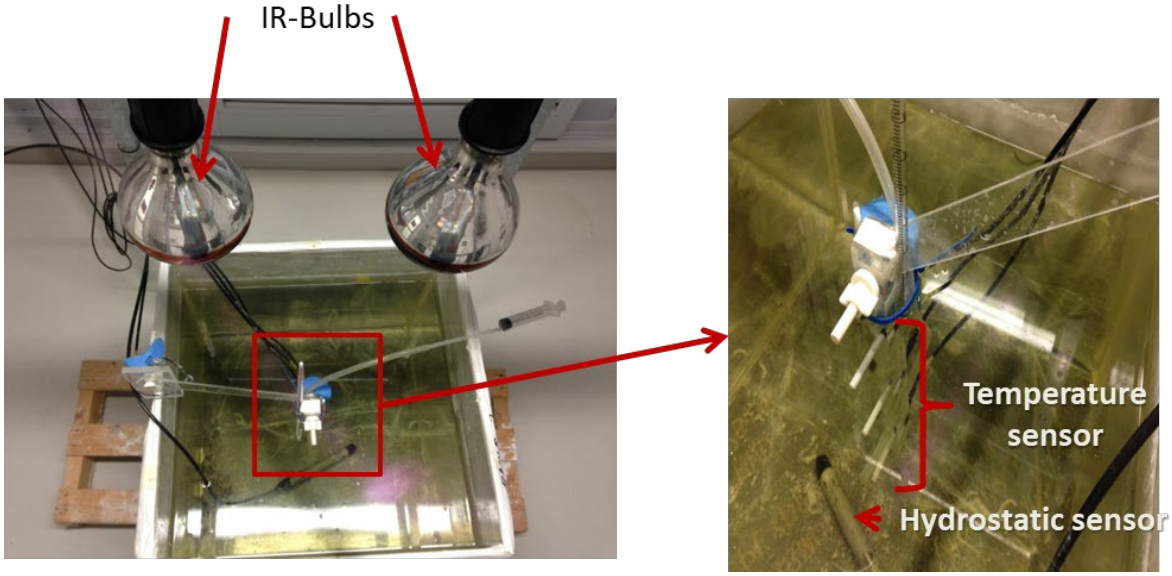

Figure 10. Pilot plant set up.

The inception of the pilot plant experiments involved filling the container with 100 L of non-sterile NuWater brine and exposing it to 12 hours per day of artificial radiation from the IR bulbs. This was done to establish a base line evaporation rate of the brine under the selected conditions. After the base line had been established, the experiment proceeded with assessing the effect of inoculating the CP5-4 culture on the brine evaporation rate.

Twenty litres (20% v/v) of CP5-4 culture were inoculated into 100 L of fresh NuWater brine supplemented with 20% (w/v) TSB medium, in the pilot plant. Two control experiments with neat NuWater brine and NuWater brine supplemented with 20% (w/v) TSB were also set up. Ten millilitres of samples were collected at four different depths; 6, 12, 18 and 24 cm below the brine surface, to measure pigment production by and growth of the CP5-4 culture during the course of the experiment. The rate of evaporation was measured as and recorded as detailed in section 3.1.3. The experiments were run for one month.

CHAPTER 4: RESULTS AND DISCUSSION

4.1. BRINE ANALYSIS

The physicochemical properties of the eMalahleni reject brine from the analyses are shown in Appendix A. The brine predominantly contained Na^+, SO_4^{2-}, K^+, Mg^{2+}, Cl^-, Si, NH_4 and Ca^{2+}, while $Fe^{2+,}$ Mo and Co^{2+}. A high level of Na^+ and SO_4^{2-} (3973 and 14 520 mg/L, respectively) indicates that the EWRP brine is a sodium sulphate type brine. It is well understood that the characteristics of the brine are influenced by the feed water, desalination process, percentage recovery and chemical additives used during the treatment (El-naas, 2015), therefore it is not unexpected for brines to have different characteristics. The high concentration of SO_4 was expected since the brine is a by-product of AMD treatment, which is laden with metals and sulphate. Metals such as Mn, Cu, Cr, Pb, Ba, Ni and Zn were found in minor concentrations, which are attributed to the brine undergoing the pre-treatment process to remove these metals prior to RO treatment. This is done to protect RO components from metals fouling (Hutton *et al*., 2009). The pH was found to be 7.76 while the EC was 21.9 mS/cm, which shows high levels of free ions due to the presence of dissolved species. Nitrite and nitrate were found to be 0.01 and 122.12 mg/L respectively, and a very low total carbon level.

4.2. ASSESSMENT OF METHYLENE BLUE FOR IMPORVEMENT OF EVAPORATION RATES

4.2.1. Evaluation of the effect of different concentrations of methylene blue on brine evaporation rates

The amount of brine lost at 6-hour intervals over the 56-hour experimental period of the evaporation studies is shown in Figure 11. No significant difference in the amount of brine lost from all the treatments was observed after the first 6 hours of the experiment. This may have been as a result of the period of time needed for water molecules to be converted to vapour as a result of exposure to heat generated by the infrared lamps. Increases in the amount of water loss and differences in these amounts started becoming apparent after 12 hours. At this point, the amount of liquid lost from the pans with MB were much higher than that lost from the control experiment, suggesting that the heat absorption by the brine increases after addition of MB under laboratory conditions. Dark coloured solutions absorb more radiant energy compared to clear solutions, which reflect all visible wavelengths and are consequently cooler (Ahmed *et al*., 2000; Rajvanshi, 1981).

Table 3. Physical and chemical properties of brine samples.

Property	eMalahleni	NuWater	Synthetic
pH; 25°C	7.76	8.2	7
Electrical Conductivity (mS/m; 25°C)	2190	1780	ND
Elements in mg/L:			
Cl^-	1072.78	539	762.565
SO_4^{2-}	14520	10871	10255.1
F^-	ND	2.9	ND
NO_3^-	122.12	0.1	ND
NO_2^-	0.01	0.4	ND
Na^+	3973	3859	3497.4
K^+	710	104	758.45
Ca^{2+}	1200	985	964.163
Mg^{2+}	459	837	223.311
Al^{3+}	0.03	<0.10	9.86409
Ba^{2+}	0.21	0.172	11.013
Cu^{2+}	0.004	<0.025	ND
Fe^{2+}	0.07	0.043	7.99594
Mn^{2+}	0.01	0.422	ND
P^+	0.03	1.64	ND
Zn^{2+}	0.008	0.571	ND

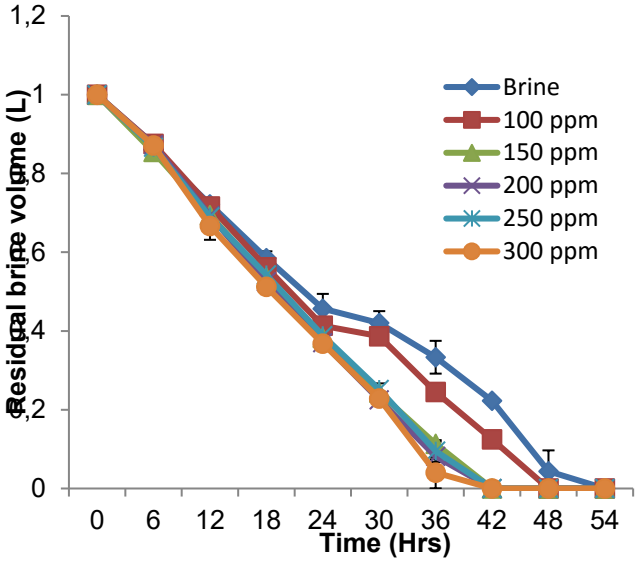

Figure 11. Synthetic brine evaporation rates at six-hour intervals from 1 L pans with different MB concentrations. Standard deviations of the triplicates are indicated on each graph.

The evaporation rate from the pans however, was not linear (especially for the brine control and 100 ppm MB experiment), which may have been as a result of the increasing salinity gradient following liquid loss. High salinity is known to affect physical parameters of brine such as surface temperature, density, resulting in reduced vapour pressure on the surface of water and increases the amount of energy needed to change water molecules from liquid to vapour state (Dama-Fakir and Toerien, 2009). Nonetheless, the addition of MB to the brine did result in an increase of the evaporation rate as compared to the uncoloured brine control (Table 4), where a maximum of 22.5% increase was obtained. Addition of MB to a final concentration of 300 ppm in the synthetic brine resulted in the most amount of liquid loss during the experiment, indicating a high evaporation rate.

Table 4. Average evaporation rates of synthetic brine with various concentrations of methylene blue dye.

Methylene blue concentration	Rate (cm/hour)	% Increase in Evaporation rate*
0 ppm	0.0289±0.053	-
100 ppm	0.0290±0.002	0.35
150 ppm	0.0343±0.060	18.68
200 ppm	0.0351±0.032	21.45
250 ppm	0.0346±0.001	19.72
300 ppm	0.0354±0.172	22.49

*Calculated as a % of the Ev cm /h obtained for the 0 ppm.

4.2.2. Reconstitution of Methylene blue

The ability to reconstitute the methylene blue from precipitated salts in successive evaporation experiments, instead of replenishing the MB, may have important environmental and economic implications. Firstly, it may aid in keeping the MB concentration at manageable levels to prevent negative environmental impacts. Secondly, this could constitute an economic saving. The effect of reconstituting MB from precipitated salt crystals by adding fresh brine, and measuring the amount of liquid lost over time is presented in Figure 12. The synthetic brine evaporation rates at six-hour intervals decreased only marginally for the reconstituted

samples, compared to the initial 200 ppm experiment; however, still yielded higher evaporation rates compared to the brine control (Figure 12). Thus repeated reconstitution of the dye from the precipitated salts resulted in comparable evaporation rates to that of 200 ppm, and should be considered as an advantage for the chemical approach. This is especially advantageous for systems where the salts are allowed to precipitate in the ponds. However, if it is the intention of the salts to be recovered, either for disposal or for downstream applications, the methylene blue represents an additional contaminant that needs to be treated.

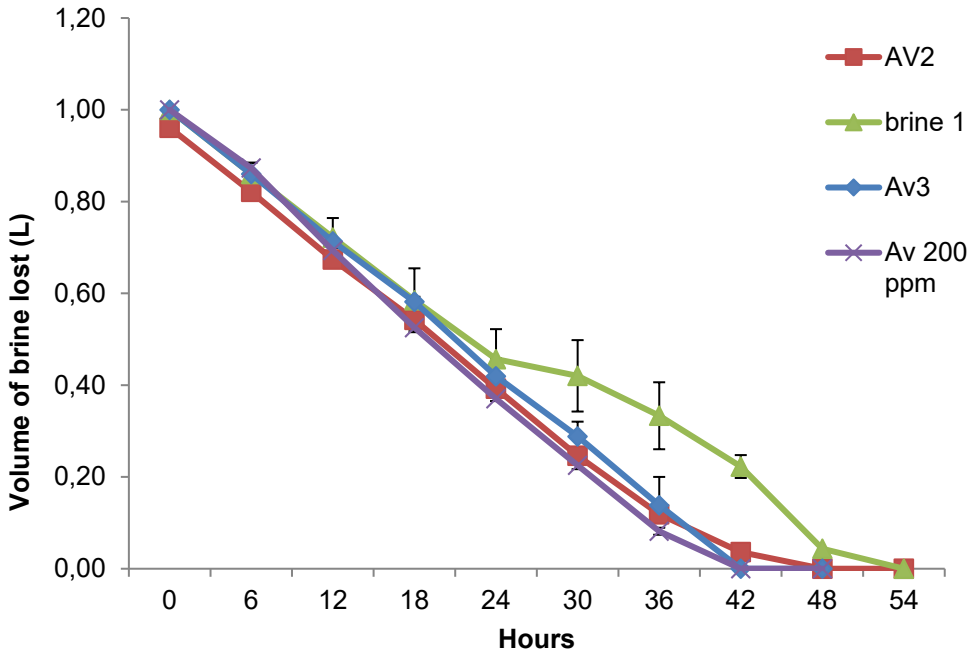

Figure 12. Brine evaporation rate at six-hour intervals from pans following methylene blue reconstitution**.** Av 200 ppm: synthetic brine with 200 ppm MB; Av2: first reconstitution of MB in the pan by adding fresh synthetic brine; Av3: second reconstitution of MB from the pan; brine 1: synthetic brine control.

