Production of mycosporine-like amino acids from *Euhalothece* sp. for use as a photoprotectant

This work is submitted in complete fulfilment of the academic requirements for the degree of Master of Applied Sciences in the Department of Biotechnology and Food Science, Faculty of Applied Sciences at the Durban University of Technology, Durban, South Africa By

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DECLARATION

I hereby declare that the work reported in this dissertation titled "Production of mycosporine-like amino acids from *Euhalothece* sp. for use as a photoprotectant" and submitted to the Faculty of Applied Sciences, Department of Biotechnology and Food Sciences at the Durban University of Technology for a master's degree is my original work. I confirm that it has not been previously submitted for a degree at any Higher Education Learning Institution.

10/12/2025

Full name **Date**

Reference Declaration in Respect of a Master's Dissertation

I, Uwais Mahmud and, Dr Ismail Rawat, Dr Trisha Mogany, Prof Faizal Bux hereby declare that in respect of the following thesis: Production of mycosporine-like amino acids from *Euhalothece* sp. for use as a photoprotectant

1. as far as we know and can ascertain:
   (a) no other similar thesis exists;
2. all references as detailed in the thesis are complete in terms of all personal communications engaged in and published works consulted.

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DEDICATION

This thesis is dedicated to my beloved family (My Wife – Fathima, my Mom – Sadiyah and Sister – Imaan). Thank you for believing in me, thank you for supporting me and for being my pillars of strength.

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PREFACE

Uwais Mahmud, Trisha Mogany, Faizal Bux, Ismail Rawat (2025) Increasing the production of natural sunscreens from novel cyanobacteria, **Journal of Sustainable Chemistry and Pharmacy** (under review).

ABSTRACT

Protecting skin from harmful UV radiation is a well-recognized health imperative. Given that UV exposure is a primary cause of sunburn, premature skin aging, and significantly increases the risk of skin cancers including melanoma, the use of sunscreens has become a vital tool in public health strategies, leading to their widespread use. Whilst effective, there are concerns about the long-term effects of synthetic sunscreen ingredients that are driving the search for natural alternatives. Cyanobacteria are constantly exposed to short-wave ultraviolet (UV) radiation as they require solar energy to perform processes such as photosynthesis and nitrogen fixation. These microorganisms have developed multiple UV defence mechanisms to protect themselves from the higher amounts of UV radiation in the environment. Some of these strategies include mat formation, active repair mechanisms, accumulation of detoxifying enzymes and antioxidants, and synthesis of photo-protectants such as mycosporine-like amino acids (MAAs) (Rastogi et al., 2014). Mycosporine-like amino acids have gained a lot of interest in cosmetics and pharmaceutical industries since they act as natural photoprotectants and have shown to aid in overcoming rashes, pigmentation, aging as well as possess anticancer properties. Mycosporine-like amino acids are secondary metabolites produced by cyanobacteria in response to environmental stress such as salinity, temperature, high light irradiance and UV wavelengths. Cyanobacteria are a good source of MAAs, can be easily cultivated and requires a very short generation time, making them an environmentally friendly source of alternate photoprotectants. This study focuses on elucidating factors affecting MAA production using indigenous halophilic cyanobacteria *Euhalothece* sp. Light, salinity, and nitrogen concentration were optimised using response surface methodology to maximise MAA production. UV light irradiance was used to further induce expression of the protein. DNA was extracted and purified for gene expression analysis targeting the *mys* gene

cluster. Purified MAAs were tested for their antioxidant activity and their stability under a range of temperatures, pH and UV irradiance to assess their potential for commercial use.

Preliminary experiments confirmed the individual influence of abiotic factors, with light and salinity significantly impacting MAA production ($p = 0,0395$ and $p = 0,0009$ respectively). Using response surface methodology (RSM), the effects of light irradiance, salinity, nitrogen concentration, and UV exposure were optimized. A significant model ($p = 0,0099$) was developed, predicting optimal MAA production under 92 hours of UV exposure, nitrogen deficiency, and high salinity (90 or 120 g/L). Using 90 g/L salt was more cost-effective, requiring 28% less salt for a 5,36% reduction in MAA production. Molecular analysis through PCR confirmed the presence of the MAA biosynthetic gene cluster utilizing the OMT and DHQS and demonstrated its upregulation in response to UV exposure through qPCR. The UV-exposed culture showed amplification around cycle 14 while the control showed amplification around cycle 26. Purified MAAs demonstrated antioxidant activity and high stability across varying conditions over 48 hours. This included room temperature and neutral pH ($p = 0,02$), 4°C ($p = 0,046$), 40°C ($p = 0,041$) and UV irradiance ($p = 0,04$). These compounds showed excellent stability across various conditions, effectively maintaining their protective abilities. While their antioxidant capacity was measured (50,15%), it was clear that the main strength of MAAs lies in their strong UV protecting ability, making them highly suitable as active ingredients.

This study demonstrates the potential of MAAs from *Euhalothece* sp. as a highly stable, effective, naturally derived, and industrially viable product. The optimized culture conditions, validated by molecular techniques, coupled with the high stability and additional beneficial properties of MAAs, position them as a compelling natural resource. Mycosporine-like amino acids can significantly

advance photoprotection strategies and offer several other valuable applications in the cosmetic and pharmaceutical industries.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF EQUATIONS

CHAPTER 1: INTRODUCTION

1.1 Background

The cosmetics market is ever expanding due to its high demand for daily applications. On average, more than 50% of individuals use one or more skin care products daily. Sunscreens are used more frequently than other cosmetic products due to its protective functions. However, while these products are highly effective, the active ingredients, which are often synthetic, have become a source of concern. The use of synthetic photoprotectants found in these products can have unintended consequences. Compounds such as xybenzone, avobenzone, and octocrylene are commonly used in sunscreen formulas due to their broad UV spectrum protection. These compounds have been found to harmful to humans by causing allergic reactions, oestrogenicity and tumorigenic effects on human skin (Zirwas 2019; Barbaud and Lafforgue 2021). Due to the widespread use of sunscreens, these compounds have found their way into natural environments due to the result of runoff directly from the skin or through pollution (Lee *et al.* 2024). The presence of inorganic filters can cause devastating effects on marine environments and have been shown to affect organisms on a molecular level as well as an ecosystem level. Synthetic filters accumulate in the tissues of marine organisms and can subsequently be passed on through the food chain (National Academies of Sciences and Medicine 2022). This has potential to cause several effects including, damage to DNA, disruption in gene expression, and behaviour alteration and an increased mortality rate in marine life (Lozano *et al.* 2020). This has caused an increase in the demand for safer natural alternatives to these synthetic compounds.

Cyanobacteria are a rich source of bioactive compounds which exhibit a broad range of biological activities. These organisms are favoured not only for their production of bioactive molecules but also for the economic and sustainable nature of their cultivation. Given their minimal growth requirements, cyanobacteria are a highly cost-effective and attractive option for commercial applications. They can grow under various conditions and withstand a variety of stress factors. Being photosynthetic organisms, cyanobacteria are exposed to UV radiation which can cause significant cellular damage and inhibit photosynthesis. To protect themselves, species have evolved to produce mycosporine-like amino acids (MAAs) which are photoprotective compounds. This unique property gives them potential for use in the cosmetics industry as natural sunscreen agents. These secondary metabolites have broad protective roles allowing several possible applications due to their antioxidant activity, anti-inflammatory and anti-aging properties, such as inhibition of protein-glycation and inhibition of collagenase activity (Kageyama and Waditee-Sirisattha 2019). Mycosporine-like amino acids from the red alga *Porphyra umbilicalis*, have led to the successful commercialisation of two different natural sunscreens, Helioguard®365 and Helionori®. Both products are marketed as natural UV-A screening compounds capable of skin protection and protect against photo-aging (Chrapusta *et al.* 2017). *Porphyra umbilicalis*, however, also finds application in the food industry and requires a coastline for growth, which limits its scalability and creates a potential conflict of interest for its use as a raw material for other products. *Euhalothece* sp., a unicellular cyanobacterium, can be cultivated in closed systems offering more control and does not compete with existing food products. Additionally, optimization studies have not been fully explored to increase MAA production, indicating a significant opportunity for further research.

This study aimed to enhance the production of MAAs in *Euhalothece* sp. by optimizing key culture conditions, including light, salinity, nitrogen availability, and UV irradiance. To investigate the genetic basis for this increase, MAAs were extracted and purified for subsequent gene expression analysis, specifically targeting the *mys* gene cluster. Finally, the purified MAAs were characterized for their antioxidant activity and their stability across a range of temperatures, neutral pH conditions, and UV exposure times to thoroughly assess their potential for commercial use.

1.2 **Aim and Objectives**

Aim

Optimisation of mycosporine-like amino acids production from *Euhalothece* sp. through alteration of cultivation conditions for possible use in sunscreens.

Objectives

- Optimisation of visible light irradiance, salinity, and nitrogen concentration for production of mycosporine-like amino acids in *Euhalothece* sp. using response surface methodology (RSM).
- Determining the impacts of UV irradiance on mycosporine-like amino acids production through gene expression analysis.
- Assessing the pH, thermostability, and antioxidant potential of purified mycosporine-like amino acids for their cosmeceutical potential.

CHAPTER 2: LITERATURE REVIEW

2.1 Sunscreens

The public has become increasingly aware of the dangers of prolonged UV exposure which can cause sunburn, accelerated aging and the potential to increase the risk of sink cancer (melanomas) which has resulted in the increased use of sunscreens. Sunscreens can be categorised into three main types i.e. organic, inorganic, and a combination of both (Engler-Jastrzębska and Wilczyńska 2021). Inorganic photoprotective compounds may have potential to harm human health as a result of the compound penetrating the skin, causing allergic reactions (Serpone 2021), and the environment by washing off the body during swimming, causing damage to coral reefs (Moeller *et al.* 2021).

/10/2025

Organic compounds function to protect the skin from oxidative stress caused by free radicals and ROS that are associated with UV exposure. Ultraviolet rays are absorbed by moving electrons from one orbital to another, allowing the energy to be released as heat. Octocrylene, avobenzone, homosalate, octisalate, octinoxate, and oxybenzone are a few examples of compounds capable of absorbing UV-A and UV-B radiation (Gadgil *et al.* 2023). Due to the molecular structure of organic compounds, they function by creating a shield, allowing UV radiation to be deflected/reflected. Inorganic compounds such as zinc oxide (ZnO) and titanium dioxide (TiO_2) are formulated into nanoparticles due to their strong UV protection and ability to not react with skin. Inorganic filters protect the skin by absorbing high-energy UV photons and converting that energy into heat. This process begins when the sunscreen molecules absorb incoming UV radiation, causing their electrons to enter an excited energy state. To return to a stable state, the molecules release the energy as heat, which is then dissipated away from the

skin. This conversion and dissipation process prevents the damaging UV rays from penetrating the skin and causing cellular damage (Gholap *et al.* 2023). Even though these compounds are considered safe, the cosmetics industry constantly seeks to improve current formulations. (Pinto *et al.* 2022).

Organisms experiencing high solar exposure develop protection mechanisms which can act as natural sunscreens. This allows the organism to mitigate photodamage through reflecting, absorbing or dissipating UV radiation. Table 2.1 summarises various natural photoprotectants and their sources.

Table 2.1: Natural photoprotective compounds and their sources

Compound	Source	Reference
Biochrome pigments	Plants	(Mendes-Silva *et al.* 2020)
Melanin	Humans and fish	(Jalili *et al.* 2022)
Scytonemin	Cyanobacteria	(Sen and Mallick 2022)
Oil with pigments	Birds	(Faux and Logsdon 2022)
Minerals	Plants and algae	(Elrefaey *et al.* 2022)
Mycosporine-like amino acids	Corals, invertebrates and cyanobacteria	(Rice *et al.* 2023); (Hartmann *et al.* 2020)

Ultra-violet radiation can be classified into three groups respective to their wavelength: UV-A radiation (315-400 nm), UV-B radiation (280-315 nm) and UV-C radiation (100-280 nm). Although UV-C radiation is the shortest wavelength, it is not considered a threat since it is completely absorbed by the atmosphere. UV-B radiation poses the biggest threat, known to alter biologically important molecules in microorganisms (Rastogi *et al.* 2010). Exposure to UV radiation can impair several biological functions such as motility, growth, pigmentation, nitrogen fixation, phycobilisome assembly and CO_2 uptake (Sinha *et al.* 2002). Prolonged exposure results in oxidative stress and produces reactive oxygen species (ROS) which damages DNA, proteins,

lipids, and other macromolecules. Cyanobacteria have evolved several defence mechanisms in response to UV radiation exposure, including the internal accumulation of solutes for osmotic balance, antioxidant production, repair mechanisms, and the production of UV-absorbing pigments (Brito *et al.* 2017; Dunaway *et al.* 2018; Mishra *et al.* 2024). Synthesis of UV absorbing compounds such as MAAs, play a vital role in protecting the cell from photo-damage.

2.2 **Cyanobacteria**

Cyanobacteria (blue-green algae) are known to have exceptional adaptive abilities allowing them to thrive in various environmental conditions. They are prokaryotic, photosynthetic organisms that have vital roles in aquatic ecology, environmental protection and agriculture. Due to the diverse capabilities these organisms, they have several biotechnological applications in respective fields such as nutrient removal (Gonçalves *et al.* 2016), treatment of wastewater (Wu *et al.* 2018), and adsorption of heavy metals (De Philippis *et al.* 2011). Cyanobacteria have also been found to be producers of high-value secondary metabolites such as phycobiliproteins and vitamins (Xiong *et al.* 2017; Khatoon *et al.* 2018). Advances in technology have allowed the application of bioengineering of cyanobacteria allowing for the production of biofuels, including ethanol (5,50 g/L) (Gao *et al.* 2012), isoprene (1,26 g/L) (Gao *et al.* 2016), polyhydroxybutyrate (1,16 g/L) (Rueda *et al.* 2020), and 3-hydroxypropionic acid (837,18 mg/L) (Wang *et al.* 2016). Due to their susceptibility to genetic manipulation and fast-growing rate, cyanobacteria are highly desirable compared to other photosynthetic microbes (Sun *et al.* 2018; Santos-Merino *et al.* 2019).

Cyanobacteria have several defence mechanisms to ensure their survival against photosynthetically active radiation (PAR) and the harmful effects of UV radiation (Pathak *et al.* 2019; Rezayian *et al.* 2019; Mishra *et al.* 2024). The 'line of defence' against photodamage can be

broken down into four parts, with non-photochemical quenching (NPQ) being the first and most vital defence. The photoactivation of photoprotective proteins such as orange carotenoid protein (OCP), soluble phycobiliproteins (PBPs), high light induced proteins (HLIPS), and fluorescence recovery proteins (FRP) enables NPQ to dissipate excess solar energy into heat energy (Kerfeld 2009; Kirilovsky 2010; Pathak *et al.* 2019). The second line of defence is the activation of antioxidative systems which can be enzymatic or non-enzymatic (Singh *et al.* 2013). Ultra-violet radiation has been found to precipitate in the formation of signalling molecules including ROS and hydrogen peroxide (H_2O_2) by activating the intricate pathways primarily linked to their photosynthetic machinery such as PSI and PSII. These pathways contain antioxidative and enzymatic scavengers such as superoxide dismutase (SOD), catalase, oxidoreductase, thioredoxins, peroxiredoxins, and alkyl hydroperoxide reductase subunit C (AhpC) (Babele *et al.* 2015). Non-enzymatic antioxidants include carotenoids, ascorbic acid (vitamin C), reduced glutathione, and tocopherols (vitamin E) (Singh *et al.* 2010; Singh *et al.* 2013; Radyukina *et al.* 2019).

When cyanobacteria are exposed to UV radiation for extended periods of time, the organism engages in the third line of defence which is the production of UV absorbing compounds (Rastogi *et al.* 2014b; Pathak *et al.* 2019). The synthesis of these compounds protect the cells from UV induced photodamage by dissipating or reflecting the UV radiation from cellular components (Zhao *et al.* 2025). Mycosporine-like amino acids and scytonemin are highly effective UV absorbing compounds protecting the organism against both UV-A and UV-B (Richa and Sinha 2011; Pathak *et al.* 2019). These photoprotective compounds can also be found extracellularly and could shield the organism from incoming UV radiation. Lastly, the incoming damage to DNA caused by UVR is repaired and new proteins are synthesised as a response. It has also been noted that cyanobacteria will employ a specific DNA repair mode known as base excision in which damaged

or inappropriate bases are excised from DNA as a free base in order to protect the cell (Pathak *et al.* 2019). Some cases may result in irreversible damage, which may cause the organism to exercise selective cell death, or programmed cell death (PCD) as a last resort in order to maintain viability of the larger population (Rastogi *et al.* 2014a).