4.3. ISOLATION AND CHARACTERIZATION OF HALOPHILIC BACTERIA

Fourteen pigmented halophilic bacteria were isolated from both the eMalahleni brine and Cerebos samples. The isolates were designated Ep or Cp respectively. Three isolates were obtained from the eMalahleni brine samples and the rest from Cerebos samples. The culturable bacterial diversity from the samples used for isolation was low. Much diversity may have been missed due to the limitations of the culture media used for isolation (Sekar *et al.*, 2014). The 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). Choosing appropriate media and growth conditions is important and published media are typically associated with a particular microbial genus or species. As with other microbial discovery research, when working with environmental samples harbouring communities of novel microbial populations, the media and growth conditions chosen will enrich for certain populations and not others (Schneegurt, 2012). In addition, bacteria from hypersaline environments are fastidious in their nutrient requirements as well as the salinity gradient they are subjected to during isolation.

Some of the isolates exhibited similarities in their phenotypic as well as in their physiological and biochemical characteristics. The 16S rRNA genes were amplified and sequenced to identify the isolates. A Blastn query of the sequenced 16S rRNA genes of the isolates revealed they belonged to the *Planococcus, Arthrobacter, Sporosarcina, Micrococcus* and *Chromohalobacter* genera. The Blastn results indicating the most closely related sequences in the database are given in Table 5.

The pigments produced by halophilic bacteria vary in colour from red, yellow, orange, etc., and play roles in pathogenesis, protection and survival. The isolated bacteria produced a range of pigments (Table 5), and generally grew in the salt range of 5-30% NaCl / $NaSO_4$ (Table 6), with an optimum requirement of about 5% salt indicating them to be moderately halophilic according to the classification of Kushner (Kushner, 1978). The isolates seem to tolerate higher Na_2SO_4 concentrations. This was not surprising for the bacterial strains that were isolated from eMalahleni brine since Na_2SO_4 are the major ions present (APPENDIX A). As for the Cerebos isolates, it was very unusual since they were isolated from NaCl-based saline water. However, studies have shown that SO_4 is the major inorganic compound in seawater and it can be found up to saturation. It is therefore, likely that these strains were already adapted to high SO_4 environments.

Table 5. Identification of the isolates.

Isolate	Pigment	Top BLASTn	Identity (%)
EP1, EP2, CP4-1, CP5-3	Peach	*Sporosarcina aquamarine*	99
CP2-1, CP2-2, CP2-3, CP5-2, CP5-4, CP5-6	Orange	*Planococcus maritimus*	98
EP3, CP5-1	Red	*Arthrobacter agilis*	99
CP5-5	Yellow	*Micrococcus yunnanensis*	99
CP5-7	Cream	*Chromohalobacter canadensis*	99

Table 6. Optimum concentrations of NaCl and $NaSO_4$ in % (w/v) required for growth of the isolates, and tolerance to a range of concentrations tested.

Isolate*	NaCl (%)		$NaSO_4$ (%)		Category
	Optimum	Range	Optimum	Range	
EP2	5	5-30	20	15-30	Moderate
EP3	5	5-30	20	5-30	Moderate
CP5-5	5	5-30	5	5-30	Moderate

* A representative isolate of each species was tested, refer to Table 5.

4.4. ASSESSMENT OF PIGMENTED ISOLATES FOR IMPROVEMENT OF BRINE EVAPORATION RATES

4.4.1. Micro-scale experiments: synthetic brine

Seven isolates (EP1, EP2, EP3, CP2-2, CP5-3, CP5-4 and Cp5-7) were evaluated for their effect on the evaporation of brine in a micro-scale (200 ml) experiment. Growth rate of, and pigment production by, the isolates were assessed together with evaluating the effect they had on the evaporation rate of the brine. Growth of the isolates during the evaporation experiment is shown in Figure 13.

Three isolates, namely CP5-4, EP3 and EP2, showed a rapid growth rate in the first 36 hours of the experiment and entered the stationary phase of growth at the 48-hour mark (Figure 13A). The second fastest growth rates were recorded for isolates EP1 and CP5-4, which reached stationary phase at 56 hours of the experiment. A number of factors that include availability of growth factors, physicochemical properties of the synthetic brine, metabolism of the isolates and change in salinity may have led to the observed growth patterns of the isolates. Nonetheless, all the evaluated isolates were able to grow in the synthetic brine and produced pigment during the course of the experiment (Figure 13B).

Isolates EP3, EP1 and CP5-4 produced the most pigment after 24 hours, 0.04, 0.02 and 0.08 Abs_{660nm}/ml, respectively. Noteworthy is the fact that isolate CP5-4 produced most of its pigment 56 hours into the evaporation rate experiment. Pigment production by isolate EP3 continued to increase during the course of the experiment. As with growth rate, various factors that include availability of nutrients, pH, inoculum size, amount of light (UV) and dissolved oxygen influence pigment production by halophilic bacteria (Goswami, Chaudhuri and Dutta, 2010; Bhat and Marar, 2015). The most important of the all the factors is the inoculum. A higher inoculum (i.e. increased biomass) results in decreased pigment production due to the utilization of critical components of the culture medium for maintenance of high bacterial biomass (Goswami, Chaudhuri and Dutta, 2010). However, this may not have been the case in this study as a relatively small and standardized inoculum was used for all the isolates. Therefore, the brine physicochemical properties may have had a greater influence on the pigment production by the isolates during the course of the experiment. The achieved evaporation rates of synthetic brine due to pigmented bacteria are shown in Figure 13C, over a 72-hour period.

Increases in the volume of brine loss during the course of the experiment were observed after 24 hours. All the inoculated experiments showed increased brine loss compared to the uninoculated control, with isolates EP3 (0.062 cm/h), EP1 (0.044 cm/h) and CP5-4 (0.050 cm/h) showing the highest rates (Table 7). Compared to the evaporation rate of the synthetic brine control, increases of 51, 6 and 22% were affected by isolates EP3, EP1 and CP5-4 respectively, and all performed better than the 200 ppm methylene blue.

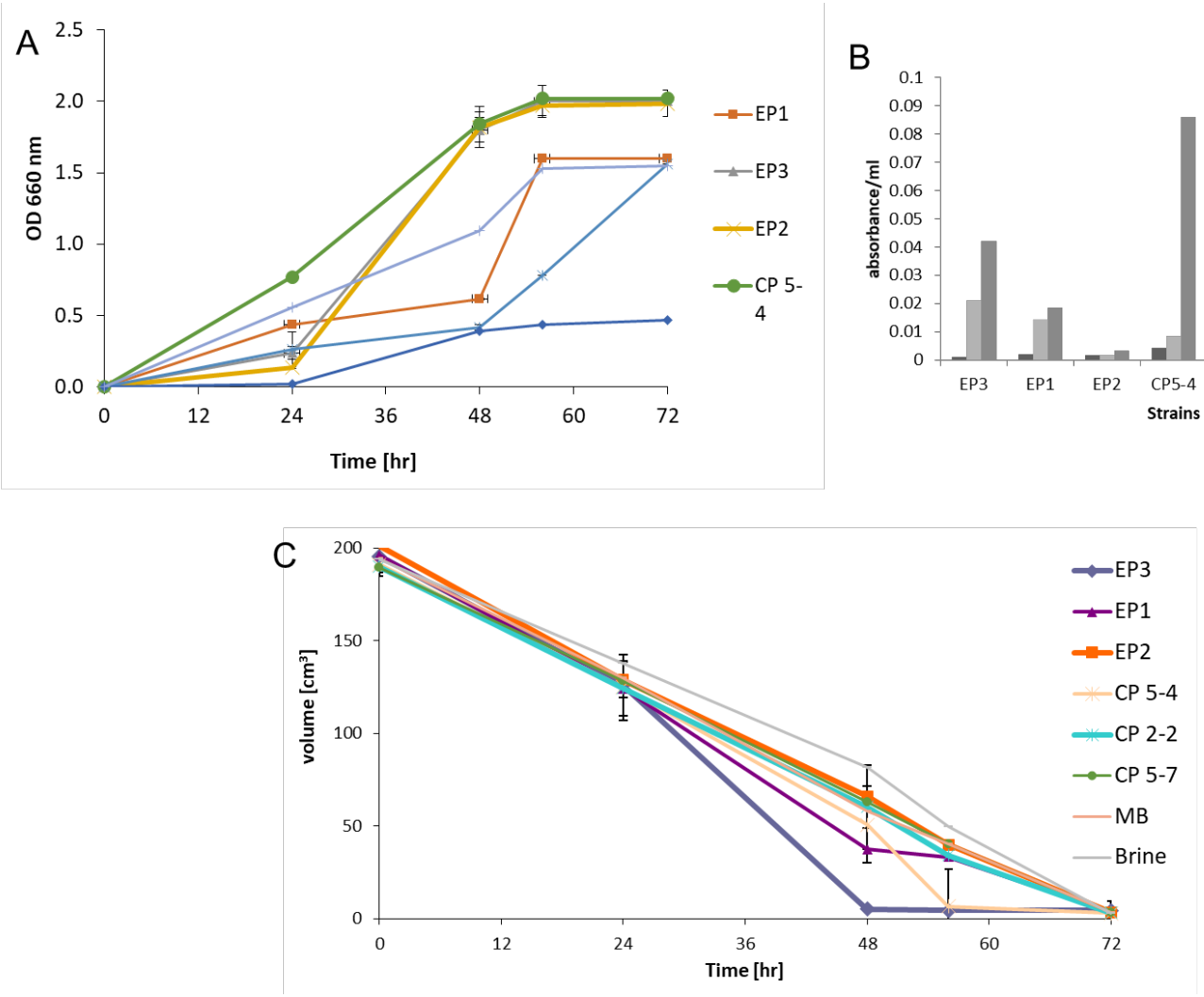

Figure 13. Assessment of Growth rate (A), Pigment production (B) and Evaporation (C) of isolates during a micro-scale (200 ml) evaporation rate experiment in synthetic brine.

Table 7. Evaporation rates of synthetic brine influenced by cultured isolates and addition of 200 ppm of methylene blue (MB).

Strains	Ev (cm/h)	% Increase in Evaporation rate*
EP1	0.044	5.70
EP2	0.043	4.94
EP3	0.062	50.95
CP 2-2	0.042	0.38
CP 5-3	0.044	6.08
CP 5-4	0.050	21.67
CP 5-7	0.041	-0.76
MB 200ppm	0.042	2.66
Brine	0.041	0

*Calculated as a % of the Ev cm /h obtained for the brine control.

4.4.2. Pan experiments: synthetic brine

Owing to the ability to grow fast in the brine, good pigment production and improving the evaporation rate of the brine at the highest rate, isolate EP3 was selected for use in the scaled-up pan (1 L) evaporation experiments. In addition, the pigment extracted from an EP3 culture was also assessed independently in the pan system.

Complete evaporation of the synthetic brine from the pans occurred 42 hours after inoculation with the EP3 culture, compared to the control (Figure 14). Addition of the pigment extracted from a 24-hour culture of isolate EP3 also resulted in the complete evaporation of the brine at 42 hours. The control reactions (without bacteria or pigment) required 54 hours for complete evaporation to occur. The calculated evaporation rates in cm^3/hr are shown in Table 8.

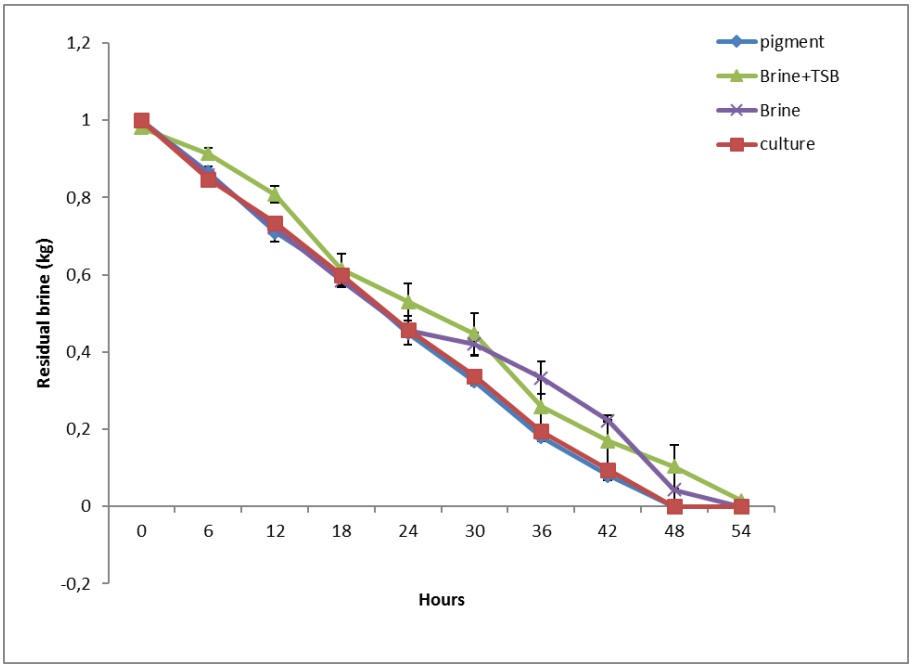

Figure 14. Evaporation of synthetic brine loss in 1 L salt pans due to isolate EP3 and its pigment.

Table 8. Calculated evaporation rates due to EP3 culture and its pigment

Sample	Evaporation rate (cm/h)	% Increase in Evaporation rate*
Brine	0.018±0.000	-
Brine + TSB	0.018±0.030	0
Culture	0.021±0.005	16.67
Pigment	0.022±0.001	22.22

4.4.3. Microscale experiments: NuWater Brine

As mentioned in section 3.3.3 NuWater brine was to be used for the pilot plant experiments. However, due to the possibility that the different composition of this brine would affect growth and pigment production, the growth of isolates EP3, EP1 and CP5-4 was evaluated in micro-scale experiments using the NuWater brine. The same experiment as presented in section 4.4.1 was performed, to enable comparison between the performance in the synthetic brine vs NuWater brine.

4.4.3.1. Assessment of growth and pigment production in NuWater brine

Growth rate and pigment production of the isolates was initially tested in 25, 50, 75 and 100% strength of the TSB in brine. The different concentrations of TSB were included to ascertain a minimum nutrient requirement to sustain the growth and pigment in an inoculated experiment. This would be important for the application of the "biological approach" in a real-world scenario, especially from an economics point of view.

The growth rate of all the isolates reached its peak after 24 hours in all the media used (Figure 15). In all cases the 75% strength TSB brine medium resulted in growth that was comparable to that of the isolates in 100% strength TSB brine medium, and to what was observed when these isolates were cultured in the synthetic brine supplemented with 100% TSB).

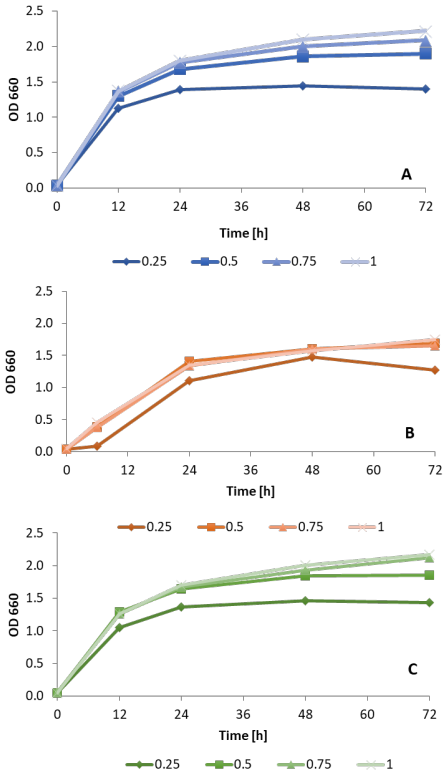

Figure 15. Growth of EP1 (A), EP3 (B) and CP5-4 (C) in TSB-NuWater brine.

It was immediately noticed that the isolates were not pigmented when cultured in the NuWater brine, unlike what had been obtained in synthetic brine. To compare the pigmentation profiles, pigment from the isolates grown in either the synthetic or NuWater brine was extracted using methanol and analysed spectrophotometrically (Figure 16, Table 9). The pigments from EP1, EP3 and Cp5-4 cultures grown in TSB-synthetic brine absorbed maximally at 470, 495 and 450 nm respectively (Figure 16A). These spectra suggested that the pigments produced are

carotenoids (Fong et al., 2001). However these profiles were not comparable to those obtained from growth in the NuWater brine, where a yellow pigment that showed maximum absorption at 400 nm was produced (Figure 16B) which is outside the carotenoid wavelength range of 450 to 650 nm. Thus, the type and colour of pigment produced by these isolates was being influenced by the physicochemical composition of the NuWater brine.

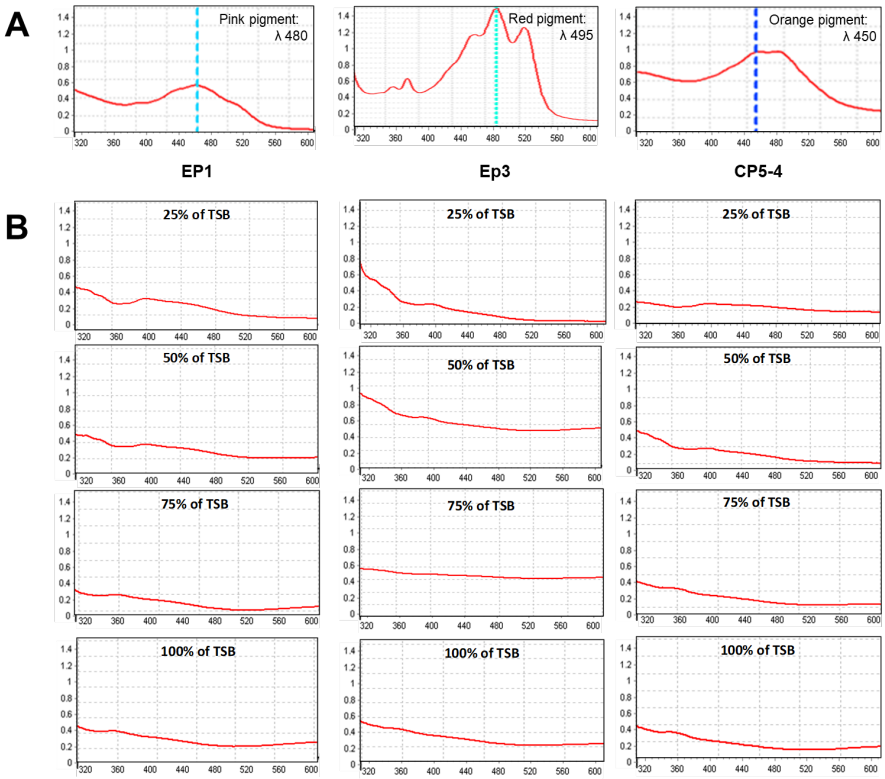

Figure 16. Absorption spectra of methanol extracts of pigment from EP1, EP3 and CP5-4 grown in (A) TSB-synthetic brine or (B) TSB-NuWater brine.

Total pigment produced by each of the isolates is shown in Table 9. The isolates produced a yellow pigment that showed maximum absorption at 400 nm, which is outside the carotenoid wavelength range of 450 to 650 nm. The growth rate of the isolates was not affected by the physicochemical composition of the NuWater brine. In fact all the isolates showed a good growth rate in the different TSB-brine media used. However, the physicochemical composition of the NuWater brine influenced pigment production by the isolates, which all produced a yellow pigment that characteristically absorbed maximally outside the carotenoid absorption spectra of 450 to 650 nm.

Table 9. Pigment production by isolates in NuWater brine supplemented with TSB.

TSB strength (%)	Time [h]	λ Max	EP1		EP3		CP5-4	
			Yield	Effic.	Yield	Effic.	Yield	Effic.
25	24	400	0.146	0.105	1.044	0.940	0.142	0.104
	48	400	0.129	0.090	0.894	0.606	0.075	0.051
	72	400	0.051	0.036	1.646	1.291	0.154	0.107
50	24	400	0.126	0.075	0.745	0.529	0.113	0.069
	48	400	0.090	0.048	0.868	0.541	0.133	0.072
	72	400	0.092	0.049	1.025	0.606	0.089	0.048
75	24	400	0.117	0.066	1.438	1.077	0.129	0.077
	48	400	0.103	0.051	0.569	0.356	0.087	0.045
	72	400	0.109	0.052	0.710	0.430	0.078	0.037
100	24	400	0.184	0.102	0.712	0.529	0.131	0.077
	48	400	0.144	0.068	0.527	0.335	0.135	0.067
	72	400	0.128	0.058	0.355	0.203	0.172	0.079

Comparison of the physicochemical properties of NuWater and EWRP brines revealed marked differences in the concentrations of chloride, sulphate and potassium (Table 3). The concentrations of the mentioned elements are much higher in EWRP brine and we conjectured that this difference may have influenced pigment production by EP1, EP3 and CP5-4 isolates. As such the effect of pH, potassium and iron concentration on pigment production by the isolates in NuWater brine was evaluated.

4.4.3.2. Effect of pH on growth and pigment production by isolates in NuWater brine

Growth of and pigment production by the isolates was evaluated in TSB-NuWater brine medium having pH values of 6, 7 and 8. Samples were obtained at 12-hour intervals for a period of 72 hours for analysis. A similar growth rate pattern was observed for all the isolates (Figure 17). All the cultures underwent exponential growth after a 12-hour lag phase in all the media with pH 6, 7 and 8. The growth of the isolates peaked at an OD_{660nm} of approximately 1.8 after 48 hours of growth.

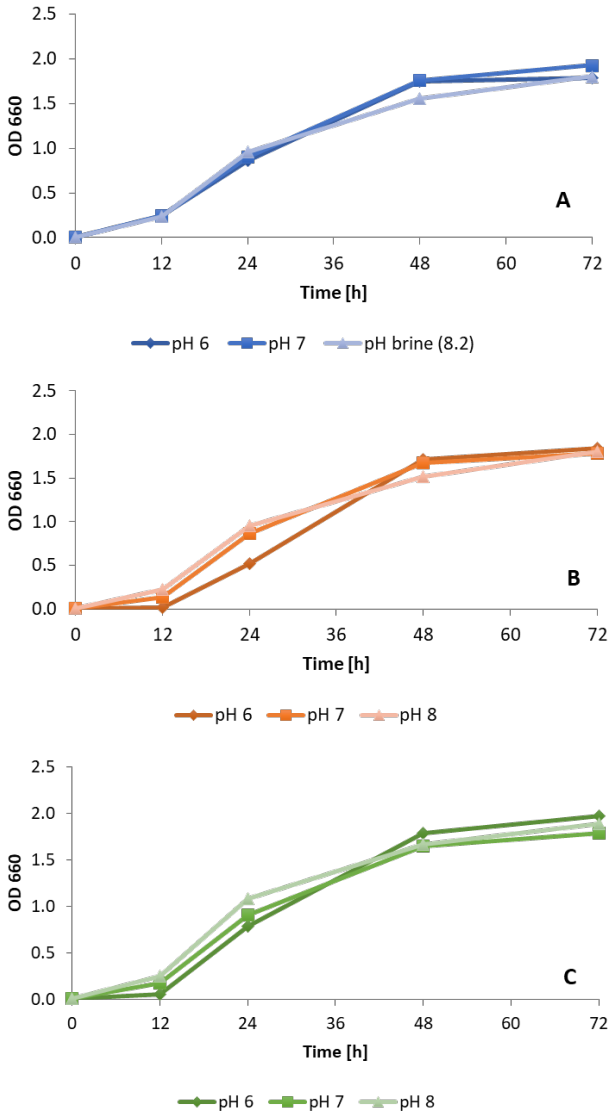

Figure 17. Effect of pH on growth rate of EP1 (A), EP3 (B) and CP5-4 (C) in NuWater brine

Methanol extracts of the pigment produced by the isolates in the pH adjusted media had absorbance maxima in the 400 to 405 nm region, indicating that pH did not stimulate the production of the native carotenoid pigment by the isolates growing in the NuWater brine. Therefore, the effect of increasing the amount of potassium in full strength TSB-NuWater brine medium was investigated next.