2.3 **Mycosporine-like amino acids**

Mycosporine-like amino acids (MAAs) are small (<400 Da), water-soluble, colourless photoprotectant molecules with high molar extinction coefficients, ranging from 28,000 to 60,000 M^{-1} cm^{-1} (Rastogi *et al.* 2020). Mycosporine-like amino acids disperse harmful UVR and release it as heat energy without the formation of reactive photoproducts. The ability of MAAs to prevent damage to the cyanobacterial cell is due to their absorption spectra which falls between 310-365 nm, covering most of the UV spectrum (295-400 nm). Given these robust UV-absorbing properties, MAAs have gained significant interest in their potential benefits in human applications. Mycosporine-like amino acids have been reported to act as a natural sunscreens and contain anti-aging effects due to their high UV absorbing property and activators of cells proliferation (Kageyama and Waditee-Sirisattha 2019). In addition, they exhibit anti-inflammatory activity, inhibition of protein-glycation, inhibition of collagenase activity and are stimulators of skin renewal (Fried *et al.* 2022; Saraiva *et al.* 2023). They have been found to effectively block the formation of thymine-dimers, a photolesion produced by UV radiation in sunlight and is considered as a potential factor causing skin cancer (Fuentes-Tristan *et al.* 2019; Pliego-Cortés *et al.* 2019). Additionally, they are known to be stable at wide ranges of temperatures and pH due to their physico-chemical characteristics however this varies based on species and type of MAA (Geraldes and Pinto 2021; Raj *et al.* 2021). The photodegradation and photophysical properties of MAAs make them excellent cosmeceutical ingredients for skincare, cosmetics, and pharmaceutical

products (Pangestuti *et al.* 2018; Hosseinabadi *et al.* 2022). All MAAs possess the same base chemical structure, a cyclohexanone or cyclohexenimine chromophore with a nitrogen substituent. Differences in the absorption spectra of MAAs are due to variations in the attached side groups and nitrogen substituents (Bhatia *et al.* 2011). There are two types of classifications of MAAs these being oxo-MAAs and imino-MAAs. Aminocyclohexenone (oxo-MAAs) are monosubstituted, and aminocyclohexene (imino-MAAs) are disubstituted. Monosubstituted MAAs contain a ketone functional group and single amino acid derivative while disubstituted MAAs contain an imine functional group, and two amino acid substituents attached to the imine nitrogen. Common types of MAAs found in cyanobacteria include mycosporine-glycine, mycosporine-2-glycine, porphyra-334 and shinorine (Llewellyn *et al.* 2020).

2.4 **Biosynthesis of MAAs**

The main pathway for the biosynthesis of MAAs and their functional genes have been elucidated in several cyanobacteria. A cluster of four main genes responsible for MAA biosynthesis includes *mysA*: 3-dehydroquinate synthase (3-DHQS), *mysB*: O-methyltransfersase (OMT), *mysC*: ATP-grasp protein and *mysD*: ligase enzyme (Ddl). In the shikimate pathway (Figure 1) 3-dehydroquinate synthase (3-DHQS) together with O-Methyltransferase (OMT) catalyzes the conversion of dehydroquinate (DHQ) to desmethyl 4-deoxygadusol (DDG), which is the core structure of MAAs (Geraldes *et al.* 2020b). Free amino acids are ligated to this core to produce the different MAAs (Bhatia *et al.* 2011; Mogany *et al.* 2022). The shikimic acid pathway was confirmed as the main pathway for MAA synthesis through the use of feedback inhibitors such as tyrosine and glyphosate. (Llewellyn *et al.* 2020). The exogenous supply of these inhibitors

suppressed MAA biosynthesis concluding that synthesis of MAAs branches off from the intermediate (4-deoxygadusol). The pentose phosphate pathway is associated with MAA production, however, is not a major pathway involved in production (Karl *et al.* 2018; Raj *et al.* 2021).

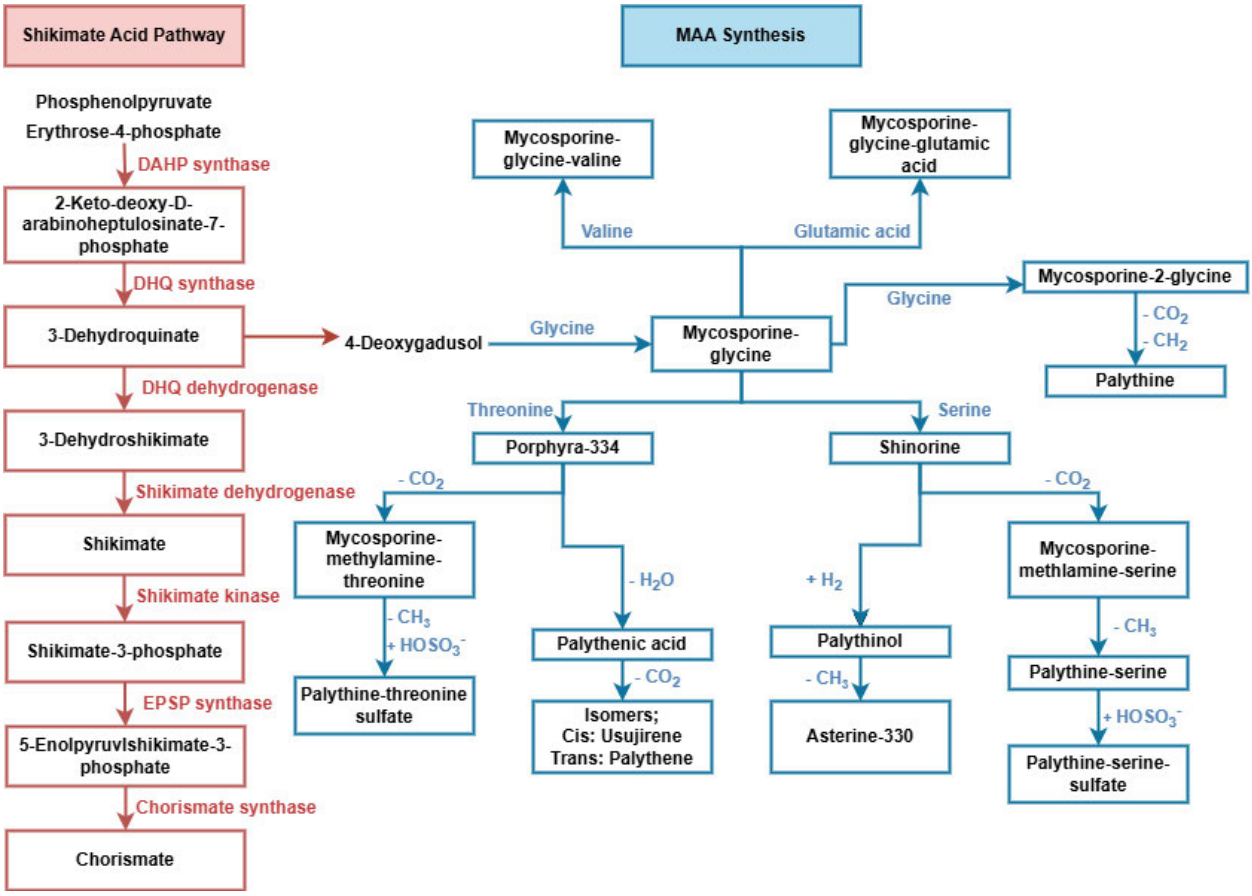

Figure 1: Shikimate acid pathway branch off point associated with MAA biosynthesis, and the combinations of amino acids with the parent compound forming a variety of structurally similar MAAs. Adapted from (Fuentes-Tristan *et al.* 2019).

2.5 **Diversity and distribution of MAAs**

To date, 12 aminocyclohexanone-type (Figure 2) and 20 aminocyclohexene imine-type (Figure 3) MAAs have been identified. When accounting for sulphated and derivatized forms, the total number of discovered MAAs exceeds 40 (Görünmek *et al.* 2024). Approximately 36% of the total number of MAAs identified have been acquired from Antarctic marine species (Wada *et al.* 2015). Environmental factors and the geographical position are known to affect the type and diversity of MAAs present within the specified organism. Mycosporine-like amino acids can be found in various other organisms including bacteria, lichens, vertebrates, and invertebrates (Mishra *et al.* 2024). Algae and cyanobacteria are the main producers of these secondary metabolites, with several MAAs identified from these organisms, including shinorine and porphyra-334 (Table 2.2) which were the first set of MAAs to be discovered and isolated form marine cyanobacteria (Sun *et al.* 2020; Peng *et al.* 2023). Mycosporine-like amino acids are widely distributed among cyanobacteria, including unicellular, heterocystous, and non-heterocystous filamentous species (Kumar *et al.* 2019). Species from hypersaline habitats, such as the genera *Halothece* and *Euhalothece*, are known to produce a variety of MAAs, including *Euhalothece* 362 and mycosporine-2-glycine (Table 2.2) (Patel *et al.* 2019; Bairwa *et al.* 2021). Cyanobacterial species such as *Gloeothece* and *Anabaena*, isolated from the desert have also shown to be producers of MAAs such as mycosporine-glycine, shinorine, porphyra-334, and palythine-serine (Table 2.2) (Bhardwaj *et al.* 2019).

Figure 2: Structure of aminocyclohexanone-type MAAs

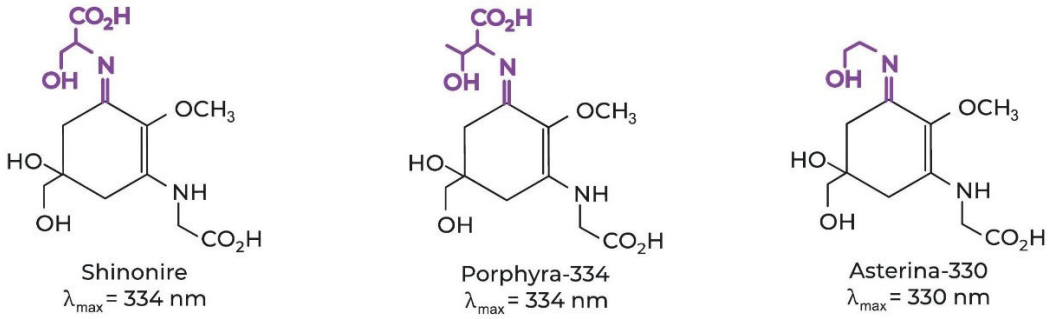

Figure 3: Structure of aminocyclohexene imine-type MAAs

Table 2.2: An overview of different MAA producing cyanobacteria (Jain *et al.* 2017)

Organism name	MAAs
Order: Synechococcales	
Synechocystis sp. PCC 6803	Mycosporine-343, M-tau, Dehydroxyl-usujirene
Order: Chroococcales	
Gloeocapsa sp. CU-2556	Shinorine, Mycosporine-307
Aphanothece halophytica	Mycosporine-2-glycine
Gloeocapsa sp.	UV-320, Mycosporine-glycine
Euhalothece sp.	*Euhalothece* 362, Mycosporine-2-glycine
Microcystis aeruginosa	Shinorine and Porphyra

Table 2.3: An overview of different MAA producing cyanobacteria (Jain *et al.* 2017)

Organism name	MAAs
Order: Oscillatoriales	
Arthrospira sp. CU2556	Mycosporine-glycine
Lyngbya sp. CU2555	Palytine, Asterina, Mycosporine-312
Leptolyngbya sp. and *Phormidium* sp.	MAA-332 nm
Lyngbya cf. aestuarii and *Microcoleus chthonoplastes*	MAA no. 3 and Shinorine
Microcoleus sp. and *Microcoleus chthonoplastes*	Shinorine
Oscillatoria spongelidae	Mycosporine-glutamic acid-glycine, Mycosporine-glycine, Usujirene, and Palythene
Trichodesmium sp.	Asterina 330, Shinorine, mycosporine-glycine, Porphyra-334, and Palythene
Order: Nostocales	
Anabaena sp.	Shinorine
Anabaena doliolum	Mycosporine-gly, Porphyra-334, and Shinorine
Anabaena variabilis PCC 7937	Shinorine, Palythine-Serine, and mycosporine-glycine
Nostoc sp.	Mycosporine-312 and mycosporine-330
Nostoc commune var. Vaucher	Extracellular MAAs
Nostoc commune	Shinorine
Scytonema sp.	Shinorine, MAA-315, stanierine, asterina-330, and mycosporine-13
Nostoc commune	Shinorine
Nostoc punctiforme ATCC 29133	Shinorine
Nostoc commune sp.	Glycosylated MAAs
Nostoc sp. HKAR-2 and HKAR-6	Shinorine and Porphyra-334
Anabaena and *Nostoc* sp.	Shinorine
Synechocystis sp., *Scytonema* sp., *Nostoc* sp., *Gloeocapsa* sp. and *Glorocapsopsis*	Shinorine, Porphyra-334, Mycosporine-glycine, and Palythiol
Nostoc commune	Glycosylated MAAs
Scytonema sp., *Lyngbya* sp. and *Nostoc* sp.	Mycosporine-taurine, Mycosporine-glycine, Asterina, and Palythiol
Nodularia baltica, Nodularia harveyana and *Nodularia spumigena*	Shinorine and Porphyra-334
Aphanizomenon flos-aquae	Porphyra-334
Chlorogloeopsis PCC 6912	Mycosporine-glycine and Shinorine

2.6 **Factors affecting mycosporine-like amino acid production**

A key factor in the induction of MAAs is exposure to UV radiation, which highlights their important photoprotective capabilities (Oren and Gunde-Cimerman 2007). However, in some organisms such as *Sphaerospermopsis torques-reginae*, MAAs can be expressed continuously due to the exposure of high levels of UV radiation, which is a constant, unavoidable factor of their ecological niche (Geraldes *et al.* 2020a; Geraldes *et al.* 2020b). The chemical makeup of MAAs directly dictates their physical and chemical properties (Woolley *et al.* 2018). Minor variations, particularly in the position of substituents on the core ring structure, define the unique functionality of each MAA. These properties, determine how effectively they perform various roles, allowing MAAs to play additional roles beyond photoprotection. This section further explores the induction of MAAs and the various roles they exert in response to environmental stressors, such as light and UV stress, osmotic stress, nutrient availability, desiccation stress, and thermal stress. The interlinking nature of these factors will also be discussed, as they often do not act in isolation but rather in combination, influencing MAA synthesis and function.

2.6.1 **Light and UV irradiance**

The ability of cyanobacteria to absorb different wavelengths of light, have been extensively studied. In response to the specific wavelength of light they receive, cyanobacteria possess the ability to alter their photosystems (PSI, PSII) and phycobilisome (PBS) components. This adaptive mechanism enables them to absorb light efficiently across the wavelength spectrum (Montgomery 2017). Korbee *et al.* 2005, demonstrated the effect of different light qualities, including white (400 – 700 nm), blue (380 – 500 nm), green (495 – 570 nm), yellow (570 – 580 nm) and red light (620

– 750 nm) on MAA production. It was observed that blue light accumulated the highest concentration of MAAs with white light showing a comparable concentration. Further observations showed that blue light had the lowest photosynthetic efficiency and growth rate while higher values were found in red and white light. The higher MAA concentration observed under blue light is a consequence of environmental stress. This acts as an adaptive response where the cyanobacterium prioritizes photoprotection at the cost of a temporary decline in photosynthetic ability and in growth. However, under white or red light, the resulting reduction in light stress allows for a more balanced and efficient absorption of light by the photosystems, lowering the demand to produce MAAs and allowing for a more efficient allocation of cellular resources towards growth. Similarly, different light wavelengths favoured the production of different MAAs. Blue light favoured production of MAAs such as porphyra-334 and palythine, while white, green, yellow and red light favoured the accumulation of shinorine only. Llewellyn *et al.* 2020, conducted a similar experiment showing the upregulation of the *mys* gene cluster under UV and far-red light in comparison to white light. MAAs can also be induced under high photosynthetically active radiation (PAR) (100 – 120 μmol photon m^{-2} s^{-1}), UV-A and UV-B radiation.