Table 10. Pigment production of isolates in NuWater brine with different pH.

pH	Time [h]	λ max	EP1		EP3		CP5-4	
			Yield	Effic.	Yield	Effic.	Yield	Effic.
pH 6	24	400	0.106	0.124	0.327	0.632	0.342	0.434
	48	400	0.567	0.324	0.183	0.107	0.434	0.381
	72	400	0.248	0.138	0.443	0.241	0.183	0.432
pH 7	24	400	0.730	0.807	0.072	0.083	0.344	0.242
	48	400	0.220	0.125	0.283	0.170	0.402	0.244
	72	400	0.342	0.177	0.278	0.156	0.222	0.294
pH brine (8.2)	24	400	0.602	0.632	0.095	0.100	0.466	0.432
	48	400	0.299	0.193	0.271	0.178	0.492	0.294
	72	400	0.275	0.153	0.226	0.126	0.435	0.230

4.4.3.3. Effect of potassium (KCl) on growth and pigment production by isolates in NuWater brine

Growth and amount of pigment produced by the isolates after addition of 1.2 and 1.6 g/L of KCl to full strength (100%) TSB-NuWater brine (Figure 18) showed a similar pattern as for the pH experiments. The lag phase of growth lasted for 12 hours followed by a 12-hour log phase. Growth peaked at the 48-hour mark at an OD_{660nm} of approximately 1.8. Methanol extracts of the pigments had absorption maxima in the 300 to 600 nm range, which was within the carotenoid wavelength. Increasing the amount of potassium and chloride in the NuWater brine by addition of KCl stimulated red and orange pigment production by EP3 and CP5-4 strains respectively; however, for isolate EP1 pink pigmentation was still not induced.

The uv/visible spectra for the EP3 methanol extract showed absorption maxima at wavelengths 375, 475 and 515 nm. The highest peak was at a wavelength of 375 nm while the 475 and 515 nm wavelengths showed smaller peaks (Figure 19, Table 11). Appearance of the peaks at 475 and 515 nm was indicative of the production of relatively small amounts of the red carotenoid pigment compared to what was seen when EP3 was grown in synthetic brine (Figure 16A). This suggests that the addition of KCl can only partially induce the pigment expression in EP3, and increasing the KCl to 1.6 g/L did not improve the yield.

Noteworthy is the fact that addition of KCl increased the yield of the orange pigment produced by CP5-4 substantially; to 0.521 Abs_{465}/g dry cell weight/ml after addition of 1.2 g/L of KCl and

0.860Abs$_{465}$/g dry cell weight/ml with 1.6 g/L of KCl (Table 11). Therefore, in the case of CP5-3, the increased concentration of KCl resulted in a higher pigment expression.

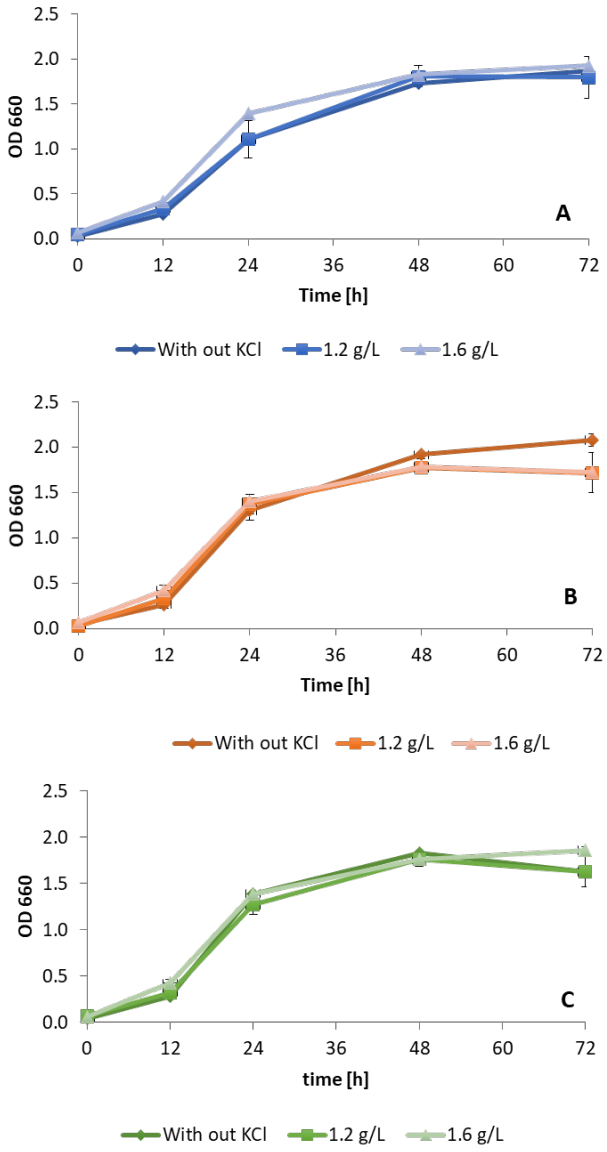

Figure 18. Effect of KCl on growth rate of EP1 (A), EP3 (B) and CP5-4 (C) in NuWater brine.

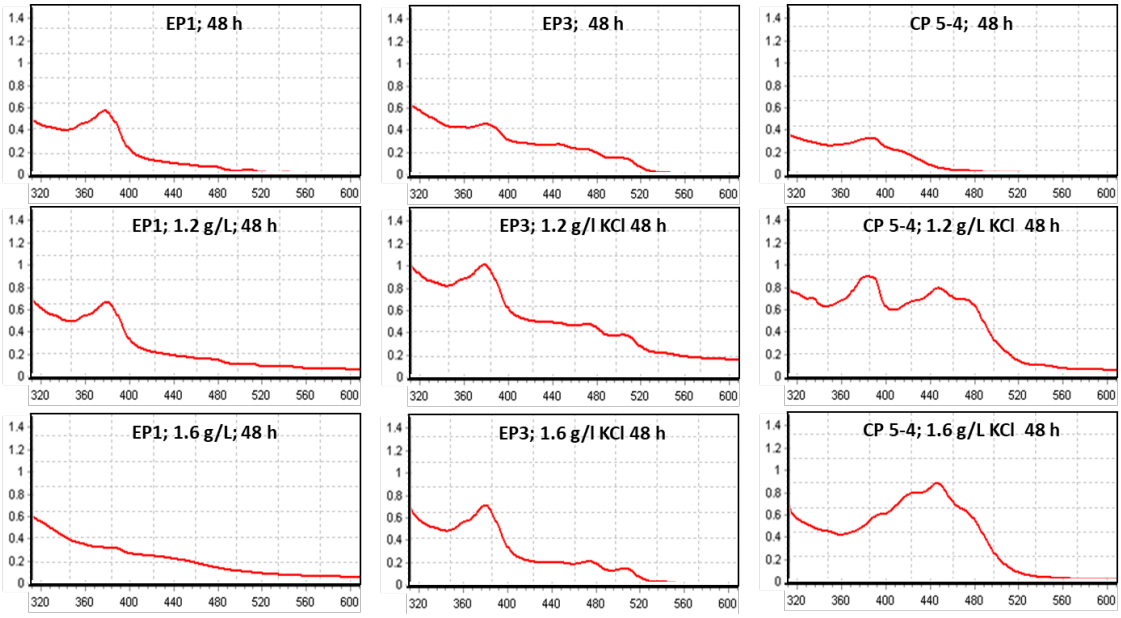

Figure 19. Absorption spectra of pigment extracts of EP1, EP3 and CP5-4 in TSB-NuWater brine with KCl supplementation after 48 hours of growth.

Table 11. Pigment production by isolates in TSB-NuWater brine supplemented with KCl.

KCl g/L	Time [h]	EP1			EP3			CP5-4		
		λmax	Yield	Effic.	λmax	Yield	Effic.	λmax	Yield	Effic.
0	24	400	0.479	0.380	400	0.17	0.127	400	0.667	0.480
	48	400	0.442	0.230	400	0.16	0.082	400	0.542	0.296
	72	400	0.313	0.162	400	0.193	0.093	400	0.375	0.230
1.2	24	400	0.279	0.248	495	0.183	0.133	465	0.603	0.478
	48	400	0.269	0.152	495	0.339	0.191	465	0.407	0.228
	72	400	0.262	0.157	495	0.255	0.148	465	0.860	0.521
1.6	24	400	0.325	0.238	495	0.227	0.163	465	0.514	0.368
	48	400	0.325	0.179	495	0.390	0.222	465	0.334	0.190
	72	400	0.226	0.118	495	0.365	0.213	465	0.512	0.275

4.4.3.4. Effect of ferric chloride ($FeCl_3$) on growth and pigment production by isolates in NuWater brine

Growth curves for EP1, EP3 and CP5-4 after addition of 0.1, 1, 10, 20 and 30 mg/L of $FeCl_3$ are shown in Figure 20. All the isolates experienced rapid growth rates during 24 hours of

incubation, reaching stationary phase of growth after 48 hours in all $FeCl_3$ concentrations evaluated.

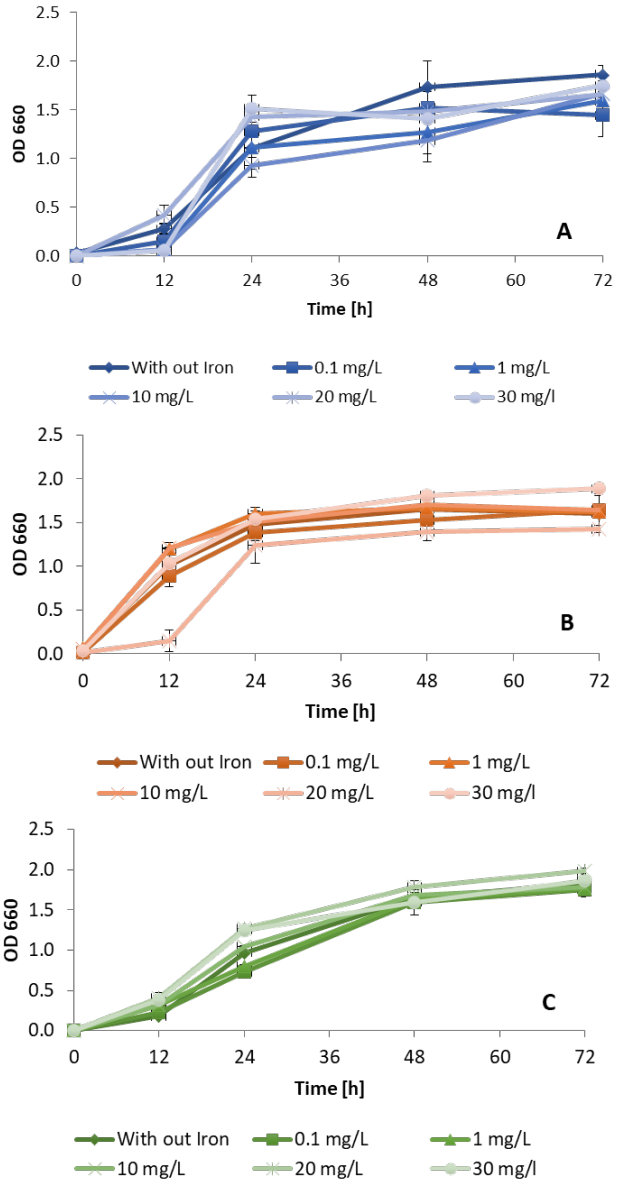

Figure 20. Effect of ferric chloride on growth rate of EP1 (A), EP3 (B) and CP5-4 (C) in NuWater brine.

Pigment spectral data from EP1, EP3 and CP5-4 methanol extracts are shown in Figure 21 and Table 12. Addition of $FeCl_3$ increased the production of the orange pigment by CP5-4 as well as the red pigment by EP3, whereas EP1 produced the lowest yield of pigment during this experiment.

Noteworthy, is the fact that addition of 20 mg/L of $FeCl_3$ to the medium resulted in high pigment yield of 3.29Abs_{495}/g dry cell weight/ml for EP3. Most importantly, maximum absorption by the methanol extract from EP3 was at 495nm which is consistent with that obtained in the synthetic brine. Interestingly, the pigment expression seems to be very tightly regulated in response to [$FeCl_3$] since concentrations above or below 20 mg/L resulted in no red pigment production. Such a scenario would make this isolate difficult to apply to a wide range of brines where the [$FeCl_3$]s would be variable.

In the case of isolate CP5-4, increasing the amount of $FeCl_3$ resulted in an increase in the yield of the orange pigment. Methanol extracts of the pigment produced by CP5-4 absorbed maximally at 465 nm.

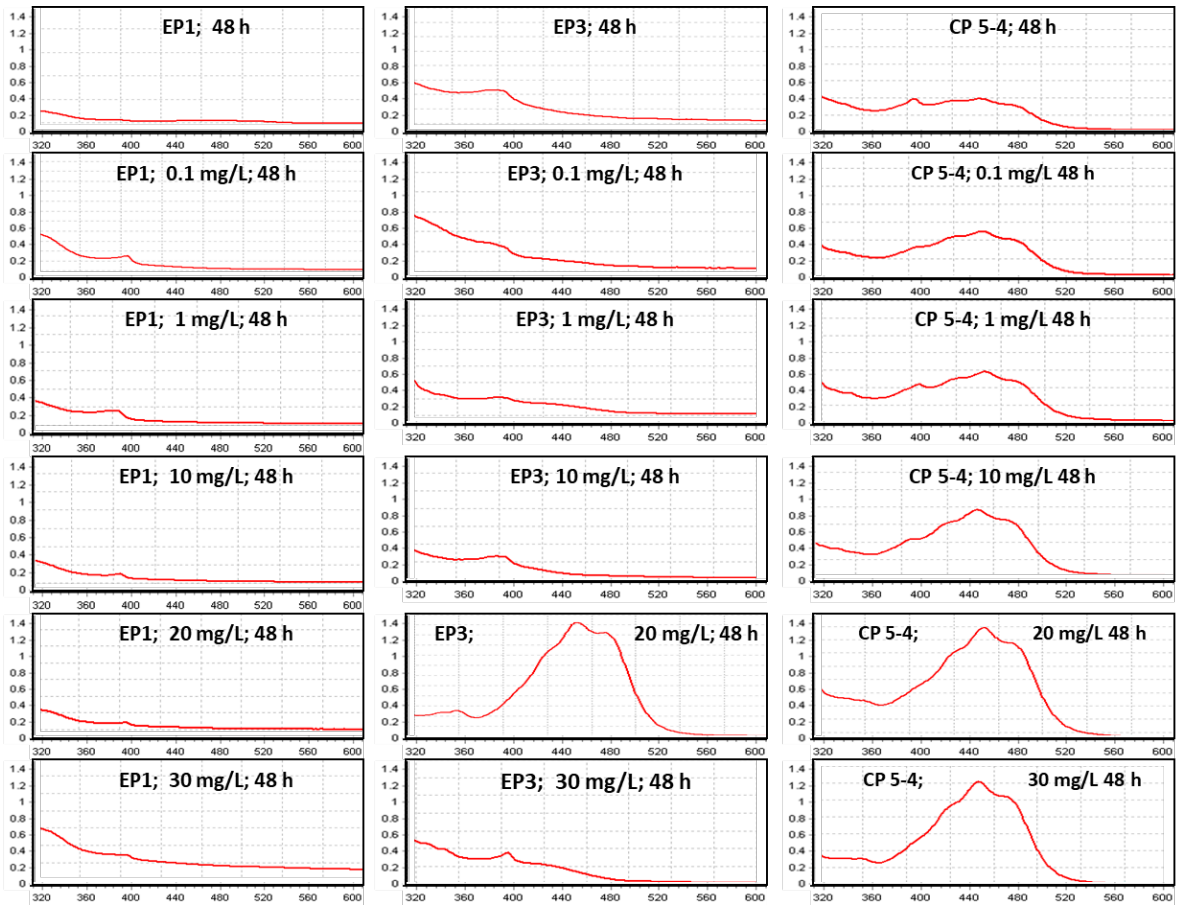

Figure 21. Absorption spectra of pigment extracts of EP1, EP3 and CP5-4 in TSB-NuWater brine with $FeCl_3$ supplementation.

Table 12. Pigment production by isolates in TSB-NuWater brine supplemented with Fe_3Cl.

$FeCl_3$ mg/L	Time [h]	λ max	EP1		λmax	EP3		λmax	CP5-4	
			Yield	Effic.		Yield	Effic.		Yield	Effic.
0	24	400	0.30	1.31	400	0.43	0.29	400	1.81	4.74
	48	400	0.20	0.37	400	1.20	0.72	465	1.46	0.88
	72	400	0.23	0.41	400	0.22	0.13	405	0.68	0.32
0. 1	24	400	0.41	0.21	400	0.07	0.04	**465**	**1.74**	**2.35**
	48	405	0.24	0.10	400	0.20	0.13	**465**	**0.50**	**0.31**
	72	400	0.21	0.10	400	0.03	0.02	**465**	**0.82**	**0.47**
1	24	400	0.91	0.57	400	0.69	0.43	**465**	**0.85**	**1.06**
	48	405	0.22	0.18	400	0.47	0.28	**465**	**2.37**	**1.46**
	72	400	0.26	0.16	400	0.12	0.07	**465**	**0.43**	**0.24**
10	24	400	0.45	0.34	400	1.28	0.84	**465**	**4.75**	**4.53**
	48	405	0.15	0.15	400	0.60	0.35	**465**	**1.54**	**0.91**
	72	405	0.24	0.14	400	1.23	0.88	**465**	**0.76**	**0.43**
20	24	400	0.62	0.28	**495**	**1.04**	**0.77**	465	**4.44**	**3.40**
	48	405	0.11	0.07	**495**	**3.29**	**2.31**	465	**1.93**	**1.09**
	72	405	0.21	0.12	**495**	**4.19**	**8.69**	465	**1.25**	**0.63**
30	24	400	0.38	0.17	400	0.93	0.58	**465**	**3.23**	**2.59**
	48	405	0.11	0.05	400	0.29	0.16	**465**	**2.65**	**1.66**
	72	405	0.12	0.05	400	0.19	0.10	**465**	**1.12**	**0.60**

Values underlined and in bold represent absorbances that correlate to the red (495nm) and orange (465nm) pigments.

4.4.3.5. Micro-scale evaluation of the effect of $FeCl_3$ supplementation on evaporation rate

Since red and orange pigmentation could be restored in NuWater brine supplemented with $FeCl_3$ for isolates EP3 and CP5-4 respectively, micro-scale evaporation rate studies were conducted with 20 mg/ml $FeCl_3$ (Figure 22).

Within the first 24-hour period, CP5-4 effected the highest evaporation in comparison to EP3 and the controls (Figure 22), displaying an evaporation rate of 4.67 cm3/hour (Figure 22B). During the same time period brine inoculated with EP3 as well as the control experiment had evaporation rates of 3.77 cm^3/hour and 3.50 cm^3/hour, respectively. During the 24-48 hour period, EP3 and the brine control showed the highest evaporation rates.

Linking evaporation rate to growth and pigment production (Figure 23) showed that CP5-4 produced the most pigment after 24 hours (0.25 Abs/g dry cell weight/ml). Pigment production remained constant through the course of the experiment, and possibly contributed to the evaporation rate effected by the CP5-4 culture. On the other hand, isolate EP3 produced 0.45 Abs/g dry cell weight/ml during the first 24 hours of the experiment, and then the production decreased thereafter, indicating that the conditions of the experiment were not conducive for constant pigment production by EP3 or that the culture started utilizing media components for biomass production in preference to pigment production.

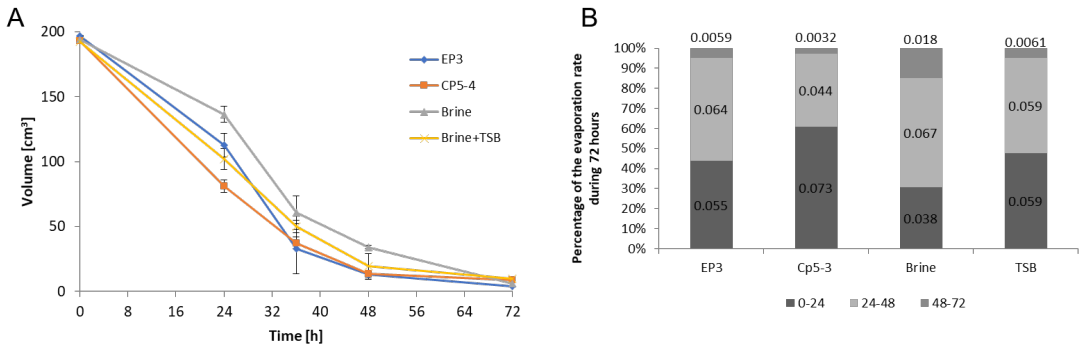

Figure 22. Effect of 20 mg/ml $FeCl_3$ on EP3 and CP5-4 induced evaporation of NuWater brine (A) and the corresponding evaporation rates (in cm/h) shown per 24-hour period (B), in a micro-scale experiment.

Despite increasing the evaporation rate of NuWater brine, the EP3 and CP5-4 did not seem to provide the level of improvements in this type of brine, compared to what was observed in the synthetic brine (compare Figure 13C and Figure 22A). To better understand the kinetics of the evaporation, the experiment was conducted in the 1 L pans, and only for CP5-4 since it effected the highest evaporation rate in the NuWater brine.

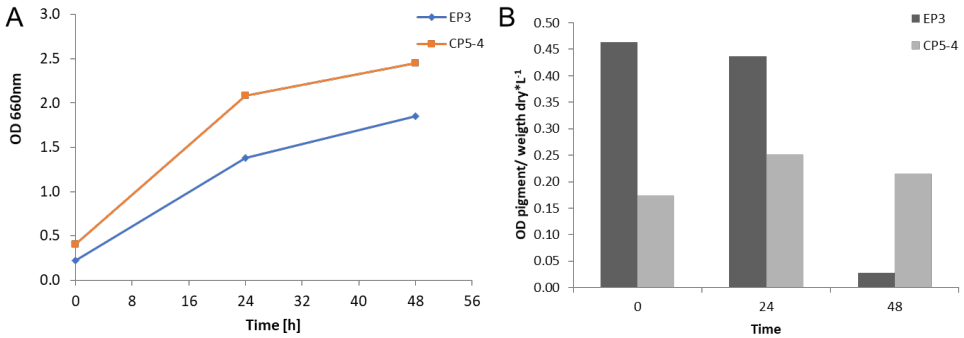

Figure 23. Growth and pigment production of EP3 and CP5-4 in TSB-NuWater brine supplemented with 20 mg/L $FeCl_3$.

4.4.4. Pan experiments: NuWater brine

Given that in the micro scale experiments CP5-4 was shown to effect the highest evaporation rate in the NuWater brine (when supplemented with TSB and $FeCl_3$), the experiment was repeated in the 1 L pan scale. The effect of the inoculum volume (10, 20 and 30%) was also determined. Figure 24 shows the evaporation curves and the evaporation results are presented in Table 13. The corresponding pigment production is presented in Figure 25. CP5-4 cultures resulted in an increased evaporation rate, and the highest was obtained with a 20% inoculum. Compared to the brine control, the 20% CP5-4 inoculum effected the highest evaporation increase of 42%.

Peak pigment production was achieved at 36 hours under all the concentrations tested, however, inoculation of 10% and 20% of CP5-4 maintained the pigment production throughout the 48-hour period being monitored.