Photosynthetically active radiation (PAR) is essential for photosynthetic organisms to convert light into chemical energy. This requirement also means they are frequently exposed to high levels of UV radiation and require sufficient protection. Experiments with light/dark cycles have revealed that photosynthetically active radiation triggers MAA production. Specifically, MAA concentrations were shown to increase during light periods, while they remained constant throughout dark periods (Neale *et al.* 1998). Exposure to UV-B had the most significant increase effect in comparison to the other wavelength ranges (Sinha *et al.* 2001). The intensity of UV

radiation also influences the concentration and composition of MAAs present within a cell (Sinha and Häder 2008).

2.6.2 **Osmotic stress**

In hypersaline environments, cyanobacteria accumulate MAAs in higher concentrations, since the high saline levels cause dehydration and the formation of ROS. The high concentration of MAAs thus acts to restore osmotic balance by storing MAAs in the intracellular space in hypertonic environments (Rosic 2019). Oren (1997), reported a halotolerant cyanobacterium having a high concentration of MAAs, making up 3% of the cells' wet weight. When the salt concentration of the environment was decreased, a significant reduction in MAAs were observed (Oren 1997). The production of MAAs can be induced under high saline concentrations which had been demonstrated by Waditee-Sirisattha *et al.* 2014. Freshwater organisms have been observed to accumulate MAAs in smaller concentrations since the environment does not possess high salt concentration (Chrapusta *et al.* 2017; Mogany *et al.* 2018).

2.6.3 **Nutrient availability**

The concentrations of nutrients available, particularly nitrogen, have been reported to affect the biosynthesis of MAAs. In studies conducted by Geraldes *et al.* 2020b and Llewellyn *et al.* 2020, it has been reported that an increased nitrogen availability has the potential to increase MAA biosynthesis due to the upregulation of genes responsible for nitrogen fixation. This upregulation was achieved through UV and far-red light exposure. An increased nitrogen concentration may influence the increased production of MAAs since nitrogen is added to 4-deoxygadusol, the core structure of MAAs (Sen and Mallick 2021). The opposite effect was observed in a study conducted by Litchman *et al.* 2002, where it was demonstrated that nitrogen deficiency in two species of

dinoflagellates showed increased MAA production. The nitrogen starved cells displayed a higher concentration of MAAs compared to when the nutrient is available in abundance. Hence the effect of nitrogen concentration on MAA production is dependent on the species and its mode of action is still unclear (Inabe *et al.* 2021).

2.6.4 **Desiccation Stress**

Cell damage caused by desiccation affects DNA and proteins present in the cytoplasm and cell membrane fluidity through the direct removal of moisture or oxidative stress. Very few studies have been conducted to demonstrate the effect of desiccation on MAA production. However, some mechanisms used by cyanobacteria to overcome the drying effect are the production of antioxidants and polysaccharides. In a study conducted by Gorbushina *et al.* 2003, it was demonstrated that desiccation stress significantly induces MAA production, and a drastic decrease in MAA concentration when the environment is rehydrated. It is thought that these compounds protect the cell by acting as an antioxidant, quenching ROS and modifying the structure of the extracellular matrix. Olsson-Francis *et al.* 2013, further suggested that MAAs form an extracellular sheath protecting the cell from desiccation. Joshi *et al.* 2018 subjected cyanobacteria *Leptolyngbya* sp. to desiccation stress and UV irradiance resulting in an increase in MAA concentration which prevented desiccation and protected the cells.

2.6.5 **Thermal stress**

Very few studies have been conducted which investigated the effect of thermal stress on MAA production. Michalek-Wagner 2001, demonstrated that MAAs were upregulated under thermal stress and when combined with UV irradiance, there was a significant increase in MAA concentration. Some studies suggest that thermal stress has no influence on the production of

MAAs, however, this can be attributed to differences between species (Portwich and Garcia-Pichel 1999; Singh *et al.* 2008).

2.7 **Applications and uses**

2.7.1 **Antioxidant activity**

Exposure of cyanobacteria to UV radiation for a prolonged period can result in the formation of free radicals. These molecules are highly reactive and are capable of damaging proteins, lipids, carbohydrates, and nucleic acids through non-enzymatic oxidation of these essential biomolecules (Gómez *et al.* 2023). Cyanobacteria have several defence mechanisms in place which quench the ROS molecule. These antioxidants fall within two broad categories, non-enzymatic antioxidants such as MAAs, and enzymatic defence mechanisms (Rai *et al.* 2021). Mycosporine-like amino acids are capable of dissipating excess UV energy that has been absorbed, into heat energy aiding in the protection of vital biomolecules against oxidative stress. This is achieved through a process called ultrafast non-radiative decay. When MAA molecules absorb a UV photon, their electrons are rapidly excited to a higher energy state. Instead of releasing this energy as damaging light or initiating harmful chemical reactions, the excited MAA undergoes internal conversion. This non-radiative process involves rapid conformational changes, such as rotations within the molecule's conjugated ring structure. As the molecule vibrates, the vibrational energy is transferred to the surrounding solvent molecules, increasing kinetic energy, leading to its dissipation as heat (Wada *et al.* 2015; Woolley *et al.* 2018). Beyond their primary role as efficient UV filters, many MAAs also exhibit significant antioxidant capabilities, offering an additional layer of cellular protection. Studies have demonstrated that MAAs, as a class, contribute to antioxidant defence, with variations in efficacy observed among different MAA compounds. A study conducted by de la

Coba *et al.* 2009, showed that the MAA mycosporine-glycine had a higher antioxidant activity when compared to shinorine and porphyra-334. Similarly, Suh *et al.* 2014, reported that mycosporine-glycine showed 8 times more antioxidant activity than l-ascorbic acid. While Athukorala *et al.* 2016, demonstrated that reducing activity and oxygen radical absorption capacity (ORAC) of MAAs such as shinorine, porphyra-223, and palythine amongst others, showed activity equivalent to l-ascorbic acid. In another study, Tarasuntisuk *et al.* 2019, demonstrated that due to the structural symmetry of mycosporine-2-glycine, it can stabilise its delocalised structure, and exhibit much higher antioxidant activity. This property is significant when comparing it to other naturally occurring antioxidants. For instance, Vitamin C is most commonly found in land plants, which when eaten by herbivores, are transferred into their system, hence vitamin C functions in diverse organisms as a general antioxidant (Paciolla *et al.* 2019).

In contrast, some antioxidants, such as anthocyanin, are produced as more unique secondary metabolites. Anthocyanin is synthesized in the tissues of specific plants primarily under stress conditions, and its various derivatives are thought to have developed in response to abiotic or biotic environmental factors (Li and Ahammed 2023). This highlights that some antioxidant systems are general, while others are specific, with unique combinations forming a direct adaptive response to environmental pressures.

2.7.2 **Protection against DNA damage**

Exposure to UV radiation for extended periods of time can cause several skin related conditions (Punchakara *et al.* 2023). The effects of UV radiation most commonly result in the formation of thymine dimers which produces other harmful products such as cyclobutene pyrimidine dimers (CPDs), and pyrimidine (6-4) pyrimidone photoproducts (6-4 PPs). Both by-products are highly

toxic and possibly mutagenic to healthy cells that are actively replicating (Kumari 2018; Wu *et al.* 2023). Generally, CPDs are considered more prevalent and a major contributor to UV-induced DNA damage than 6-4 PPs, particularly under UVB exposure. While CPDs can form under UVA radiation, their production rate is significantly lower compared to UVB-induced formation. The rate of photoproduct production directly correlates with the level of harm to cells; a higher rate means higher DNA damage, potentially overwhelming the cell's natural repair mechanisms (Pfeifer 2020). This increases the likelihood of errors during DNA replication, leading to mutations, impaired cell function, and potentially initiating processes that can result in skin cancer or premature aging. Misonou *et al.* 2003, demonstrated that several MAAs such as shinorine, porphyra-334 and palythine are capable of blocking DNA damage as a result of UV radiation. In a study conducted by Cheewinthamrongrod *et al.* 2016, mycosporine-2-glycine was shown to protect DNA damage through direct UV absorption and energy dissipation, and ROS scavenging. Furthermore, the MAA mycosporine-taurine contains structural similarities to that of mycosporine-glycine, which was found to contribute to its effectiveness in DNA protection (Zhang *et al.* 2007).

2.7.3 **Anti-inflammatory activity**

Inflammation, often triggered by the formation of reactive oxygen species (ROS), is a factor that can induce stress on the immune system. Mycosporine-like amino acids (MAAs) possess significant anti-inflammatory activity, by effectively scavenging ROS and modulating key inflammatory signalling pathways. In a study conducted by Becker *et al.* 2016, the anti-inflammatory effect of MAAs were demonstrated, more specifically shinorine and porphyra-334. Both these MAAs were able to play a role in nuclear factor kappa B (NF-κB) activity, which is responsible for complex signalling and maintaining homeostasis (Albensi 2019). Oxidative stress

induces NF-κB activity significantly, making it a target pathway when pursuing anti-inflammatory compounds (Asehnoune *et al.* 2004). Tarasuntisuk *et al.* 2019, tested mycosporine-2-glycine in vitro and was shown to exhibit anti-inflammatory properties by reducing the formation of nitric oxide from lipopolysaccharides. In another study conducted by Suh et al., (2014), MAAs were tested on HaCaT under exposure to UV irradiance. This resulted in a decrease in COX-2 mRNA which regulates biological processes and are a symptom of inflammation in the skin (Roelofs *et al.* 2014; Suh *et al.* 2014).

2.7.4 **Photo-aging and wound healing ability**

Mycosporine-like amino acids demonstrate potential beyond photoprotection, with implications for mitigating photo-aging and promoting wound healing. During the aging process, advanced glycation end products (AGEs) are known to occur. These molecules occur naturally and are known to cause intracellular inflammation and oxidative stress once bound to the cell membrane receptors (Hechtman 2020). The increase in AGEs can lead to further health risks including neurodegenerative diseases, diabetes, liver disease cardiovascular complications and cancer (Rungratanawanich *et al.* 2021). The damage done to collagen by AGEs can be significant enough to cause the total loss of elasticity of collagen, increasing skin associated complications (Görünmek *et al.* 2024). In a study conducted by Orfanoudaki *et al.* 2020, MAAs were shown to inhibit the formation of AGEs and inhibit collagenase activity during the healing process. Hartmann *et al.* 2015 demonstrated the effectiveness of specific MAAs such as shinorine, porphyra and palythine isolated from cyanobacteria against skin aging by suppressing collagenase activity. Schmid *et al.* 2003 formulated mycosporine-like amino acids isolated from *Porphyra umbilicalis* into a cream that improved skin quality by 10% and reduced wrinkles by 20%. Mycosporine-like amino acids such as shinorine, mycosporine-glycine, and porphyra have also demonstrated their wound

healing properties by accelerating the focal adhesion kinase (FAK) pathway. The FAK pathway is involved in the regulation of cell proliferation and cell survival, increasing the rate at which wounds heal with acceleration in the pathway (Choi *et al.* 2015).

2.7.5 **Potential as anti-cancer agents**

Torres *et al.* 2004 tested MAAs extracted from a photobiont, and demonstrated to effectively prevented the formation of thymine dimers, which in turn inhibited cell destruction and erythema caused by UV overexposure, highlighting the potential of MAAs in skin cancer prevention. In a study highlighting MAAs' role in skin protection, Kim *et al.* 2014, tested MAAs extracted from seaweed on UVB-irradiated HaCaT cells. The results showed that the MAA extract notably increased the viability of cells that had been damaged, and simultaneously enhanced the apoptosis of these compromised cells, suggesting a protective and quality-control mechanism.

Table 2.4: Different bioactive properties of MAAs adapted from (Punchakara *et al.* 2023)

Compound	Bioactive effect	Source
Mycosporine-glycine	Antioxidant Property	*Palythoa tuberculosa, Lichina pygmaea, Chlamydomonas hedleyi*
	Anti-inflammatory Activity	*Chlamydomonas hedleyi*
	Anti-aging	*Chlamydomonas hedleyi*
	Wound Healing Property	*Chlamydomonas hedlyei, Porphyra yezoensis*
Shinorine	Anti-inflammatory Activity	*Chlamydomonas hedleyi*
	Anti-aging	*Chlamydomonas hedleyi*
	Anti-adipogenic Effect	*Porphyra denta*
	Wound-healing Property	*Chlamydomonas hedlyei, Porphyra yezoensis, Bostrychia scorpiodes*
	Anti-viral	PubChem database

Table 2.5: Different bioactive properties of MAAs adapted from (Punchakara *et al.* 2023)

Compound	Bioactive effect	Source
Porphyra-334	Anti-aging	*Chlamydomonas hedleyi*
	Anti-adipogenic Effect	*Porphyra dentata*
	Wound-healing Property	*Chlamydomonas hedlyei, Porphyra yezoensis*
		Bostrychia scorpiodes
Usujirene	Antioxidant Property	*Porphyra yezoensis*
Asterina-330	Antioxidant Property	*Gelidium corneum*
Palythine	Antioxidant Property	*Gelidium corneum*
Bostrychine-B	Wound-healing Property	*Bostrychia scorpiodes*
Mycosporine glycine-valine	Anti-viral	PubChem database

The diverse characteristics of MAAs have allowed for possible non-medical applications as additives to protect plastics, paints and varnishes against UV radiation consisting solely of natural compounds. These materials are biocompatible, photo-resistant, thermo-resistant and provide an efficient protection against UV radiation (Fernandes *et al.* 2015). With the expansion of the cosmetics industry and increasing market, the application of MAAs is highly promising as an efficient UV blocker in sunscreen formulations.

CHAPTER 3: OPTIMISATION OF CULTURE CONDITIONS USING RESPONSE SURFACE METHODOLOGY (RSM)

3.1 Introduction

Sunscreens have become an essential product that sees use daily. However, with the increasing awareness of the harmful effects of synthetic compounds to human health and eco-systems, eco-friendly products have become more attractive to the average consumer (Galamgam *et al.* 2018; Schneider and Lim 2019; Santiesteban-Romero *et al.* 2022). With the demand for these natural compounds increasing, MAAs have been identified as a promising alternative to synthetic chemicals in sunscreens, having the most beneficial effect on skin and the environment (Suh *et al.* 2014; Hartmann *et al.* 2015; Sen and Mallick 2021). Since cyanobacteria are efficient producers of MAAs, it is driving the cosmetics industry to invest in the development of these compounds (Ariede *et al.* 2017). Mycosporine-like amino acids have been used in mixtures which coat the skin of fruits and vegetables protecting them against scalding while also being safe and biodegradable (Pedrosa *et al.* 2022). Since it is essential to investigate parameters affecting MAA production, optimisation of culture conditions is an essential step to enhance MAA production in producing organisms.

Studies have shown that culture conditions significantly impact MAA production in cyanobacteria. One of the primary factors enhancing MAA synthesis is exposure to UV irradiance, as organisms naturally upregulate these photoprotective compounds in response to harmful UV radiation (Figueroa *et al.* 2003). Additionally, nutrient availability in particular, nitrogen, plays a crucial role, influencing both the concentration and composition of MAAs. However, in some instances

nitrogen limitation can sometimes induce higher MAA accumulation as these compounds can serve as a nitrogen reserve. (Gharib *et al.* 2020; Hosseinabadi *et al.* 2022).

However, it's essential to recognize the specific factors and the degree of influence on MAA production they possess. This can vary considerably due to the environmental adaptation of different cyanobacterial species. An organism isolated from a high-salinity environment might respond differently to salt stress in terms of MAA production compared to one from freshwater. This inherent variability necessitates a tailored approach to optimizing production for specific strains.