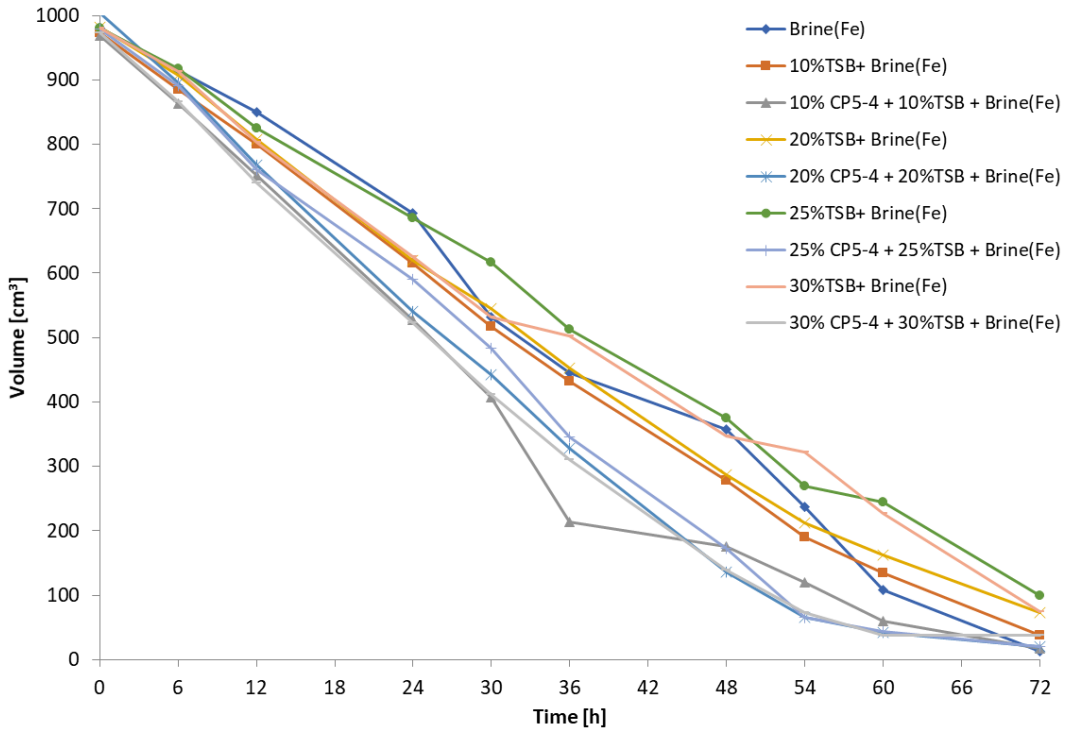

Figure 24. Evaporation of TSB-NuWater brine (supplemented with $FeCl_3$) loss in 1 L pans inoculated with 10, 20, 25 and 30% CP5-4 culture volumes.

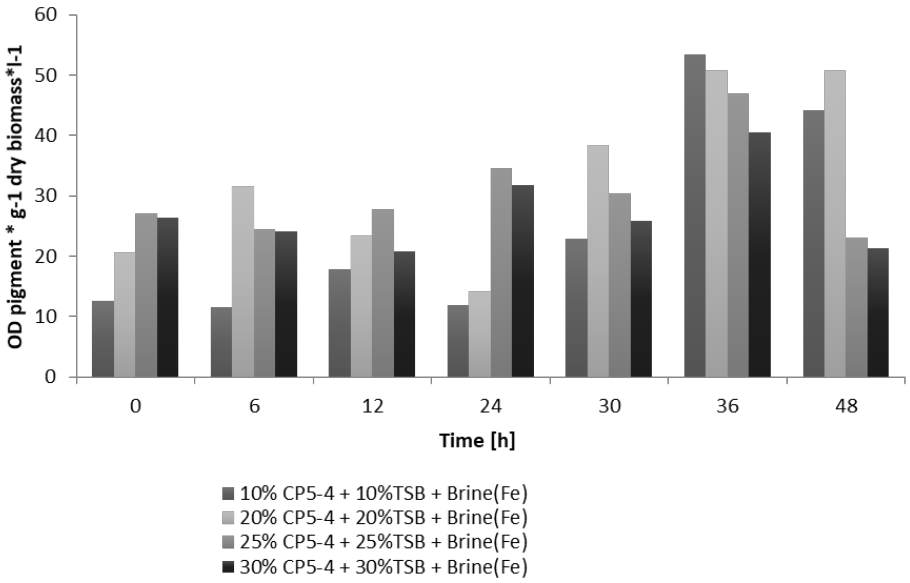

Figure 25. Yield of CP5-4 pigment during evaporation assay**.**

Table 13. Evaporation rate of TSB-NuWater+$FeCl_3$ brine with CP5-4 cultures in 1 L pans.

Conditions	EV [cm/h]	%
Brine(Fe)	0.024	
10%TSB+ Brine(Fe)	0.027	12.5
10% Cp5-4 + 10%TSB + Brine(Fe)	**0.033**	**37.5**
20%TSB+ Brine(Fe)	0.027	12.5
20% Cp5-4 + 20%TSB + Brine(Fe)	0.034	**41.7**
25%TSB+ Brine(Fe)	0.022	0.0
25% Cp5-4 + 25%TSB + Brine(Fe)	0.031	29.2
30%TSB+ Brine(Fe)	0.023	0.0
30% Cp5-4 + 30%TSB + Brine(Fe)	0.032	33.3

Generally, carotenoid production occurs in response to environmental conditions such as growth temperature, light and salt concentration, and the investigation of the regulatory mechanisms has provided insight into the adaptation of bacteria to their respective environment (Sutthiwong et al., 2014). For example, red-carotenoids have been proposed to increase the resistance of heterotrophic bacteria to environmental stress as being cryo- and solar radiation protectants (Dieser et al., 2010). In this study the EP3 and CP5-4 pigment production was not constitutive, where pigmentation was absent when cultured in the NuWater brine, whereas pigmentation was observed in the synthetic brine supplemented with TSB. Schobert and Jahn (2002) proposed that changes in environmental conditions could lead to

this as a result of an adaptation of the energy conserving electron transport chain and cofactors of various enzymes and thus causing significant changes in pigment production (Sutthiwong et al., 2014). The biological roles of ions such as potassium, magnesium, sodium, calcium and other transition elements like metal ions in microbes are difficult to study because they interact weakly with carrier ligands (Bhosale, 2004). However, similar to what we observed, supplementation of inorganic salts to the culture medium was reported to affect or stimulate carotenogenesis in *Haematococcus pluvialis* and *Rhodotorula*.

The pigment production behaviour observed in ion supplemented medium was different for each isolate. Addition of iron in the form of $FeCl_3$ promoted pigment production for isolate CP5-4, which led to increased evaporation rates, however the same effect was not observed for EP3, which showed decreased pigment production under the conditions tested. Several studies suggest that hyper-accumulation of astaxanth in by *Haematococcus pluvialis* induced by ferrous iron was due to the generation of hydroxyl radicals from the fenton reaction, which stimulates cellular carotenoid synthesis (Kobayashi et al., 1992; Tjahjono et al., 1994; Bhosale, 2004). In the same way, Bhosale and Gadre (2001) reported that *Rhodotorula* showed a marked improvement in the production of carotenoids due to a stimulatory effect of copper, zinc and ferrous iron on carotenoid synthesizing enzymes or to the generation of oxygen radicals in the culture broth. In this regard, these metals have the same effect as ionizing radiation on the cultures. The observation that each isolate produced two possible pigments with different absorbance spectra as well as different responses to the addition of iron, could point to different modes of regulation and/or biosynthesis.

4.4.5. Pilot scale experiments

Having established that a 20% CP5-4 culture inoculum results in the most improved evaporation in NuWater brine supplemented with $FeCl_3$, we proceeded with the pilot scale experiment, using a 100 L brine volume. Since a much larger volume of culture (2 L) was to be used for seed culture production, the growth of the CP5-4 in a Minifors™ bioreactor was assessed. Assessing the performance in the bioreactor would also be important for any future applications where >100 L operations are considered which would require several batches of bioreactor preparations.

4.4.5.1. Seed culture production for application in pilot plant experiments

At first, culturing in the bioreactor was conducted using the growth conditions established in the previous experiments (NuWater brine+20 mg/L $FeCl_2$+ 100% TSB) (Figure 26A). Although high biomass was achieved, the culture did not produce the orange pigment (Figure 27). It is well-known that secondary metabolite expression (which includes carotenoids) can respond differently to changes in culturing protocols, therefore it was not surprising that changing to bioreactor vs shake-flask growth affected the expression of pigment. Supplementation with 40, 50 and 60 mg/L of $FeCl_3$ however, restored the pigment production, with the optimum yield at 50 mg/L (Figure 26A, Table 14). In addition, bacterial growth reached a maximum $OD_{660\ nm}$ of 4.5 indicating high biomass production. Thus, addition of $FeCl_3$ to a final concentration of 50 mg/L was regarded as the best condition for optimal biomass production and maximum pigment production for seed culture preparation.

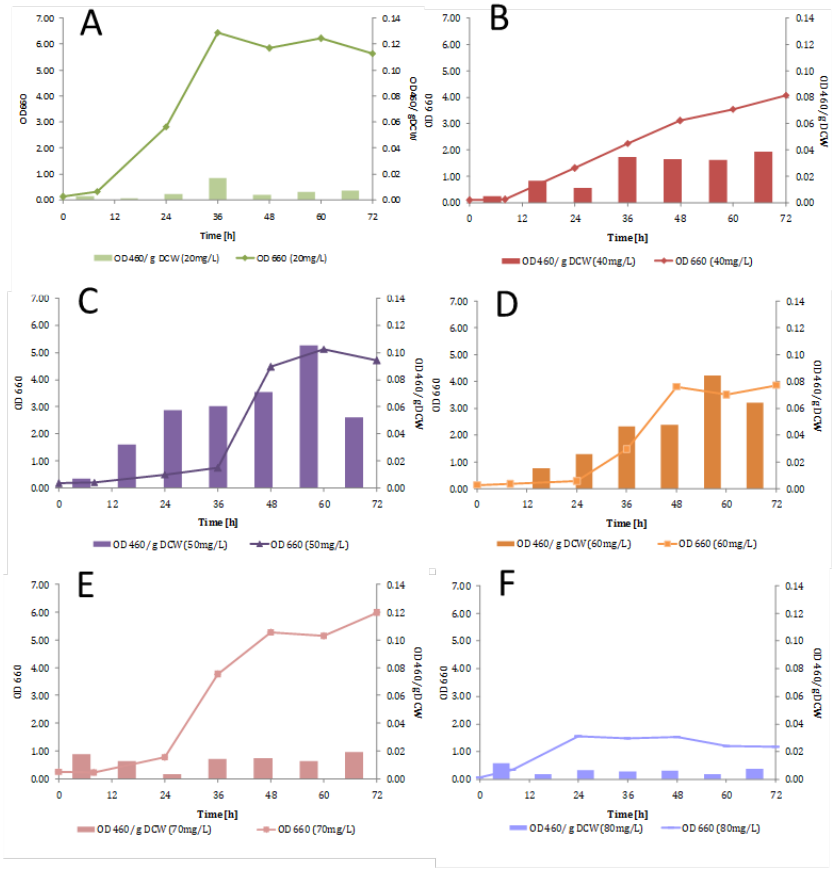

Figure 26. Evaluation of the growth ($OD_{660\ nm}$) and pigment production ($OD_{460\ nm}$/g dry cell weight) by CP5-4 in a bioreactor.

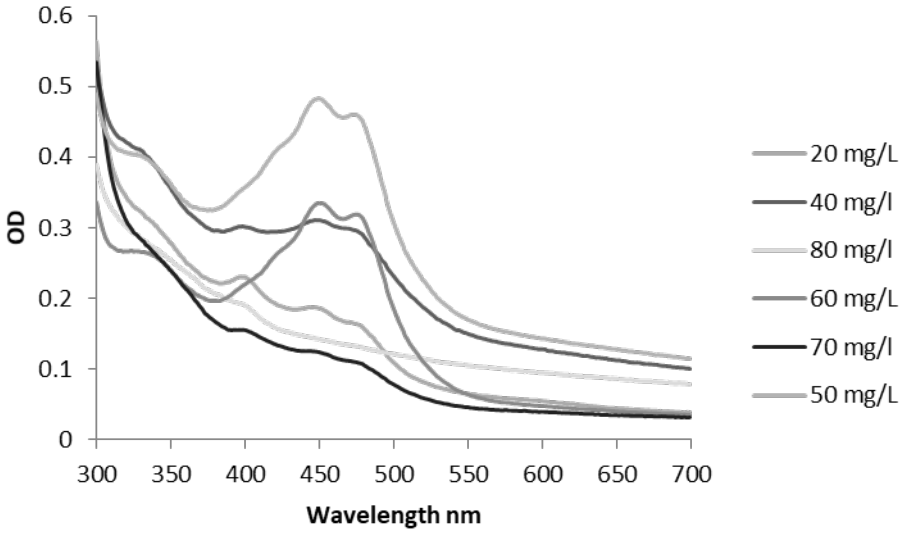

Figure 27. Absorption spectra of pigment produced by CP5-4 in different $FeCl_3$ concentrations when cultured in the bioreactor.

Table 14. Cell biomass and pigment concentrations from CP5-4 after 72 hours growth in the bioreactor.