Therefore, the objective set out was to investigate how various factors influenced MAA production both individually and in combination. This comprehensive approach, beyond single-factor analysis, allowed investigation of potential synergistic effects between different environmental parameters. The goal was to determine the optimal culture conditions for enhanced MAA production. Response Surface Methodology (RSM) was employed to achieve this. This experimental design and analysis process enabled the refinement of factor concentrations, leading to an accurate model that could predict and identify the most efficient combination of culture conditions for maximizing MAA yield. With the growing availability of cyanobacterial genome sequences, it's become significantly simpler to investigate the biosynthetic pathways involved in MAA synthesis. Comparative analysis on the available genome sequences has allowed for the elucidation of four genes present in the *mys* gene cluster (*mysA*, *mysB*, *mysC* and *mysD* or *mysE*) (Llewellyn *et al.* 2020). This chapter further looks at confirming the presence of the DHQS (*mysA*) and/or the OMT (*mysB*) genes, due to their key involvement in MAA synthesis. The *mys* genes were identified using PCR techniques and the upregulation of the gene under UV irradiance

was compared to standard light. This served as a secondary confirmation since MAAs are photoprotective compounds.

3.2 **Materials and Methods**

3.2.1 **Culturing of isolate**

An euryhaline cyanobacterium, *Euhalothece* sp. was cultivated according to Mogany *et al.* 2019. Briefly, the isolate was grown in standard BG11 media made up with 120 g/L sea water, in 1 L Erlenmeyer flasks with a light:dark period of 16:8 hours under the luminescence of 18 W Sylvania GroLux bulbs (75 µmol photon m^{-2} s^{-1}). Biomass was determined by optical density at 750 nm and gravimetrical on the days of extraction.

3.2.2 **Optimisation of culture conditions**

Optimisation of light, salinity and nutrients were conducted under standard conditions (BG11 in 120 g/L salt at 75 µmol photon m^{-2} s^{-1}). Light optimisation used an altered irradiance level of 150 µmol photon m^{-2} s^{-1} for 7 days with daily extraction of MAAs. Salinity optimisation was conducted at 0 g/L, 30 g/L, 60 g/L, 120 g/L and 150 g/L salt for 7 days with daily MAA extractions. Nitrogen optimisation utilized 0 g/L, 0,25 g/L, 0,5 g/L, 1 g/L, 1,5 g/L, and 2 g/L $NaNO_3$ for 7 days. The isolate was cultivated under UVB lamps (UVP34-0039-01, 15 W, Sylvania). The induction experiment occurred over a 3-day period, with extractions occurring at 0, 24, 30, 48, 54, and 72 hours.

3.2.3 **Extraction of mycosporine-like amino acids**

Mycosporine-like amino acids were extracted according to Chandra *et al.* 2020. Briefly, samples (10 ml) were centrifuged at 3500 x *g* for 5 minutes at 4°C using a Hermle Labortechnik GmbH

Z 326 K centrifuge. The pellet was washed in 6 ml distilled water and centrifuged using the Eins-Sci E-C15-24.2PR microcentrifuge at 10000 x *g* for 5 minutes at 4°C. The pellet was resuspended in 1 ml of methanol and incubated overnight at 4°C. The crude sample was centrifuged at 10000 x *g* for 5 minutes at 4°C and the supernatant was read using a full spectrum scan from 200-800 nm in the UV/Vis spectrophotometer using UV cuvettes (BrandTech 759150 Disposable UV-Cuvettes, Semi-Micro). Distinctive peaks between the range of 310-365 nm were noted indicating the presence of MAAs. The samples were dried in a vacuum desiccator overnight for purification.

3.2.4 **Partial purification of mycosporine-like amino acid**

Partial purification of MAAs was conducted according to Chandra *et al.* 2020, with slight modifications. Briefly, 600 µl sterile distilled water was added to the dried sample and centrifuged at 10000 x *g* for 5 minutes at 4°C. The supernatant was then transferred into a new Eppendorf tube, to which 50 µl chloroform was added. The mixture was vortexed untill the solution was mixed thoroughly, and centrifuged at 10000 x *g* for 5 minutes at 4°C. The upper aqueous layer of sample was carefully pipetted out and filtered through a 0,22 µm nylon syringe filter to remove any remaining impurities. The supernatant was read in the UV/Vis spectrophotometer between the range of 200 - 400 nm.

3.2.5 **Response surface methodology (RSM) design**

Central composite design (CCD) was used to determine the optimum concentrations of salinity, nitrogen and light irradiance for maximum MAA production. In this design, each nutrient had three levels i.e., low, centre, and high that corresponded to the coded values – 1, 0, and +1, respectively. In this experiment, six replicates of the centre points and fourteen non-centre points resulting in a total number of twenty experiments were conducted.

Table 3.1: Actual CCD design looking at the low (-1), centre (0) and high (+1) points, consisting of fourteen non-centre points and six replicates

		Factor 1	Factor 2	Factor 3	Response 1
Std	Run	A: Salinity	B: Nitrogen	C: UV exposure	MAA
		g/L	g/L	Hours	nm
1	4	90	0	30	0,493
2	2	120	0	30	0,688
3	17	90	0,5	30	0,729
4	12	120	0,5	30	0,634
5	10	90	0	92	0,921
6	1	120	0	92	0,986
7	16	90	0,5	92	0,907
8	15	120	0,5	92	0,576
9	5	79,7731	0,25	61	0,762
10	18	130,227	0,25	61	0,679
11	7	105	-0,170448	61	0,632
12	11	105	0,670448	61	0,78
13	3	105	0,25	8,86442	0,367
14	8	105	0,25	113,136	0,983
15	20	105	0,25	61	0,735
16	9	105	0,25	61	0,811
17	6	105	0,25	61	0,666
18	13	105	0,25	61	0,869
19	19	105	0,25	61	0,644
20	14	105	0,25	61	0,663

3.2.6 **DNA extraction and Polymerase chain reaction (PCR)**

The total algal DNA was isolated using Macherey-Nagel NucleoMag DNA/RNA Water kit as per the kit protocol using the DynaMag-2 magnetic separator with slight modifications. Briefly, 40 ml of the sample was collected and centrifuged at 3000 x *g* for 15 minutes. The supernatant was discarded, and the pellet was washed with phosphate buffer solution (PBS) to remove excess salt. The washing step was repeated until the resulting supernatant was clear. The pellet was then

resuspended in 10 ml of PBS and aliquoted into MN Bead Tubes Type A (5 ml). The tubes were placed in the Bead Ruptor 12 (Omni International, Inc.) at medium speed for 2 - 3 minutes. From the tube, 1,5 ml of sample was removed and placed into a 2 ml Eppendorf tube which was centrifuged for 30 s at 11000 x *g*. The supernatant was placed into a separate tube and 25 µl of NucleoMag B-Beads and 475 µl of Binding Buffer MWA2 were added. Using a pipette, the sample was thoroughly mixed and allowed to incubate at room temperature for 5 minutes. Using the magnetic separator, the sample was allowed to stand for 5 minutes, and the clear supernatant was then discarded. The sample was then washed with 850 µl MWA3, resuspended for 2 minutes and allowed to separate for 5 minutes. Lastly, the sample was washed using 850 µl MWA4, resuspended for 2 minutes and allowed to separate for 2 minutes. The magnetic beads were allowed to air-dry for 20 minutes and resuspended in 100 µl RNase-free H_2O. The beads were incubated on a DLAB HB 120-S heating block at 56°C for 10 minutes and separated for 2 minutes on the magnetic separator. The concentration and purity of the DNA was determined using the Implen 80 nanophotometer. The primers and PCR conditions were followed as per Hu *et al.* 2015. The primers were purchased from Inqaba Biotechnical Industries (Pty) Ltd and were prepared as per the supplier's instructions. A polymerase chain reaction mixture was prepared in the BioBase PCR800 PCR Cabinet**.** All pipettes and tips were sterilized under the UV lamp for an hour prior to preparation. The PCR mixture was prepared using the EmeraldAmp GT PCR Master Mix and was run using the Bio-Rad T100 Thermal Cycler for 35 cycles. The resulting products were run on a 1% gel electrophoresis which was prepared by dissolving 1 g of LE multi-purpose agarose in distilled H_2O and microwaving the solution till the solution was clear. The solution was allowed to cool at room temperature and once cool enough 2 µl of ethidium bromide was added and mixed into the solution. The agar was then poured out into a mould to

allow it to solidify. The gel was placed into a gel electrophoresis tank, which was connected to a Bio-Rad Powerpack Basic, and submerged in TAE buffer. To 4 µl of each sample, 1 µl of loading dye was added and the total volume (5 µl) was loaded into each well respectively. The gel was run at 70 V for 30 minutes and was carefully removed and viewed in the Bio-Rad Molecular Imager, ChemicDoc XRS+. The image was obtained with the Image Lab Software which came standard with the gel viewing station.

Table 3.2: DHQS and OMT primers targeting the *mys* gene cluster in *Euhalothece* sp.

	DHQS	**OMT**
F'	ACTGACGCAGCATAGGAGGA	GTAGCATCTAGGGCGACGAG
R'	TCGATCGCATAAACCTGTCC	CTGGATTCGCCGTATTTTGT

Table 3.3: PCR master mix recipe (x1 run)

Item	**Volume (µl)**
Master mix	12,5
dH2O	6,5
Forward primer	2
Reverse primer	2
DNA	2

Table 3.4: PCR conditions

Step	**Temperature (°C)**	**Time**
Initial denaturation	98	5 min
Denaturation	95	30 sec
Annealing	60	30 sec
Elongation	72	2 min
Final elongation	72	10 min

3.2.7 **Quantitative real-time PCR**

Real-time qPCR was carried out according to conditions set out by Hu *et al.* 2015. Briefly, the samples were prepared similarly to the PCR master mix, using the Bio-Rad iTaq Universal SYBR Green Supermix. Two samples were prepared, a control grown in standard conditions (120 g/L salt, 1,5 g/L NaNO3, 75 µmol photon m^{-2} s^{-1}) and the experimental grown in the modified conditions stipulated by the RSM output (90 g/L salt, 0 g/L NaNO3, UV exposure for 92-hours). DNA was extracted from both samples using the method previously mentioned, and a comparative CT value experiment was performed to identify the expression level of the *mys* gene cluster. The qPCR was run using QuantStudio3 and the experiment run was designed and analysed using QuantStudio Design & Analysis Software. The products were run on a gel using the SYBR Safe DNA Gel Stain.

Table 3.5: qPCR master mix recipe (x1 run)

Item	**Volume (µl)**
Supermix	10
dH2O	5
Forward primer	1,5
Reverse primer	1,5
DNA	2

3.3 **Results and Discussion**

Preliminary experimentations were conducted to gain a broad understanding of the factors that affect MAA production for *Euhlothece* sp. to enable appropriate factor selection for RSM. Irradiance is a crucial factor for MAA production as MAA's are light harvesting compounds which protect the cell from photoinhibition.

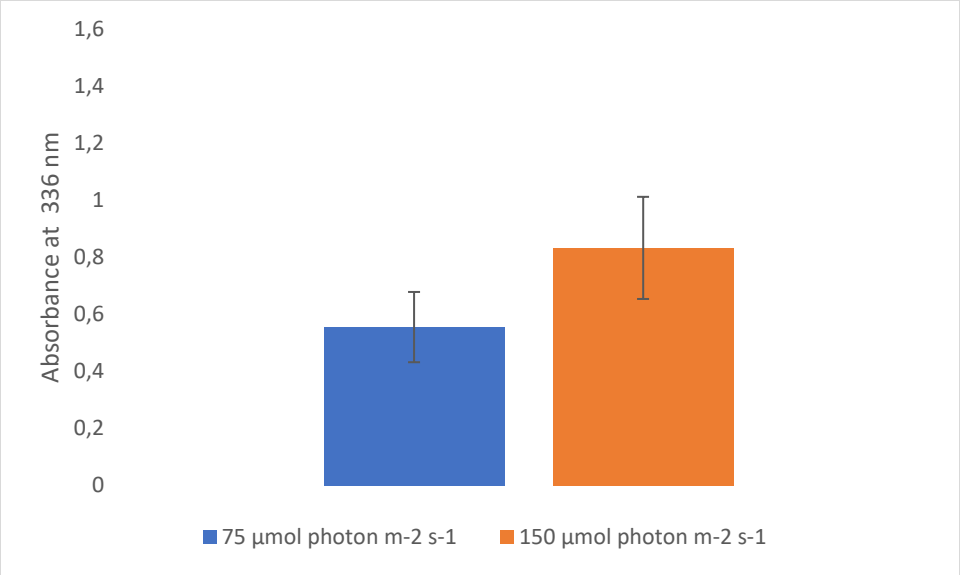

Figure 4: Comparison of MAA production in *Euhalothece* sp. using saline BG11 medium (120 g/L NaCl) under exposure to low light irradiance at 75 µmol photon m^{-2} s^{-1} vs high light 150 µmol photon m^{-2} s^{-1} after 16 days. Error bars represent the standard deviation across triplicate samples

*Note: y-axis maximum value is set to 1,6 to allow all graphs (in this chapter) to be read and compared uniformly.

Figure 4 highlights the effect of low light (75 µmol photon m^{-2} s^{-1}) and high light (150 µmol photon m^{-2} s^{-1}) on MAA production over a period of 16 days. It is important to note that while 150 µmol photon m^{-2} s^{-1} is considered high in the laboratory setting, these intensities are relatively low compared to natural sunlight. The results showed that production increased by 28,76% when exposed to higher the irradiance level. Light is an important factor for normal cellular processes in cyanobacteria. Changes to light irradiance may affect pigments that are directly related to light-harvesting mechanisms and thus will affect secondary metabolite production that is coupled with photosynthesis (Khatoon *et al.* 2018). Mycosporine-like amino acids are secondary metabolites that function as photoprotectants. Due to their protective nature, an increase in irradiance levels directly influences their production.

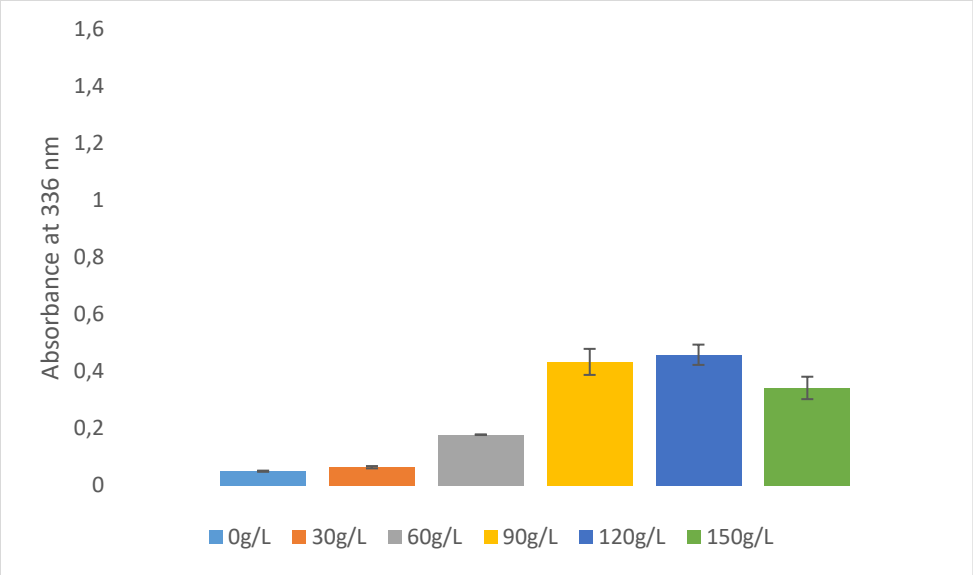

Figure 5: Comparison of MAA production in *Euhalothece* sp. using saline BG11 media with ranging salinity concentrations under exposure to 75 µmol photon m^{-2} s^{-1} after 7 days. Error bars represent the standard deviation across triplicate samples

Figure 5 shows the effect of salinity on MAA production at 75 µmol photon m^{-2} s^{-1} light irradiance level. A range of stock concentrations were prepared, and the isolate was inoculated and allowed to grow for 7 days. Starting from 0 g/L to 120 g/L, a steady increase in MAA production is observed, with the maximum production of MAAs occurring at the standard concentration (120 g/L = 0,457 Au). At 90 g/L (Au = 0,434), the production of MAAs is comparable to 120 g/L, suggesting that the salt concentration can be decreased to provide comparable results. Gharib *et al.* 2020, showed a similar trend, in that increasing the salt concentration increases MAA production, since these compounds are known to play a role in regulating osmotic balance. The standard concentration of a saline environment is 35 g/L. *Euhalothece* sp. has the capability to grow in a concentration 3,5 times more than the standard concentration. This is a direct correlation that cyanobacteria in higher saline environments produce MAAs in higher concentrations since there was an increase in MAA concentration as the salinity increased from

0 – 120 g/L. However, at 150 g/L salt, a decline in MAA production is observed (0,342 Au) providing insight that salt concentrations exceeding 120 g/L hinders MAA production. While *Euhalothece* sp. is a hypersaline strain, the increase in concentration of inorganic ions can cause strain on cells due to osmotic and ionic stress (Singh *et al.* 2022). The combined stressors can lead to essential biological processes becoming impaired, which can lead to the decline in the production of some secondary metabolites, including MAAs.