FeCl3 Concentration (mg/L)	Cell Biomass (g/L)	Pigment concentration ($OD_{460\ nm}$/L)
20	4.80	0.36
40	9.52	1.73
50	**7.72**	**1.97**
60	7.64	1.96
70	9.46	0.42
80	6.23	0.44

4.4.5.2. Pilot plant evaporation rate studies

Evaluation of the feasibility of applying the biological approach in real world scenarios was done in 50*50*50 cm^3 Perspex pilot plant equipped with three temperature sensors as well as insulated with reflective material to minimize the impact of external conditions on the experiment. Twenty percent v/v of CP5-4 seed culture was inoculated in 100 L of NuWater brine contained in the pilot plant and the amount of brine lost on a daily basis measured in

cm^3/day. However, after five days the brine had turned black and was emitting a foul odour (Figure 28).

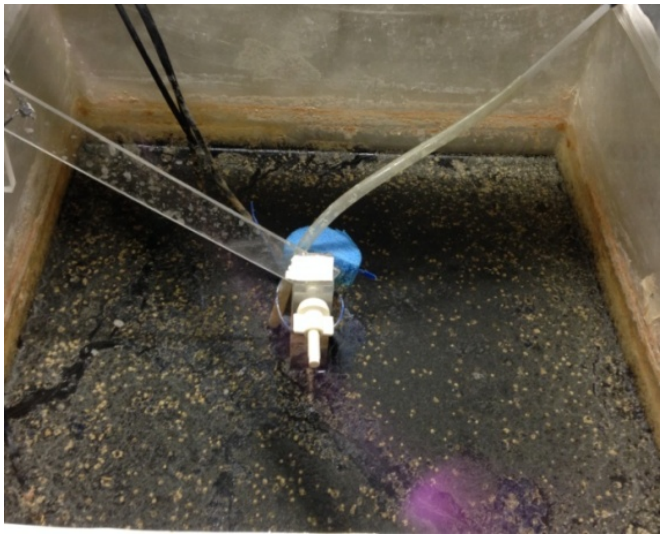

Figure 28. Appearance of NuWater brine after 5 days in pilot plant.

The odour was attributed to the growth of sulphate reducing bacteria (SRB) in the brine. SRBs (and archaea) obtain energy by oxidizing organic compounds or molecular hydrogen (H_2) while reducing sulphate to hydrogen sulphide (H_2S) (Barton and Fauque, 2009), for anaerobic respiration. The black colour may have been the result of H_2S reacting with the ferric ion (Fe^{3+}) to produce iron sulphide (FeS_2) as shown below.

$$\mathbf{2FeCl_3 + 2H_2S \rightarrow FeS_2 + FeCl_2 + 4HCl}$$

Within a depth of 50 cm, an oxygen gradient developed resulting in condition conducive to the growth of the SRBs. Proliferation of sulphate reducing bacteria was prevented by supplying air to the pilot plant to preserve aerobic conditions. Successive experiments in the pilot plant were carried out with steady air supply to the brine through a pump. The amount of brine lost at 5-day intervals is shown in Figure 29. Not much difference was observed in the volume of brine lost when comparing the 20% (w/v) TSB control (2501 cm^3/day) vs the 20% (w/v) TSB+CP5-4 experiment (2332 cm^3/day), although both showed a 40% increased evaporation compared to the brine control (1739 cm^3/day).

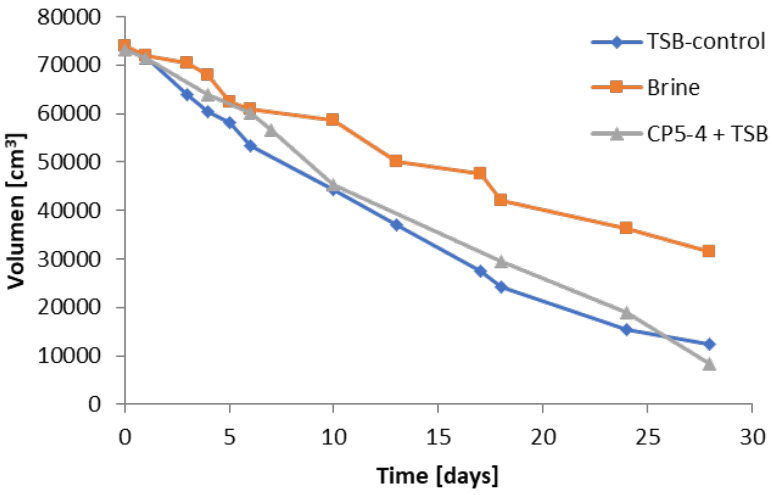

Figure 29. Volume of NuWater brine lost in the pilot plant.

Noteworthy is the fact that there was some bacterial growth in the NuWater brine + 20% TSB control during the course of the experiment. This bacterial growth may have been as a result of native or indigenous microflora utilising the TSB as nutrients for growth. As a consequence, the growth of these bacteria may have contributed to the observed increased evaporation rate for the NuWater brine + 20% (w/v) TSB control (Figure 29).

Samples were obtained from the pilot plant for microbial growth and pigment production analyses. Sampling was at four depths; H1, H2, H3 and H4 at 6, 12, 18 and 24 cm below the brine surface respectively. Microbial growth was determined through total viable counts (TVCs) as the number of colony forming units per ml (CFU/ml) of brine sampled. Pigment production was measured spectrophotometrically following methanol extraction of the pigment from the samples. The TVCs of heterotrophic bacteria growing in both the NuWater + 20% (w/v) TSB control and the NuWater + 20% (w/v) TSB + CP5-4 seed culture treatment are illustrated in Figure 30. The graphs labelled alphabetically illustrate the TVCs of bacteria from the samples obtained at the four depths below the brine surface as follows:

A. TVC from sample obtained at H1 in pilot plant with NuWater + 20% (w/v) TSB control.
B. TVC from sample obtained at H1 in pilot plant with NuWater + 20% (w/v) TSB + CP5-4 seed culture.
C. TVC from sample obtained at H2 in pilot plant with NuWater + 20% (w/v) TSB control.
D. TVC from sample obtained at H2 in pilot plant with NuWater + 20% (w/v) TSB + CP5-4 seed culture.
E. TVC from sample obtained at H3 in pilot plant with NuWater + 20% (w/v) TSB control.
F. TVC from sample obtained at H3 in pilot plant with NuWater + 20% (w/v) TSB + CP5-4 seed culture.
G. TVC from sample obtained at H4 in pilot plant with NuWater + 20% (w/v) TSB control.
H. TVC from sample obtained at H4 in pilot plant with NuWater + 20% (w/v) TSB + CP5-3 seed culture

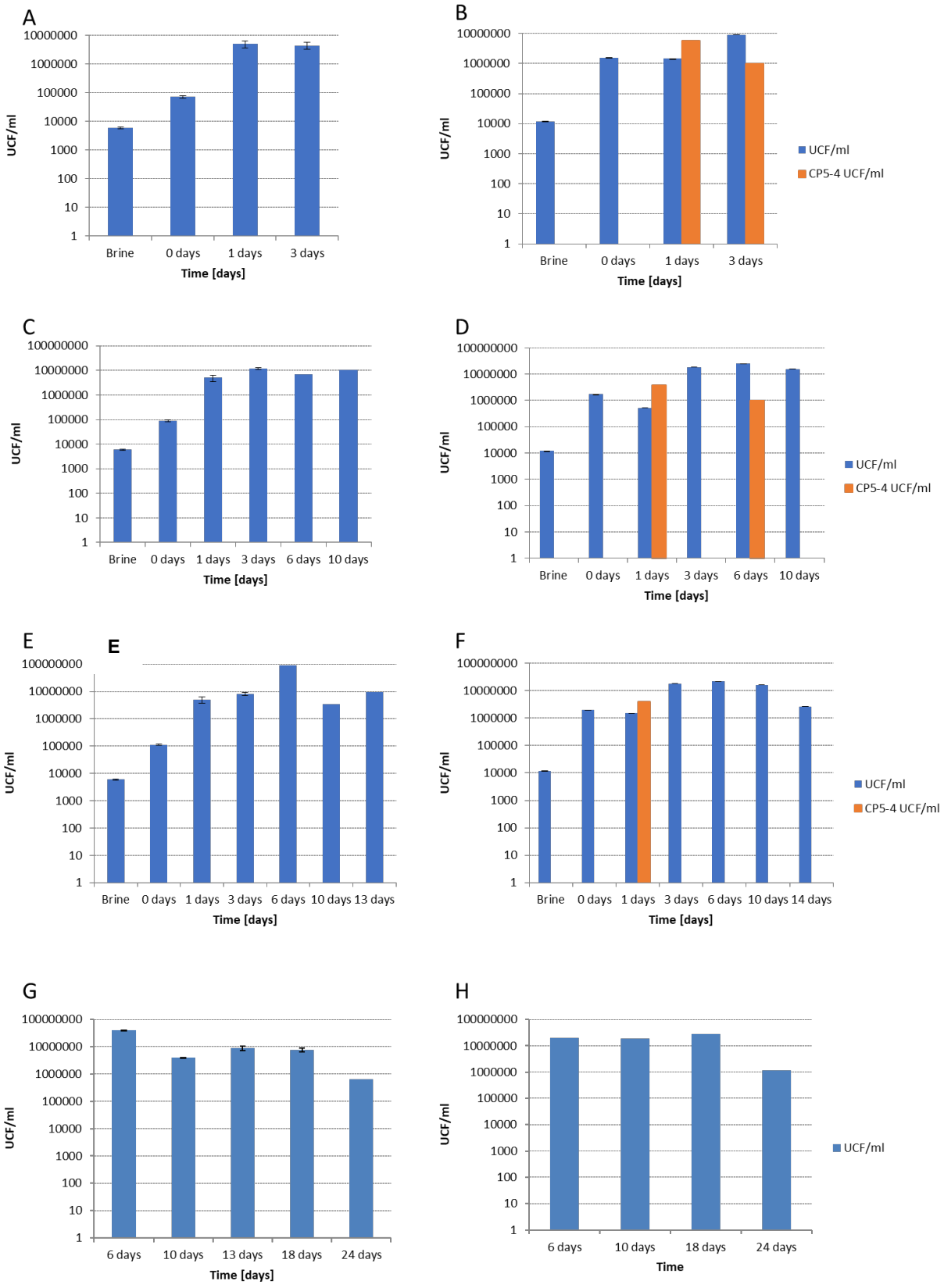

Figure 30. Total viable counts of bacteria growing in the pilot plant.

It is apparent from Figure 30 that the TVCs of bacteria increased with time in the pilot plant. Noteworthy is the fact that over time the CP5-4 seed culture was out grown by bacterial flora indigenous to the NuWater brine (Figure 30 F and H). Phenotypically the bacteria indigenous to the NuWater brine were pigmented. The isolated 'NuWater brine' strains as well as the absorption spectra of the methanol extracts of their pigments are shown in Figure 31.