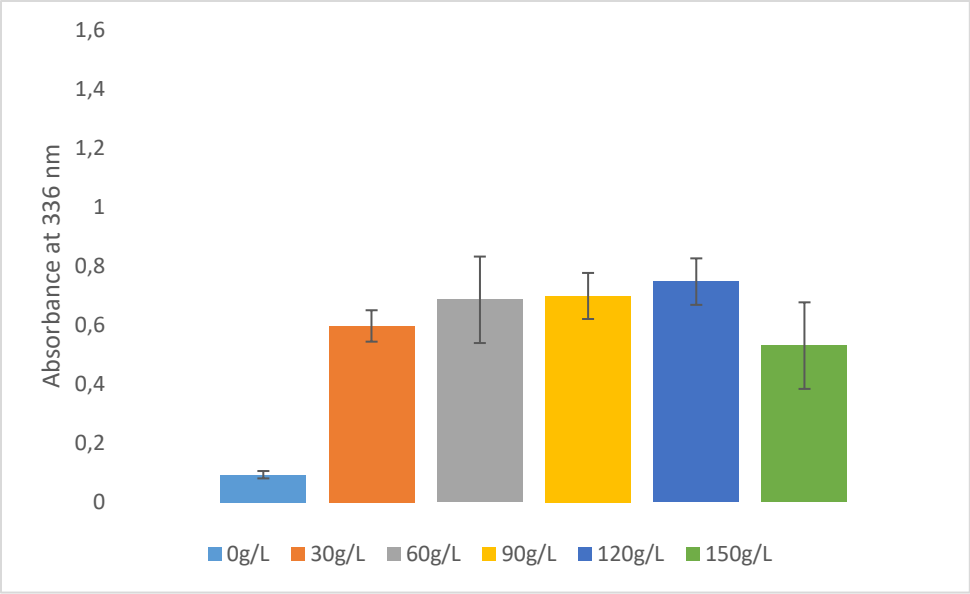

Figure 6: Comparison of MAA production in *Euhalothece* sp. using saline BG11 media with ranging salinity concentrations under exposure to 150 µmol photon m^{-2} s^{-1} after 7 days. Error bars represent the standard deviation across triplicate samples

Similarly, Figure 6 employs the same experimental procedure, with the only modification being a high light irradiance level to further investigate MAA production trends, following the observation in the previous study that higher light intensities led to increased MAA concentrations. The same trend observed of MAA production in Figure 5 was observed in Figure 6. From 0 g/L to 120 g/L a steady increase was observed with 120 g/L (0,748 Au) having the highest concentration ($p = 0,0043$). Doubling the irradiance level increases the MAA concentration by 48,26%. Notably, at

60 g/L and 90 g/L, the concentration of MAAs is comparable (0,686 Au and 0,699 Au respectively), suggesting under high light exposure, using a concentration of either 60 g/L or 90 g/L does not significantly (p = 0,0486) affect MAA production. Similarly, at 150 g/L there is a decline in MAA production (0,531 Au), due to the excess amounts of solutes present.

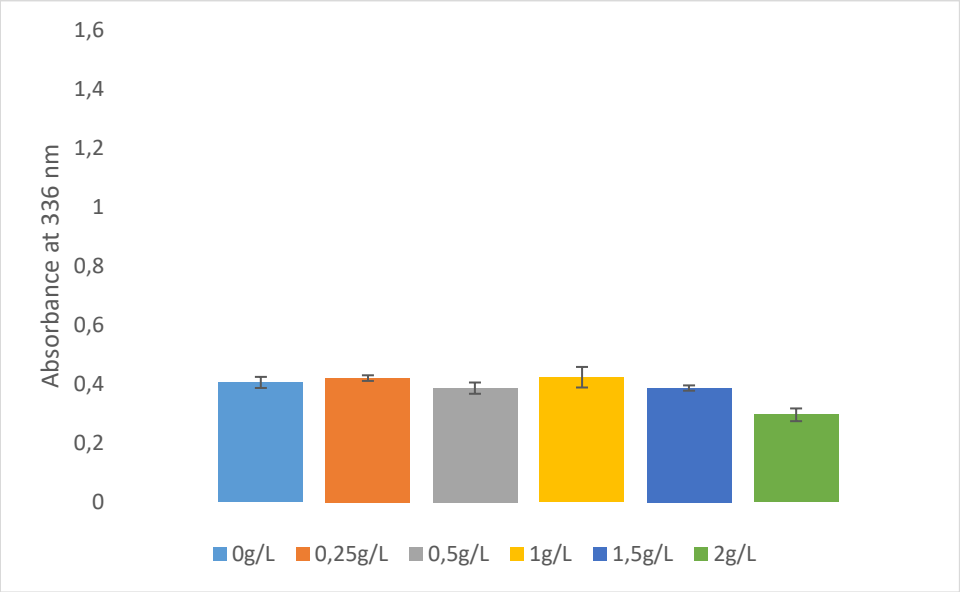

Figure 7: Comparison of MAA production in *Euhalothece* sp. using saline BG11 media with ranging nitrogen concentrations under exposure to 75 µmol photon m^{-2} s^{-1} after 7 days. Error bars represent the standard deviation across triplicate samples

Figure 7 highlights the effect of nitrogen on the production of MAAs. A range of concentrations were prepared from 0 g/L –2 g/L, and extraction for MAAs were done after 7 days. The isolate showed that at lower concentrations (0 g/L - 1 g/L), production of MAAs were significantly higher (p = 0,0304) as compared to the stock concentration of 1,5 g/L. Furthermore, 2 g/L showed a significant decrease in production (p = 0,0062) which differs from literature. Since MAAs contain

a nitrogen substituent, it is thought that an excess of nitrogen present increases MAA production by upregulating the genes responsible for MAA production (Raj *et al.* 2021). However, the results demonstrate that a nitrogen deficient medium increases production. Salehian *et al.* 2023, observed that cyanobacteria *Lyngbya purpureum* produces more MAAs when nitrogen levels are low, compared to *Glenodinium* sp. Studies conducted by Loza *et al.* 2014, and Lalegerie *et al.* 2020, found that in certain species of cyanobacteria, nitrogen has been found to be a limiting factor for MAA production. A nitrogen atom is required to produce the core MAA structure and thus could influence the regulation of the nitrate transport genes associated with MAA synthesis. However, the influence of nitrogen on MAA production varies across species due to differences in nutrient requirements and growth patterns.

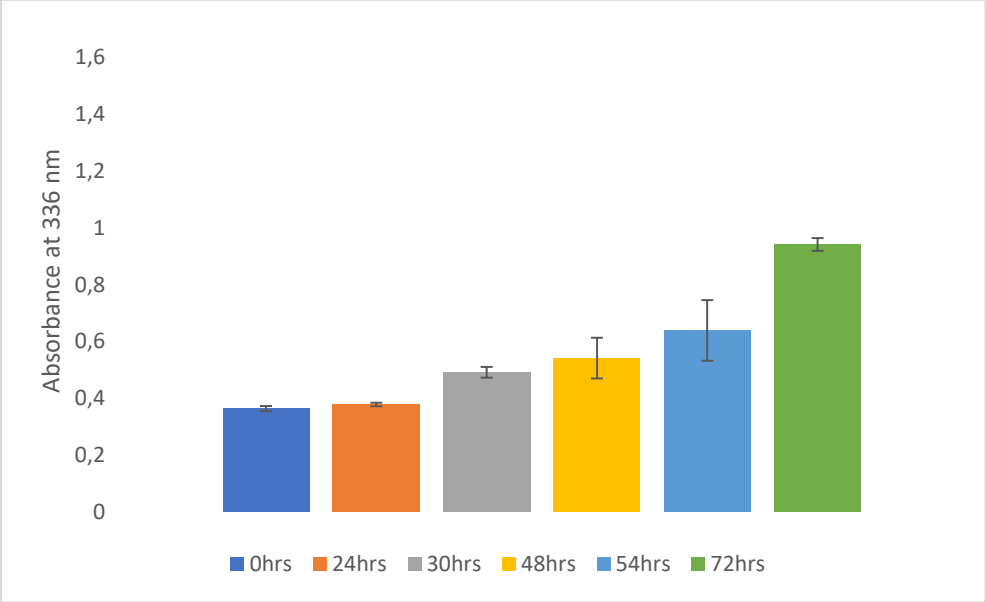

Figure 8: Comparison of MAA production in *Euhalothece* sp. using saline BG11 media (120 g/L) after 72-hours of low-level exposure to UV irradiance. Error bars represent the standard deviation across triplicate samples

Figure 8 and Figure 9 and show the effect of UV irradiance on MAA production, under low and high irradiance levels respectively. Literature suggests that as UV irradiance increases, MAA production increases (Garcia-Pichel *et al.* 1993; Sinha *et al.* 2001). This was confirmed with preliminary experimentation as doubling the irradiance level increased MAA production by 97,4% over a 3-day period. A noticeable trend in the *Euhlaothece* sp. showed that MAA production remained low from 0 hours to 48 hours.

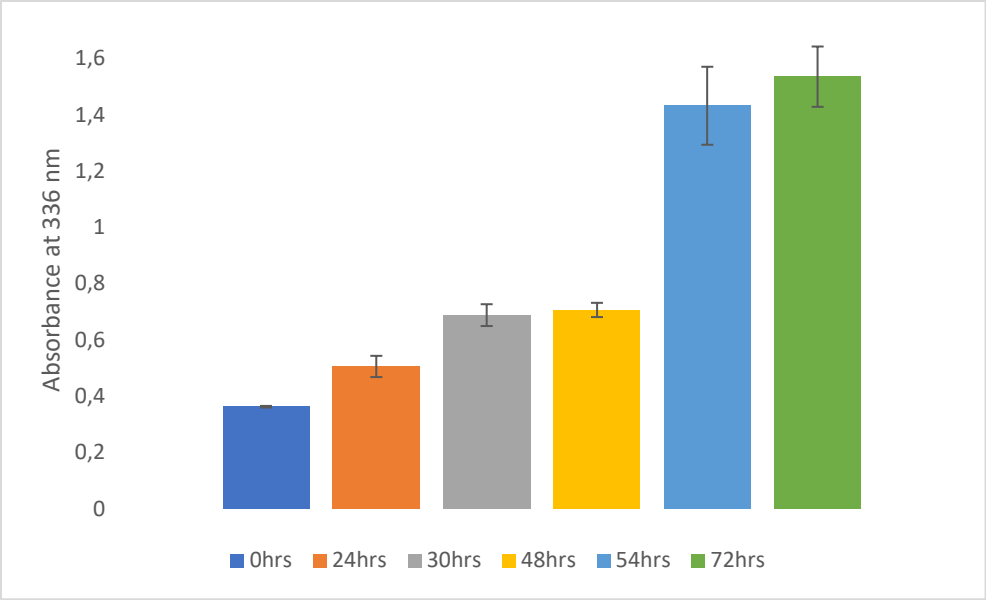

Figure 9: Comparison of MAA production in *Euhalothece* sp. using saline BG11 media (120 g/L) after 72-hours of high-level exposure to UV irradiance. Error bars represent the standard deviation across triplicate samples

After the 48-hour mark, production significantly ($p = 0,0167$) increased, meaning that the isolate needed a minimum of 48-hours of UV exposure for a better yield of MAAs. The culture remained viable after a 72-hour period of ultraviolet (UV) exposure, suggesting it could tolerate longer periods of UV exposure. However, prolonged exposure to high UV irradiance is known to cause

severe oxidation, leading to cell death. The 72-hour period was selected to balance the need for MAA induction with the critical goal of preserving the culture's viability.

Each factor was assessed individually to determine their impact on MAA production and to determine appropriate experimental ranges for the RSM. The experiment ran for a total of 113 hours, with extractions occurring at defined timestamps. The ANOVA Table 3.6 for the central composite design showed the model to be significant (p = 0,0099) and the lack of fit to be insignificant (p = 0,4648). This indicates that the chosen factors and the ranges tested can significantly affect MAA production.

Table 3.6: Analysis of variance (type III) for a quadratic model for MAA production

Source	Sum of squares	df	Mean square	F-value	p-value	
Model	0,3899	9	0,0433	4,96	0,0099	Significant
A- Salinity	0,0068	1	0,0068	0,7825	0,3971	
B- Nitrogen	3,492E-06	1	3,492E-06	0,0004	0,9844	
C- UV exposure	0,2593	1	0,2593	29,68	0,0003	
AB	0,0588	1	0,0588	6,73	0,0267	
AC	0,0167	1	0,0167	1,92	0,1964	
BC	0,0459	1	0,0459	5,25	0,0449	
A^2	0,0004	1	0,0004	0,0458	0,8349	
B^2	2,937E-07	1	2,937E-07	0,0000	0,9955	
C^2	0,0017	1	0,0017	0,1930	0,6698	
Residual	0,0874	10	0,0087			
Lack of fit	0,0455	5	0,0091	1,09	0,4648	Not significant
Pure error	0,0419	5	0,0084			
Cor total	0,4773	19				

Out of the three factors, UV irradiance was shown to have the most significant (p = 0,0003) effect on MAA production when acting as a single source. Factors acting in combination with each other including salinity and nitrogen, and nitrogen and UV irradiance show significance (0,0267 and 0,0449 respectively) on MAA production. Factors acting in combination with each other better influence MAA production than when acting alone.

The equation provides an estimate of the level of MAA's (y) as a function of salinity (X_1), nitrogen (X_2) and UV exposure (X_3). The response from the experiments can be written as an equation as follows:

$$Y\ (Au) = -0{,}54812 + 0{,}005309 \times X_1 + 2{,}99811 \times X_2 + 0{,}018593 \times X_3 - 0{,}022869 \times X_1 \times X_2 - 0{,}000098 \times X_1 \times X_3 - 0{,}009774 \times X_2 \times X_3 + 0{,}000023 \times X_1^2 + 0{,}002284 \times X_2^2 - 0{,}000011 \times X_3^2$$

Table 3.7: Detailed experiment report for the central composite design with the predicted and actual values

Run order	Standard order	Actual	Predicted	Residual	Leverage	Internally studentized residuals	Externally studentized residuals	Cook's distance	Influence on fitted value DFFITS
1	6	0,9860	0,9552	0,0308	0,670	0,573	0,553	0,067	0,787
2	2	0,6880	0,6196	0,0684	0,670	1,273	1,320	0,329	1,879
3	13	0,3670	0,4676	-0,1006	0,607	-1,717	-1,940	0,456	-2,412
4	1	0,4930	0,4014	0,0916	0,670	1,706	1,922	0,590	2,738
5	9	0,7620	0,7825	-0,0205	0,607	-0,350	-0,334	0,019	-0,415
6	17	0,6660	0,7299	-0,0639	0,166	-0,749	-0,732	0,011	-0,327
7	11	0,6320	0,7295	-0,0975	0,607	-1,664	-1,857	0,428	-2,309
8	14	0,9830	0,9311	0,0519	0,607	0,886	0,875	0,121	1,089

9	16	0,8110	0,7299	0,0811	0,166	0,950	0,945	0,018	0,422
10	5	0,9210	0,9200	0,0010	0,670	0,019	0,018	0,000	0,026
11	12	0,7800	0,7312	0,0488	0,607	0,833	0,819	0,107	1,019
12	4	0,6340	0,6006	0,0334	0,670	0,622	0,601	0,078	0,856
13	18	0,8690	0,7299	0,1391	0,166	1,629	1,803	0,053	0,806
14	20	0,6630	0,7299	-0,0669	0,166	-0,784	-0,768	0,012	-0,343
15	8	0,5760	0,6332	-0,0572	0,670	-1,065	-1,073	0,230	-1,528
16	7	0,9070	0,9410	-0,0340	0,670	-0,632	-0,612	0,081	-0,872
17	3	0,7290	0,7254	0,0036	0,670	0,068	0,064	0,001	0,092
18	10	0,6790	0,7072	-0,0282	0,607	-0,482	-0,462	0,036	-0,575
19	19	0,6440	0,7299	-0,0859	0,166	-1,007	-1,008	0,020	-0,450
20	15	0,7350	0,7299	0,0051	0,166	0,059	0,056	0,000	0,025

Table 3.7 provides a detailed summary of the CCD and shows possible errors that may have occurred with individual runs that may influence the significance of the model. In the design set up, run 3, 4 and 7 are highlighted since they have an actual value that differs from the predicted value. Based on the preliminary experiments employing UV irradiance, it was clear that yield of MAAs increased after 48 hours (Figure 9). The extractions for runs 3 and 4 were conducted at 8 hours and 30 hours respectively as per the experimentation design which could have led to lower MAA production.