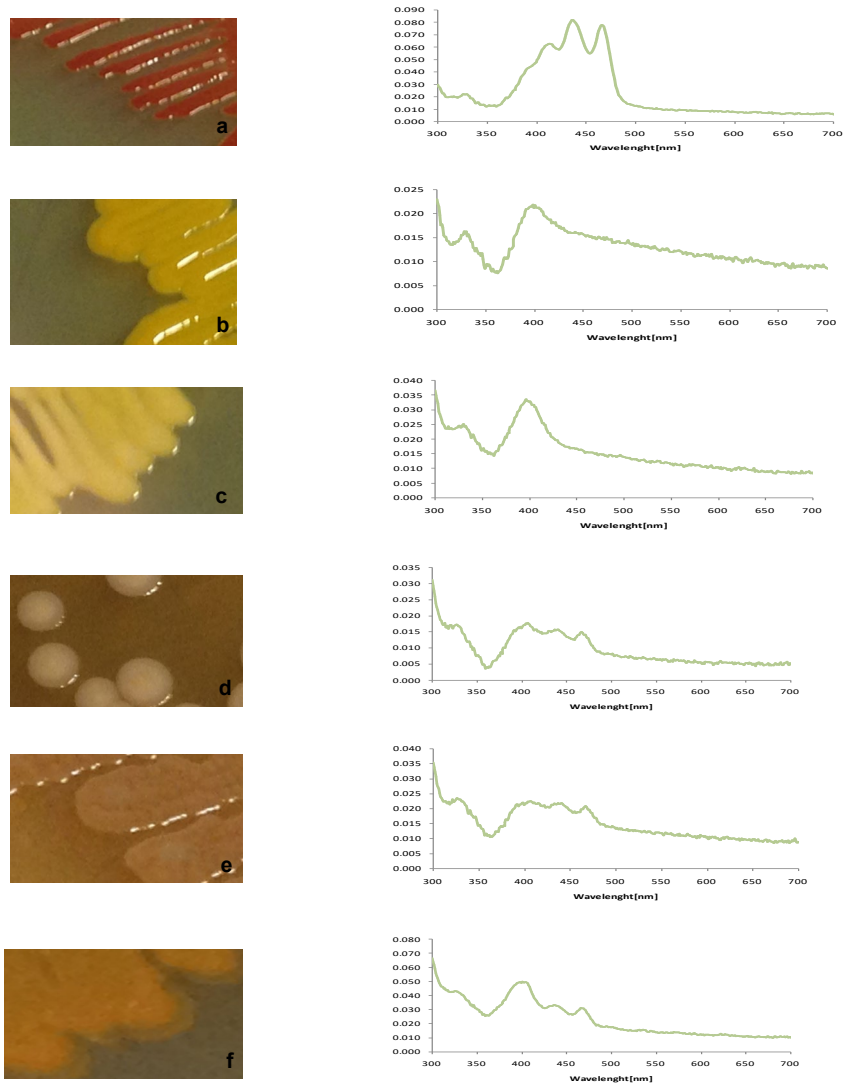

Figure 31. NuWater brine isolates and their pigment absorption spectra.

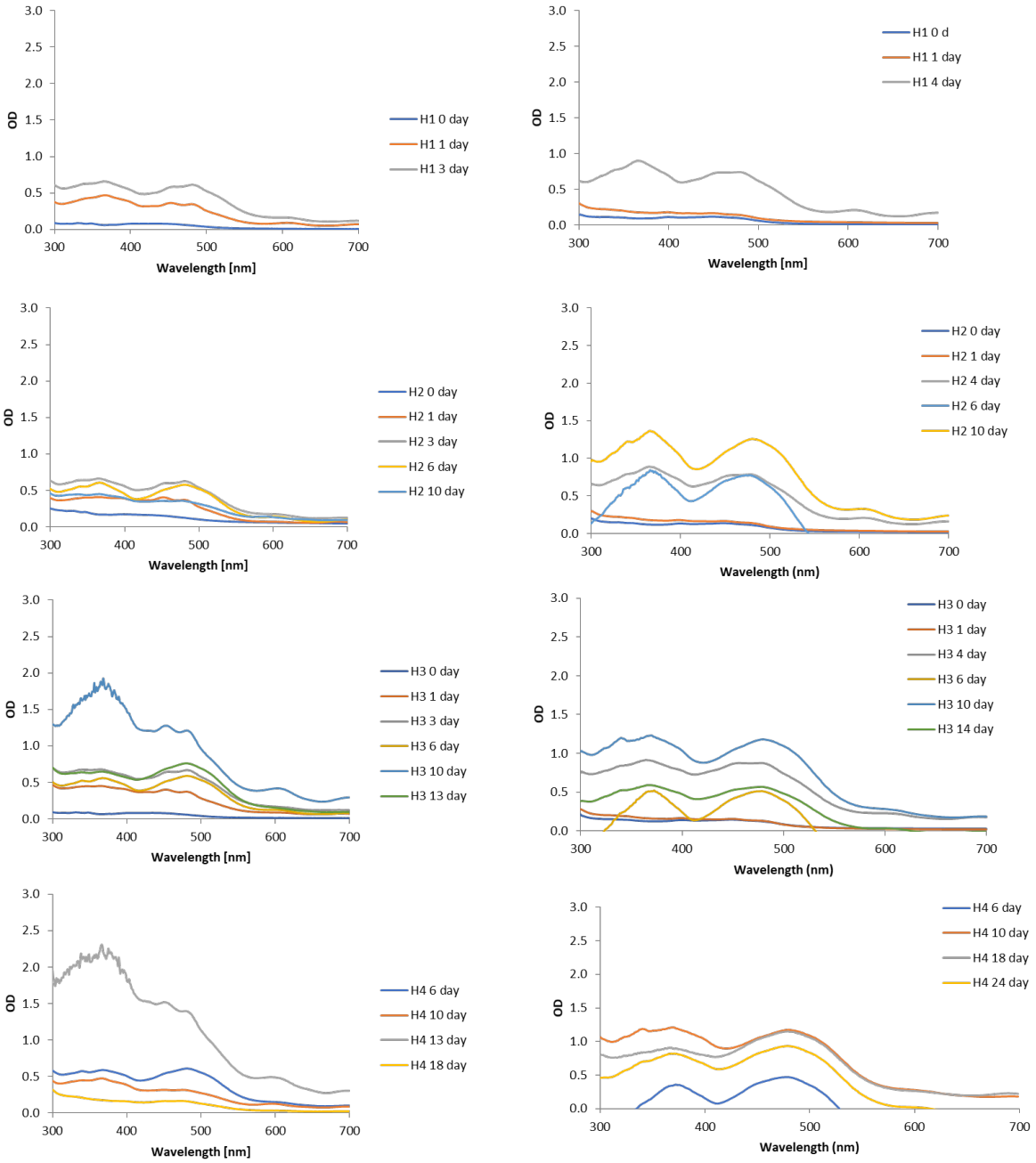

Figure 32. Pigment production in the pilot plant, measured at the 4 different depths (H1-4). All panels on the left represent the NuWater+20% TSB control; panels on the right represent the NuWater+20% TSB+CP5-4 seed culture treatment.

Six pigmented isolates were obtained from the NuWater brine treatments in the pilot plant and it was concluded that they may have contributed to the higher evaporation rates experienced by the NuWater brine + 20% (w/v) TSB control and the NuWater brine + 20% (w/v) TSB + CP5-4 seed culture compared to the unaltered brine. Furthermore, pigment production in the pilot plant was monitored over 24 days at the various depths for the NuWater brine + 20% (w/v) TSB control and the NuWater brine + 20% (w/v) TSB + CP5-4 seed culture (Figure 32). Pigment was detected after 4 days of treatment and was maintained until the end of the assay. Three different pigments were observed and measured at the following respective wavelengths; 375nm (yellow), 460nm (orange) and 490nm (red).

The experimental pilot plant model suggested that TSB increases the evaporation rate by up to 44%, by stimulating the growth and pigment production of the indigenous halophilic bacteria, and inoculating with CP5-4 did not improve this any further. Therefore, in a real-world application, there would be no control of the type of halophilic bacteria that may colonise the evaporation pond if it were to be supplemented with TSB. However, the data suggests that, as long as the pond is aerated, the supplementation with nutrients would stimulate the growth of pigmented bacteria which would ultimately result in increased evaporation, and as such can be considered as a biological and appropriate remediation technology for wastewater brine treatment. A longer-term study would have to be conducted to assess the survival rate of the pigmented organisms. Eventually the nutrients would be exhausted, therefore to maintain the pigment production continuous supplementation may be required.

CONCLUSIONS AND RECOMMENDATIONS

The most economical method of wastewater brine management currently relies on the evaporation of these brines, the effectiveness of which is dependent on climate and the composition of the brine (the type and concentration of dissolved salts). Increased evaporation rates would make the process more economical and increases the lifespan of an evaporation pond, which normally has a large footprint and is expensive to build. The focus of this project was therefore two-fold: to assess two different strategies − one chemical and the other biological, that could increase brine evaporation and preferably in an environmentally friendly and sustainable manner. Both approaches are premised on the application of a dye/pigment to the brine to trap more solar radiation, increase temperature and vapour pressure and thus increasing the rate of evaporation. In this study we assessed the addition of methylene blue dye and the application of pigmented halophilic bacteria autochthonous to brines.

Improvement of evaporation rates was achieved ranging between 17-51%. Some of the major factors identified which influenced the evaporation included:

- The experimental design (volume being evaporated and depth). It has been established in previous studies that a dye maximises the absorbance of solar energy in saline effluent in the thin upper layer (~2 cm), thus raising the surface temperature of the brine (Rajvanshi, 1981). Using the shallow 1 L pan system, this was more easily achieved and thus it could be expected that the improved evaporation rates in our study represent an over-estimate compared to an industrial evaporation pond. Therefore the results cannot necessarily be extrapolated to real-world evaporation pond sizes. Furthermore, these evaporation rates represent best case scenarios, since the experimental setup employed in this study was under very controlled conditions, where solar energy was maintained 24 hours a day, and was not subjected to changing climatic conditions as is experienced in an industrial scenario.
- The method of pigment application (the biological vs chemical approach). A 21% increased evaporation rate was achieved following addition of methylene blue dye at a concentration of 200 ppm, whereas the highest increase (51%) was achieved with the biological approach. In the biological approach it made no difference whether the pigmented organism or its extracted pigment was applied, as comparative evaporation rates were achieved with each. This confirms that the enhanced evaporation was solely a result of the pigment absorbing radiant energy as opposed to the combined effect of bacterial biomass increase and pigment production.

- The source of biological pigment. Each pigmented organism (due to its pigment and metabolism) influenced the evaporation rate differently. The highest rate was achieved with the red-pigmented organism, the pigment is presumed a carotenoid.
- The brine composition. Pigment production was not constitutive and was influenced by the brine composition. Depending on the concentration of certain salts and other elements the microbes alter their metabolism which can result in different pigments being produced. Such an organism-specific response to the brine composition makes executing the biological approach difficult and the outcome unpredictable.
- Nutrient supplementation. Since the brines in an industrial setting are non-sterile, the requirement to supplement with nutrients to support the microbial pigment production could result in the target microbe being outcompeted by a non-pigmented and faster-growing microbe.
- Aeration. Aeration and agitation of the brine are necessary to promote the growth of heterotrophic pigmented halophilic bacteria instead of sulfate reducing bacteria. Thus, in a real world application there is no control over the type of bacteria that may colonize after addition of a pure culture of the target pigmented bacteria, although the ultimate outcome would still be enhanced evaporation rate as long as the pond is aerated and supplied with some form of nutrient supplementation. A 40% increase in the evaporation rate was achieved which was directly attributed to the activity of pigmented bacteria in the brine.

While this study has demonstrated that evaporation rates can be improved with the application of dyes/pigments, consideration of the above-mentioned factors needs to be balanced against the cost of application. A very crude cost analysis is presented for a 1 million L capacity pond. At a current cost of R882 per 25 g of methylene blue, a 200 ppm application would cost approximately R7 056 000 per full application. At a current cost of R785 per 500 g of TSB, a 20% application would cost R314 000. There are several additional factors which would have to be considered. For example, methylene blue may only require a single application, whereas nutrient supplementation may have to be replenished on a continuous basis. However, different sources of nutrients could be considered which may offer cheaper alternatives. Furthermore, depending on the depth of the ponds, infrastructure to enable aeration of the ponds would be required for the biological approach.

The observation that pigment production was not constitutive, where the isolates responded differently to the different brine compositions, advocates the need to develop a genetically engineered bacterium. The constitutive expression of pigment production in a host that does

not require nutrient supplementation would offer a huge advantage to the application of the biological approach. We propose investigating the development of the *P. maritimus* CP5-4 organism identified in this study as an expression host. We have already established an efficient and reproducible transformation protocol for CP5-4, which is in itself an important achievement since such capabilities for halophilic bacteria are lacking, and this could be applicable to other moderately halophilic bacteria. We would also recommend engineering CP5-4 to express and produce a dark pigment; for example, the violacein pathway (detailed in the introduction section) so as to achieve the maximum improvement in evaporation rate possible. The ability to develop such an engineered bacterium would not only be important for realising the initial aim of this project, it would also contribute significant advancements in the area of genetic manipulation in halophilic bacteria.

Another interesting prospect for further consideration relates to the potential for generating products of value such as food colouring, halophilic enzymes, compatible solutes, etc. from pigmented halophilic bacteria isolated from the brine. Halophilic microorganisms are becoming well understood as producers of polyhydroxyalkanoates (PHA), ectoines, biosurfacant/bioemulsifiers and several other chemicals. If the use of these organisms in enhancing brine evaporation rates can be successful then the benefits from having a natural bioreactor for the production of the above-mentioned products may help offset costs of brine storage and disposal.

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