The ANOVA Table 3.6 highlighted that salinity and nitrogen are factors that, when in combination with each other, have a significant effect on MAA production. For run 7, the predicted values for low nitrogen and higher salinity (105 g/L salt) were higher than the actual value obtained. This was likely due to the salinity of 105 g/L being in the ideal range. The lower nitrogen however has a larger effect than salinity which resulted in a lower actual value.

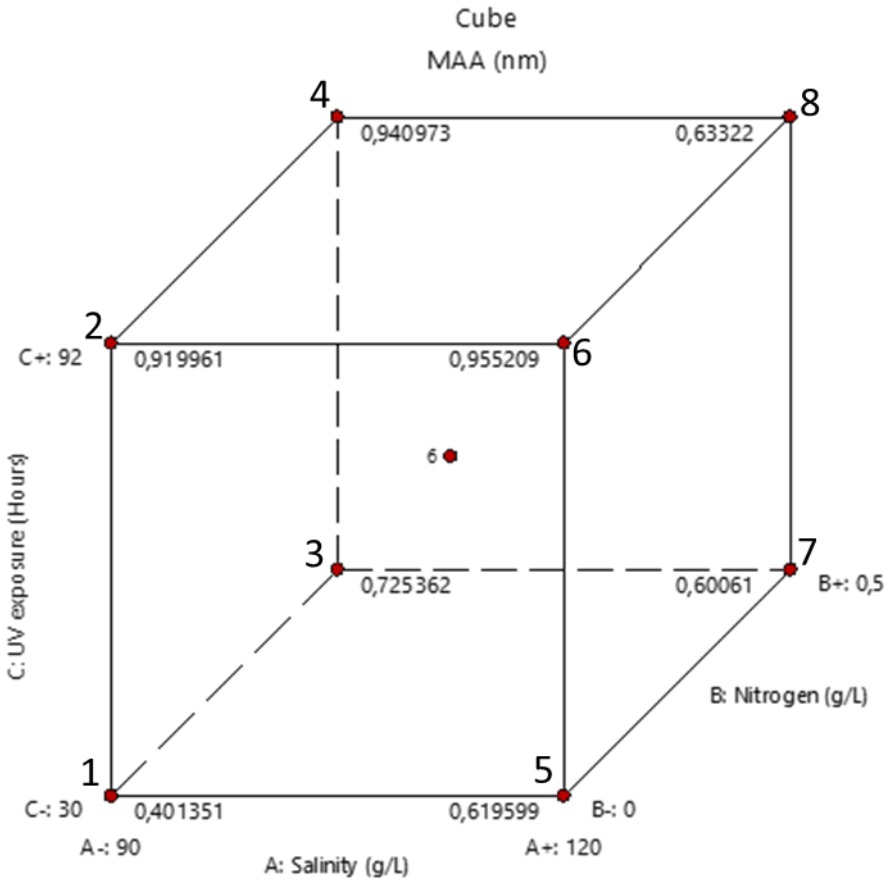

Figure 10: Cube plot showing the predicted values from the coded model for the combinations of the -1 and +1 levels of salinity, nitrogen and UV exposure

The cube plot shows 8 possible solutions represented by each corner. Horizontal lines (length) indicate salinity concentration from 90 g/L to 120 g/L, while the vertical lines indicate the UV exposure time from 30 hours to 92 hours. The other horizontal lines (breadth) indicate the nitrogen concentration from 0 g/L to 0,5 g/L. Corner points represent the minimum predicted value at 336 nm that should be observed under the specified conditions. Identifying corner points with the highest predicted output stipulates the conditions required for maximum output. According to point 1, cultivation in 120 g/L of salt in a nitrogen deficient media, exposed to 92-

hours of UV irradiance will result in a peak of 0,955 at 336 nm. Table 3.8 provides a simplified view of the solutions given by the cube plot.

Table 3.8: Summary of combinations of the -1 and +1 levels of salinity, nitrogen and UV exposure

	Salinity (g/L)	Nitrogen (g/L)	UV exposure time (hours)	MAA response (nm)
1	90	0	30	0,401351
2	90	0	92	0,919961
3	90	0,5	30	0,725362
4	90	0,5	92	0,940973
5	120	0	30	0,619599
6	120	0	92	0,955209
7	120	0,5	30	0,60061
8	120	0,5	92	0,63322

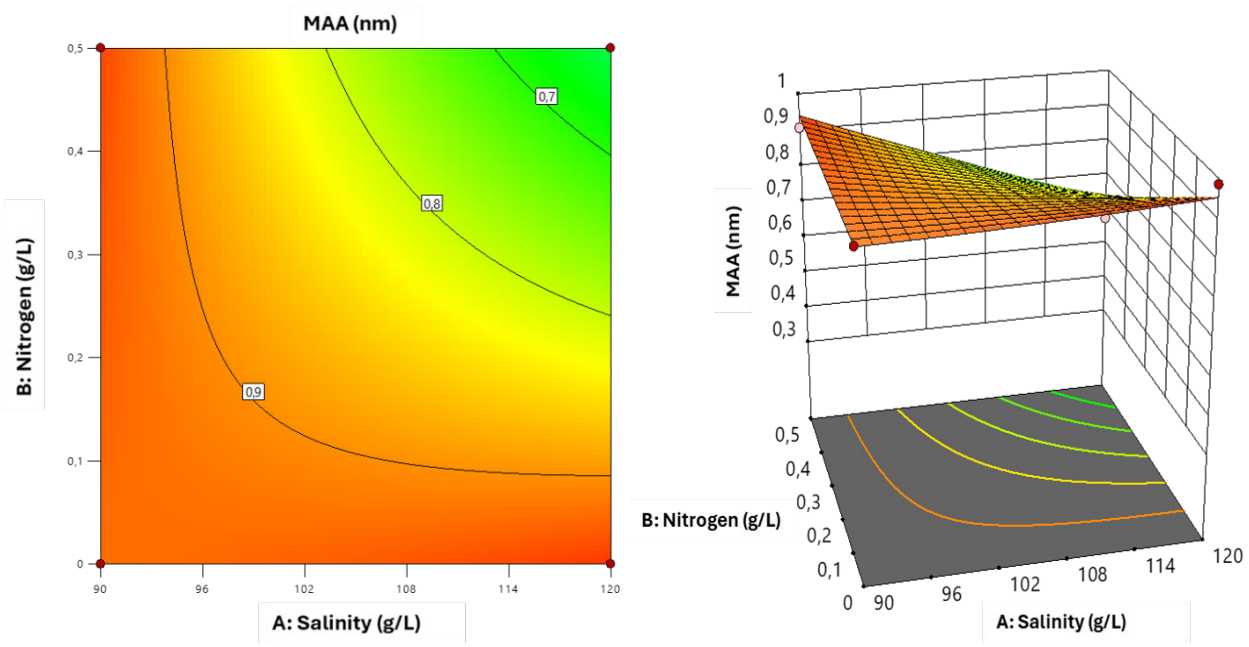

Figure 11: Contour plot showing significant interactions between nitrogen and salinity at different concentrations that influence MAA production in *Euhalothece* sp. exposed to high UV irradiance level for 92-hours

Extrapolating from the graph (Figure 11), there are three possible solutions: (i) 0 g/L $NaNO_3$ in 90 g/L NaCl, (ii) 0,5 g/L $NaNO_3$ in 90 g/L NaCl, and (iii) 0 g/L $NaNO_3$ in 120 g/L NaCl. Although all three solutions are viable, solution (iii) is predicted to yield the highest MAA concentration (0,955 Au). In the solutions, UV exposure was kept constant at 92 hours since MAA production was the highest when exposed for this period. Further exposure would result in the culture becoming at risk of photobleaching. Solutions using 90 g/L NaCl were also considered because using a lower salt concentration could offer other economic benefits, such as reduced costs associated with production requirements. Therefore, a balance between maximizing MAA concentration and minimizing production costs was investigated.

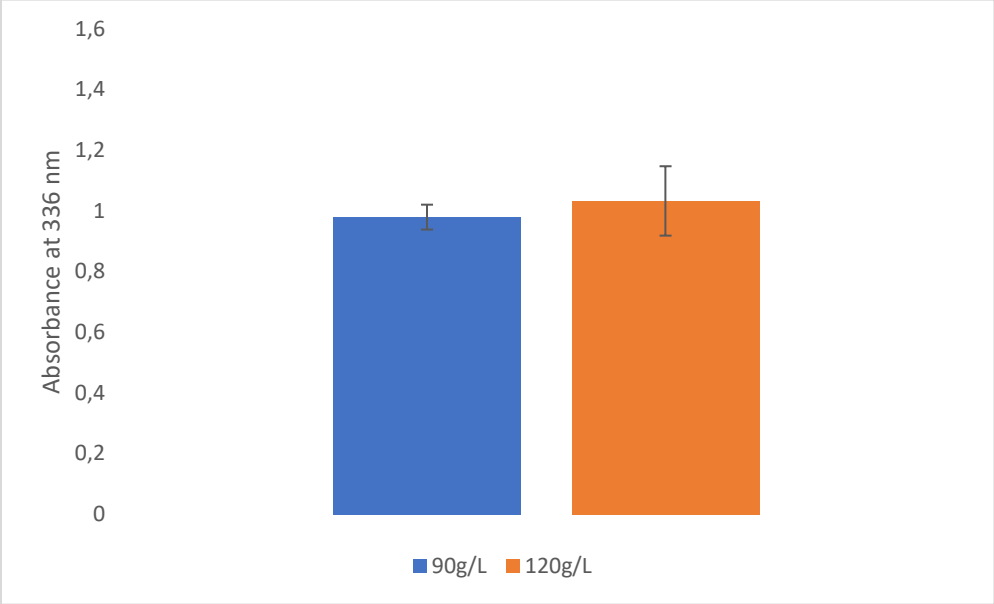

Figure 12: Comparison of RSM outputs of MAA production in *Euhalothece* sp. using altered salinity concentrations of 90 g/L vs 120 g/L in a nitrogen deficient media exposed to high UV irradiance for 92 hours. Error bars represent the standard deviation across triplicate samples

The solution for the maximum production of MAAs included that the isolate be grown in nitrogen deficient media, in 120 g/L salt, and exposed to UV irradiance for 96 hours. While this solution is suggested, the heatmap and cube plot show a second possible solution where the only

modification is a 90 g/L salt media. The suggested response for at 120 g/L should give a maximum MAA yield of 0,955 Au and at 90 g/L should give a maximum response of 0,919 Au. Figure 11 shows the comparison between the two responses and shows that both conditions had improved readings than those predicted. At 120 g/L the maximum MAA response was 1,033 Au and at 90 g/L the response was 1,01 Au. While there is a higher concentration of MAAs at a salt concentration of 120 g/L, dropping the concentration to 90 g/L shows no significant decrease ($p = 0,2375$) in MAA yield. While increased salinity can enhance production, a cost-benefit analysis suggests alternative strategies. Increasing the salt concentration from 90 g/L to 120 g/L requires a 33% increase in salt, providing a relatively low increase in production of MAAs of only 5.53%.

The presence of MAAs were only detected through spectroscopic analysis, hence validation of the result by another method was required. The presence of the *mys* gene cluster in *Euhalothece* sp. was investigated using PCR.

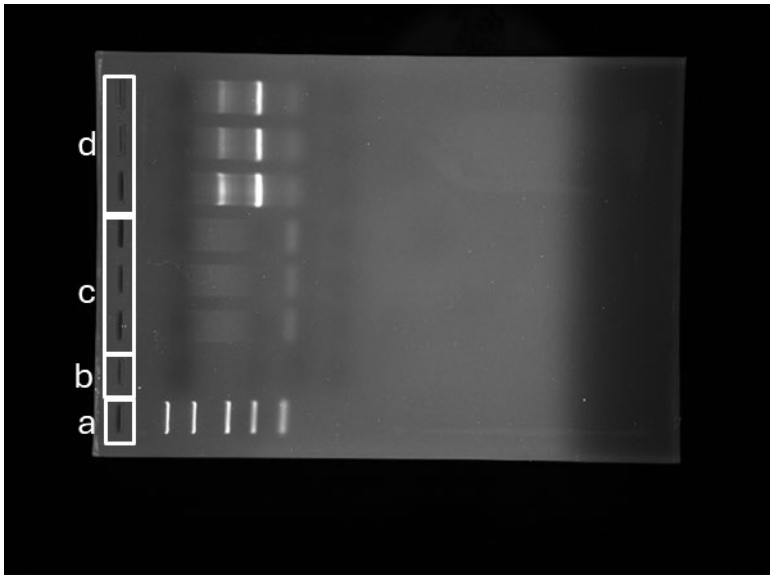

Figure 13: Gel electrophoresis showing PCR products with wells loaded as follows: (a) middle range DNA ladder (100 bp – 5 kb); (b) negative control; (c) DHQS primer; and (d) OMT primer using ethidium bromide gel stain

There are four main steps involved in the production of MAAs. The first step involves the conversion of desmethyl 4-deoxygadusol (DDG) and 4-deoxygadusol (4-DG) which is catalysed by dehydroquinate synthase (DHQS) *(mysA*) in the shikimic acid pathway or by desmethyl-4-deoxygadusol synthase (DDGS, a DHQS homolog) in the pentose phosphate pathway (Gao and Garcia-Pichel 2011b); (Balskus and Walsh 2010); (Pope et al. 2015). O-methyltransferase (*mysB*) then converts DDG to 4-DG and using ATG grasp (*mysC*), the first nitrogen substituent is incorporated. This results in the formation of mycosporine-glycine which acts as a branch point for the formation of all other MAAs. Non-ribosomal peptide synthetase-like enzymes (NRPS) (*mysD* or *mysE*) allow for the conversion of mycosporine-glycine to other imino-mycosporines (Portwich and Garcia-Pichel 2003); (Challis and Naismith 2004). In some instances, a D-alanine ligase homolog (*mysD*) replaces the NRPS-like (*mysE*) enzymes (Gao and Garcia-Pichel 2011a). Both the DHQS and OMT primers were run on a gel to determine the presence of the *mys* gene cluster in the *Euhalothece* strain. The gel shows clear amplification of the OMT primer (d) while the DHQS primer (c) shows minimal amplification. The successful amplification of the OMT primer provides an indication of the presence of the gene cluster since it is responsible for catalyzing the conversion of DHQ. This is the starting point of MAA synthesis, forming the core structure of these compounds (Chrapusta *et al.* 2017).

Going forward, only the OMT primer was used. After confirming successful PCR amplification, the next crucial step was to evaluate if UV irradiance led to the upregulation of the target gene cluster.

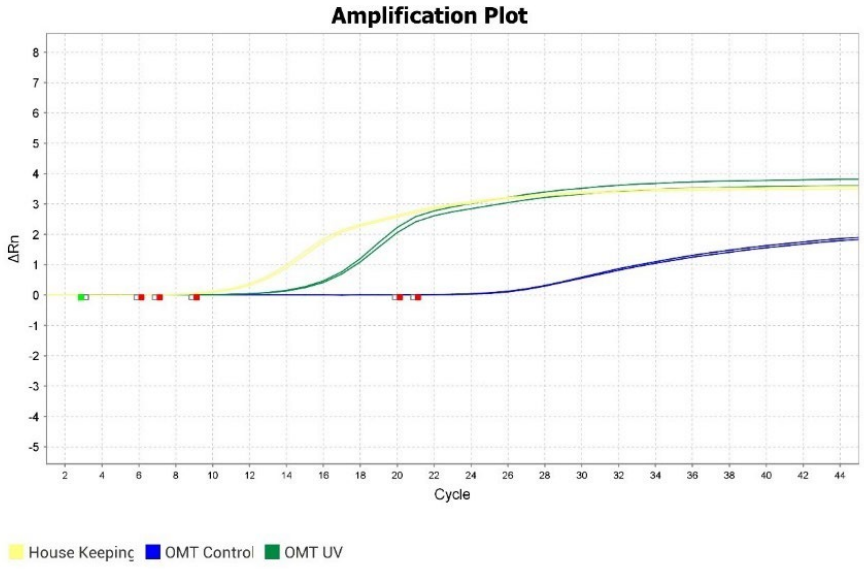

Figure 14: qPCR amplification plot showing the comparative CT values of the housekeeping gene vs control vs UV exposed samples

A comparative CT value test was conducted comparing the control to the experimental (conditions given by RSM output) since the control lacked UV radiation. The comparative study showed the UV exposed culture to amplify around cycle 14 while the control only showed amplification around cycle 26. Since MAAs are photoprotective compounds, when exposed to UV radiation, production is increased to protect cells. Since amplification of the UV exposed culture occurs at an earlier cycle, it provides an indication that the gene cluster is being upregulated under UV exposure as compared to the control. This correlates with the high MAA concentration obtained in the single experiment and reflects the long exposure time in the RSM output.

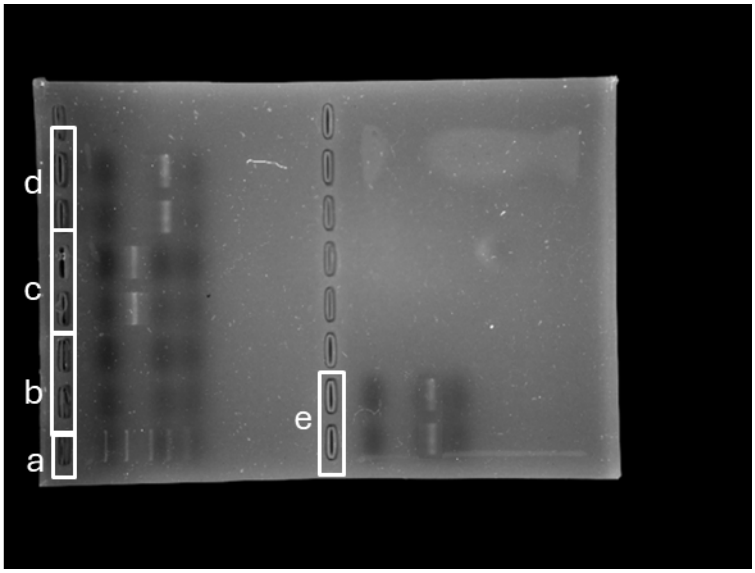

Figure 15: Gel electrophoresis showing qPCR products with wells loaded as follows: (a) ladder; (b) negative control; (c) housekeeping gene; (d) OMT gene of *Euhalothece* sp. grown in RSM conditions; and (e) OMT gene of *Euhalothece* sp. grown in standard conditions stained using SYBR Safe gel stain

The qPCR results were then run on a gel for visualisation. The gel shows clear bands for the products produced during qPCR, however, do not appear as bright as the standard PCR gel due to a different gel stain used.

3.4 **Conclusions**

The preliminary experiments demonstrated how each abiotic factor influenced MAA production individually. While these results conformed with findings in literature, it was notably observed that higher production occurred when two factors were combined. This synergistic effect was evident in the salinity experiments and the UV experiments whereby combining high saline conditions with a high irradiance level nearly doubled MAA production. The RSM results from

these optimized conditions not only confirm the potential for maximizing MAA production but also highlighted the significant savings on incurred costs.

Through gene expression analysis, the presence of the *mys* gene cluster was successfully confirmed. The results correlate with the solvent extraction results in terms of confirming that the increase in the peaks that were observed, were that of MAAs. While quantification is possible through molecular methods, identifying the compounds would be a necessary next step since different MAAs display different properties.

CHAPTER 4: STABILITY AND ANTIOXIDANT ACTIVITY OF MYCOSPORINE-LIKE AMINO ACIDS

4.1 Introduction

Natural antioxidants are low molecular weight compounds that have the potential to supress the formation of ROS and prevent the damage caused by the oxidative effects of the biomolecules. Oxidative stress can be caused by various factors, some of these include UV radiation, desiccation, and toxicity caused by harmful materials. Natural antioxidants can be classified into five categories: organosulfur compounds, flavonoids, phenolic compounds, carotenoids, and vitamins (Flieger *et al.* 2021). Due to the adaptation to environmental conditions of organisms, it has facilitated for the presence of a variety of antioxidants.

Mycosporine-like amino acids function primarily to protect the cell against UV radiation; however, they have been found to be novel antioxidants. Since these compounds function as UV protectants, prolonged exposure to UV radiation can cause devastating effects. This includes degradation of pigments, singlet oxygen generation (under longer wavelengths) and production of free radicals (under shorter wavelengths) (Wada *et al.* 2015). Thus, it is generally accepted that MAAs possess antioxidative roles since UV radiation is one of the strongest sources of oxidative stress. Mycosporine-like amino acids play a role against a variety of environmental stresses since their production can be influenced by osmotic stress, desiccation and thermal stress. These environmental factors often enhance oxidative stress, and the antioxidant properties of MAAs are therefore crucial for their protective functions in vivo (Jomova *et al.* 2023). Mycosporine-like amino acids are known to function as excellent antioxidants as their concentration has been found to increase under oxidative stress (Torres *et al.* 2018). Studies conducted in vitro suggest

that the antioxidant capacity of MAAs vary from high to low depending on the MAAs found within the organism (Figueroa *et al.* 2003; Isla Naveira *et al.* 2024).

4.2 **Materials and Methods**

4.2.1 **Stability testing**

The stability of the purified MAA extract was tested according to Singh *et al.* 2022. The stability parameters were strategically chosen to provide a rapid, comprehensive assessment of the MAA extract's robustness across critical conditions. The purified MAA extracts were incubated for 48 hours at temperatures of 4°C, 25°C and 40°C, pH 7 and, under exposure to UV radiation respectively. The extracts were read in the UV/Vis spectrophotometer at 336 nm at 0, 3, 24 and 48 hours to monitor the stability of the samples.

4.2.2 **Antioxidant activity**

Antioxidant potential was conducted according to Rastogi *et al.* 2016. Briefly, a standard curve using an ascorbic acid stock was prepared (Figure 21). To 1 mg of ascorbic acid, methanol was added, bringing the total volume to 1 ml. A stock solution of DPPH (2,2-diphenyl-1-picrylhydrazyl) was prepared by mixing 3,94 mg into 100 ml of methanol. The calibration curve was prepared by using four different volumes of the standard solution (1,25 µl, 2,5 µl, 5 µl and 10 µl) in each cuvette respectively and bringing the total working volume to 1 ml using DPPH. The cuvettes were incubated in the dark for 30 minutes. The spectrophotometer was blanked with methanol followed by a reagent blank, which the cuvette contained 1 ml of DPPH and no sample. The absorbance was read at 517 nm. The purified MAA extracts were tested for their antioxidant

potential using a concentration of 10 µl and topped up to 1 ml using DPPH. The absorbance was expressed as a percentage using the following:

Equation 1: Antioxidant potential expressed as a percentage

$$\frac{(A_{cs} - A_{as})}{A_{cs}} \; x \; 100$$

Where *Acs* is the absorbance of the control and *Aas* is the absorbance of the samples.

4.3 Results and Discussion

Ascorbic acid (vitamin C) standard was prepared due to its high antioxidant activity. It is also highly soluble in water and can be easily quantified. Due to its ideal properties and its extensively studied properties, ascorbic acid is generally prepared as a standard for any antioxidant research, serving as a benchmark for potential antioxidants.

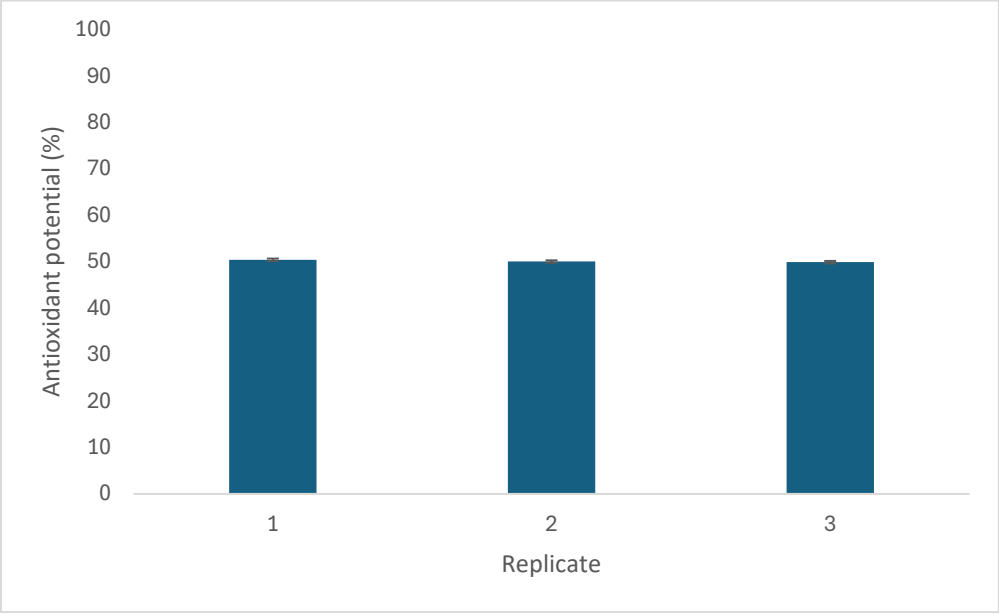

Figure 16: Antioxidant potential of MAAs tested using DPPH, optical density measured at 517 nm and expressed as a percentage (%). Error bars represent the standard deviation across triplicate samples

The antioxidant potential of MAAs in *Euhalothece* sp. were measured at 517 nm, with the three replicates showing an average absorbance of 0,779. Using Equation 1 the absorbance was calculated to be an average antioxidant activity of 50,15%. The antioxidant activity of 10 µl of ascorbic acid was calculated to be 98%, showing a difference of 48% in antioxidant activity compared to MAAs. This means that a higher concentration of MAAs is required to quench ROS as compared to ascorbic acid. In a study conducted by Punchakara *et al*. 2023, shinorine and mycosporine-glycine have showed to have a higher antioxidant activity as compared to asterina-330 and palythine, with mycosporine-glycine having the highest antioxidant activity, measuring 8-fold more than ascorbic acid (Punchakara *et al.* 2023).

Mycosporine-like amino acids possess a unique chemical structure that provides exceptional stability. This inherent stability allows them to effectively withstand various environmental stressors like UV radiation and temperature fluctuations. To assess the stability of MAAs, the purified extracts were incubated at different temperatures, neutral pH and under UV exposure to assess their ability to remain viable over a period of 48 hours. The 48-hour timeframe provided a practical and relevant short-term benchmark for assessing the sample's stability, reflecting conditions common in initial product development and short-term storage.

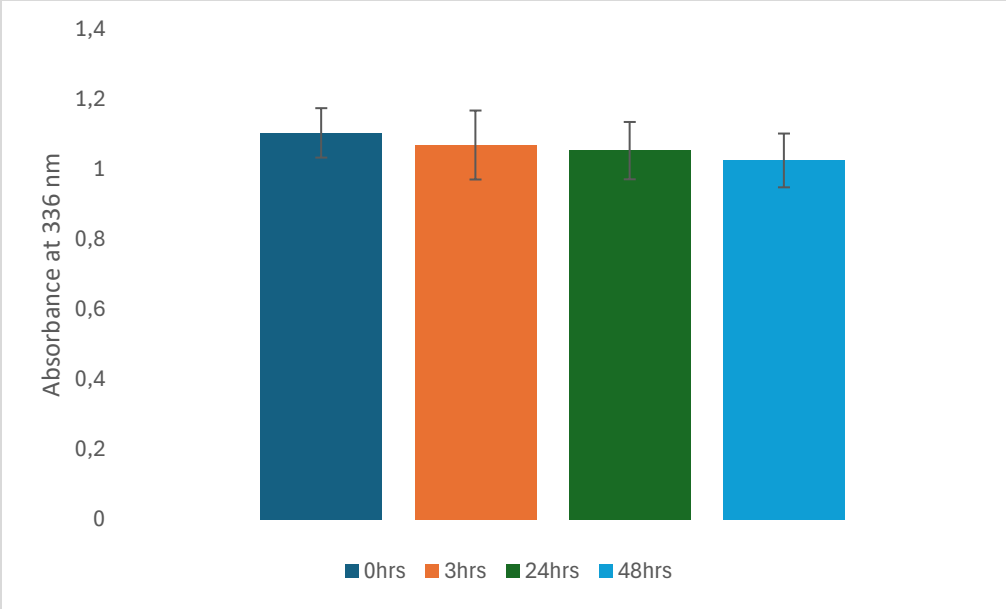

Figure 17: Stability of MAAs at room temperature and neutral pH measured at 336 nm over a period of 48 hours. Error bars represent the standard deviation across triplicate samples

*Note: y-axis maximum value is set to 1,4 to allow all graphs (in this chapter) to be read and compared uniformly.

The purified samples incubated at room temperature and neutral pH show significance (p = 0,02) in the stability of the sample. Over 48 hours, there was only a 7,38% decrease in the overall stability of the sample. This demonstrates the excellent stability of MAAs under room temperature and neutral pH, which was confirmed by de la Coba *et al.* 2019.

Similarly, when the samples were incubated at 4°C, there was no significant decrease (p = 0,046) in the stability of MAAs over the 48-hour period. During this time, there was only 6,47% decrease in the overall stability.

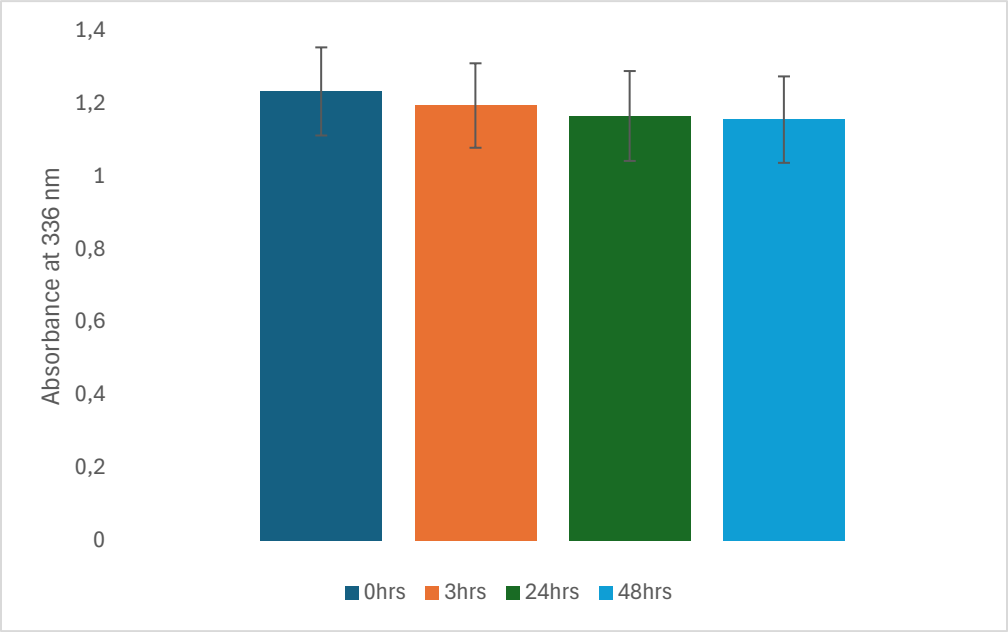

Figure 18: Stability of MAAs stored at 4°C measured at 336 nm over a period of 48 hours. Error bars represent the standard deviation across triplicate samples

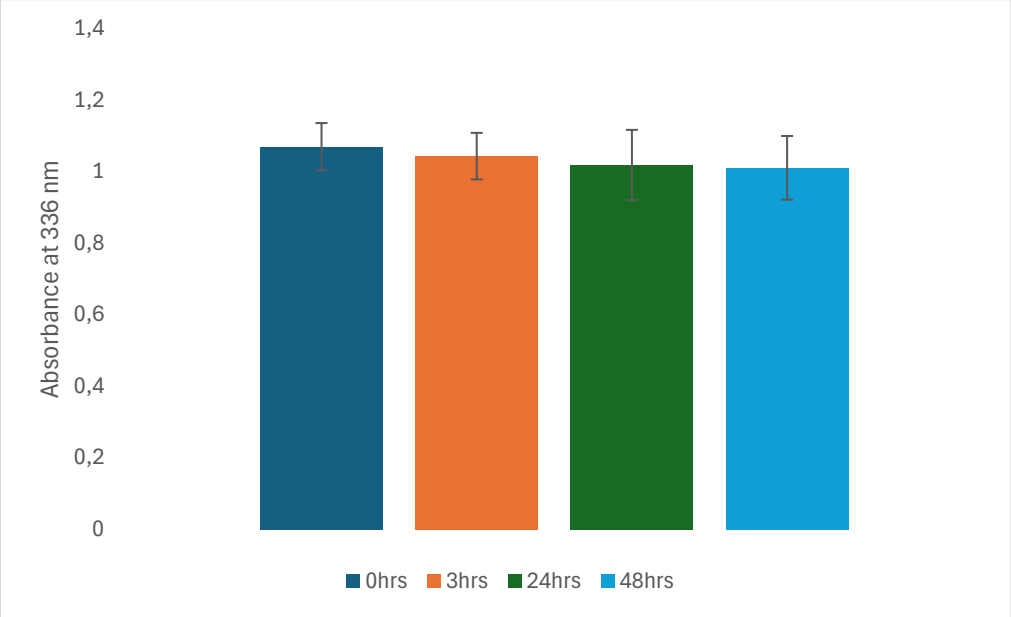

Figure 19: Stability of MAAs incubated at 40°C measured at 336 nm over a period of 48 hours. Error bars represent the standard deviation across triplicate samples

The purified samples were also incubated at 40°C to determine their ability to withstand higher temperatures. After 48 hours, there was no significant decrease (p = 0,041) in the overall concentration of MAAs present. Additionally, the concentration of MAAs only decreased by 5,67%, proving their ability to maintain viable under heat. This strongly suggests that MAAs do not significantly degrade under heating to 40°C, indicating they can maintain their structural integrity and protective functions. The results obtained by de la Coba *et al.* 2019, showed that MAAs can withstand temperatures as high as 85°C. These compounds are highly thermo-stable, unless the pH becomes highly alkaline, which will cause the compounds to rapidly degrade.

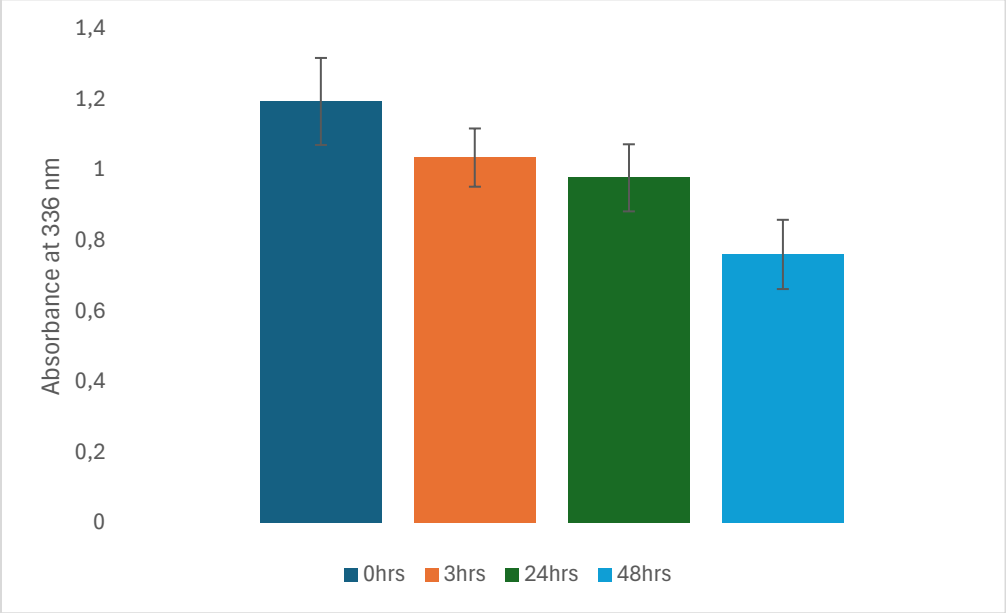

Figure 20: Stability of MAAs exposed to high UV irradiance measured at 336 nm over a period of 48 hours. Error bars represent the standard deviation across triplicate samples

Ultra-violet irradiance showed to have a significant (p = 0,04) effect on sample degradation between 0 – 48 hours, decreasing the total concentration by 45,33%. Under UV exposure, sample concentration decreased by 14,9% within the first 3 hours of exposure and a further

5,44% decrease was seen after 24 hours. Mycosporine-like amino acids serve a primary photo-protective role, and it takes 3 hours to see a slight reduction in its concentration.

The stability of a compound reflects its ability to retain its properties and characteristics from manufacturing, storage, to the time it is used. Since these compounds are being sought after as a natural replacement, they need to remain viable under UV exposure over a 3 - 6-hour period. Mycosporine-like amino acids have been found to be stable under abiotic stress factors and strong photosensitising agents (de la Coba *et al.* 2009). Under exposure to UV irradiance (Figure 20), the harmful effects of UV exposure directly reflect on the stability of the compounds. After 3 hours of exposure, the concentration of MAAs decreased by 14,9%. While the concentration of MAAs dropped under UV irradiance, the compounds show viability after 48 hours of exposure. While there is an overall decrease of 45,33%, the concentration of the compounds has not completely been depleted. According to the World Health Organization (WHO) sunscreen should be reapplied every 2 hours especially after sweating, swimming or being outdoors. The stability data shows promise for their application in sunscreens.

4.4 **Conclusions**

The data collected demonstrates the high stability of MAAs and their natural antioxidative capacity. While their antioxidant potential may be lower than that of ascorbic acid, their main function serves to act as photoprotectants. Hence, the additional properties these compounds display, are added benefits that may have possible application. The main interest lies within the stability MAAs exhibit. Stability of a compound determines how well it can resist alteration and reactive. The MAAs displayed a high stability and remained viable in room temperature and neutral pH (7,38% MAA decrease), incubated at 4°C (6,47% MAA decrease), 40°C (5,67% MAA

decrease), and under UV irradiance (45,33% MAA decrease). Under majority of the stability tests, the compounds were able to retain 90% viability, providing an indication of how well suited these compounds are for application. Under UV irradiance, there was a significant decrease in concentration, however, this was constant exposure over a 48-hour period. The ability of the compound to not decrease below 50% provides an indication of how strong these compounds are at protecting against UV radiation. Mycosporine-like amino acids are ideal compounds to be used in sunscreens and while the antioxidant activity of each of these compounds vary, they can still find application as natural antioxidants.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The investigation on MAAs provides evidence of their high stability and significant potential as natural photoprotectants for future application. A key finding from experimentation is the synergistic effect on MAA production when combining specific abiotic factors. This means that by strategically pairing environmental conditions, MAA yields can be significantly boosted. These finding are not just academically valuable but also highlight the potential for achieving significant cost efficiencies in large-scale MAA cultivation, making their industrial application more viable. At a fundamental level, the genetic analysis played a vital role in validating the understanding of MAA biosynthesis. The presence of the *mys* gene cluster was successfully confirmed, which provided a strong molecular foundation to produce these compounds. While identifying MAAs through molecular methods was vital, research suggests the next crucial step would be identifying and quantifying the specific MAA compounds present. Different MAA structures possess unique properties and identifying them accurately will be key to fully leveraging their diverse functions in various applications.

Furthermore, the study demonstrates the resilience of MAAs across various environmental conditions. Their ability to maintain viability and concentration under a range of temperatures, pH level, and prolonged exposure to UV radiation is particularly noteworthy. The high stability demonstrates their suitability for developing natural sunscreen formulations that can withstand diverse environmental stressors. While MAAs' primary function is photoprotection, their inherent antioxidant capabilities represent a valuable secondary benefit.

The project's findings strongly support the viability of MAA production with potential from commercial application. The ability to significantly enhance MAA yields through optimised, synergistic culturing conditions directly translates to higher productivity, which is a fundamental requirement for industrial scale. The exceptional stability of MAAs under various environmental stressors, including heat and prolonged UV exposure, is a critical advantage for product formulation, shelf-life, and distribution, reducing concerns about degradation. By offering benefits such as superior photoprotection and natural antioxidant activity, MAAs present a strong value proposition in the cosmeceutical and pharmaceutical markets. Further research into the precise identification and characterization of individual MAA compounds will be instrumental in unlocking their full market potential, allowing for the development of highly targeted and effective products. Due to their numerous advantages and broad potential, MAAs represent a highly promising natural resource for environmental utilisation.

5.2 **Recommendations**

Future efforts should focus on scaling up MAA production by implementing bioreactor systems that precisely control key abiotic factors. Given that high saline conditions combined with high UV irradiance significantly boost MAA yield, bioreactor designs should facilitate uniform and intense UV exposure while maintaining optimal salinity levels. Utilizing hypersaline cyanobacterial strains, which naturally accumulate MAAs at higher concentrations, would be a strategic starting point. For economic viability, the comparable production observed at 90 g/L salt concentration, being more cost-efficient than 120 g/L, should be prioritized in initial scale-up designs. Furthermore, exploring continuous culture systems, as opposed to batch processes, could ensure sustained high productivity and more consistent MAA output over time, crucial for meeting industrial demands.

The inherent properties of MAAs, such as their water solubility and high stability across a wide range of temperatures and pH, present significant advantages for extraction and purification processes. Recommendations include developing and optimizing robust, energy-efficient extraction methods that capitalize on their thermal and pH resilience, potentially reducing processing costs. Given that different MAA compounds possess varied properties, future work should also focus on refining purification techniques to isolate specific MAA types. This targeted purification would enable the development of specialized products tailored to leverage the unique benefits of individual MAAs, maximizing their market value.

To overcome potential limitations in natural MAA accumulation and tailor specific MAA profiles, significant potential lies in applying genetic and metabolic engineering strategies. This could involve manipulating the *mys* gene cluster in cyanobacteria to increase expression levels. Engineering approaches could also focus on optimizing precursor pathways, reducing competing metabolic pathways, or enhancing MAA transport and storage within the cells. Such advancements could lead to significantly improved productivity and the ability to produce specific MAA compounds on demand.

For MAAs to successfully transition into commercial products, especially in the cosmetic and pharmaceutical sectors, rigorous safety testing and adherence to regulatory guidelines are required. This includes comprehensive toxicology studies, allergy assessments, and environmental impact evaluations. Establishing clear regulatory pathways and obtaining necessary approvals will be critical for market entry and consumer trust. Collaboration with regulatory bodies and industry experts early in the development process can streamline this phase and ensure that MAA-based products meet all required safety and quality standards.

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APPENDIX A: SUPPLEMENTARY DATA

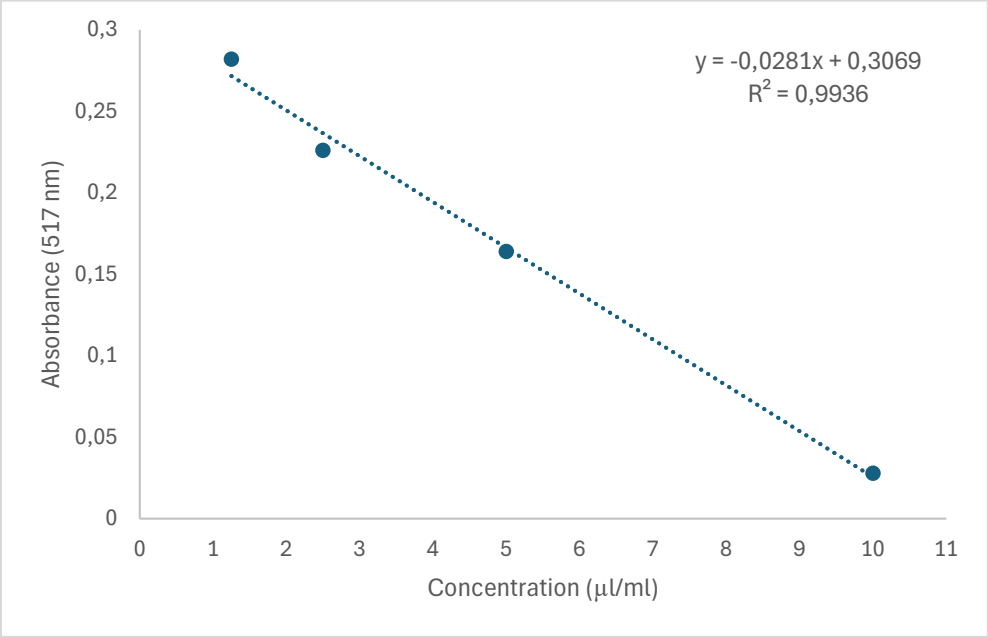

Figure 21: Antioxidant calibration curve of ascorbic acid reference standard measured using DPPH at 517 nm

APPENDIX B: RECIPES

Preparation of BG11 media

The following chemicals were measured, and added to 900 ml of distilled H_2O

Chemical	Quantity
Sodium nitrate ($NaNO_3$)	1,5 g
Dipotassium phosphate K_2HPO_4	0,04 g
Magnesium sulphate ($MgSO_4$)	0,075 g
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	0,036 g
Sodium carbonate (Na_2CO_3)	0,006 g
Ferric ammonium citrate	0,006 g
Disodium EDTA dihydrate	0,001 g
Citric acid monohydrate	0,02 g
Sodium chloride (NaCl)	120 g

The final volume of the solution was brought up to 1 L and sterilized by autoclaving.

In a separate Schott bottle, the following chemicals were measured, and added to 450 ml of distilled H_2O

Chemical	Quantity
Boric acid (H_3BO_3)	2,86 g
Manganese (II) chloride tetrahydrate ($MnCl_2.4H_2O$)	1,81 g
Zinc sulphate heptahydrate ($ZnSO_4.7H_2O$)	0,22 g
Sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$)	0,39 g
Copper (II) sulphate pentahydrate ($CuSO_4.5H_2O$)	0,079 g
Cobalt (II) nitrate hexahydrate (Co $(NO_3)_2.6H_2O$)	49,4 mg

The final volume was brought up to 500 ml. The final working solution was sterilized using a 0,22 µm filter, and the trace metal solution was stored 4°C and ready for use. When the media cooled down, 1 ml of trace metal solution was added and the media was stored in a cool place, ready for use.

Preparation of TAE (Tris Acetate EDTA) buffer

Diluted 50x concentrated TAE buffer 50-fold with ultrapure water.

Preparation of 1% agarose gel

Measured out 1 g of agarose powder and mixed into 100 ml 1x TAE buffer solution. The solution was microwaved under high heat for 1-3 minutes until the agarose powder fully dissolved. It is essential to ensure that the agarose does not boil over as this affects the final concentration of agarose present in the gel. Once the solution was cool, 2 µl of ethidium bromide was added. The solution was poured into a casting tray and allowed to set.

Preparation of DPPH (2,2-diphenyl-1-picrylhydrazyl)

Measure 3,94 mg of DPPH and add to 100 ml of methanol. It is essential to work in a dimly lighted area as DPPH is light sensitive and prone to degradation when exposed to light. Schott bottles were covered with foil and the solution was stored at 4°C.