BIOCHEMICAL AND GENOMICS-BASED STUDIES ON *Oxynema aestuarii*, A POTENT PRODUCER OF BIOACTIVE COMPOUNDS

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JADAVPUR UNIVERSITY

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BIOCHEMICAL AND GENOMICS-BASED STUDIES ON *Oxynema aestuarii*, A POTENT PRODUCER OF BIOACTIVE COMPOUNDS

A thesis

Submitted in partial fulfilment of the requirements for the award of the degree of
Master of Technology in Environmental Biotechnology

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by

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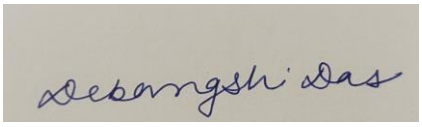
DECLARATION

I hereby declare that the work presented in this thesis report title **"Biochemical and Genomics-based Studies on *Oxynema aestuarii*, a Potent Producer of Bioactive Compounds"** submitted to Jadavpur University, Kolkata in partial fulfilment of the requirements for the award of the degree of Master of Technology in Environmental Biotechnology is a bonafide record of the research work carried out under the supervision of Prof. Joydeep Mukherjee. The contents of Thesis report in parts, have not been submitted to and will not be submitted by me to any other Institute or University in India or abroad for the award of any degree or diploma.



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CERTIFICATE

This is to certify that the Thesis Report titled "**Biochemical and Genomics-based Studies on *Oxynema aestuarii*, a Potent Producer of Bioactive Compounds**", submitted by Debangshi Das (002130904001) to Jadavpur University, Kolkata for the award of the degree of Master of Technology in Environmental Biotechnology is a bonafide record of the research work done by her under my supervision. To the best of my knowledge, the constituents of this report, in full or in parts, have not been submitted to any other Institute or University for the award of any degree or diploma.

Prof. Joydeep Mukherjee

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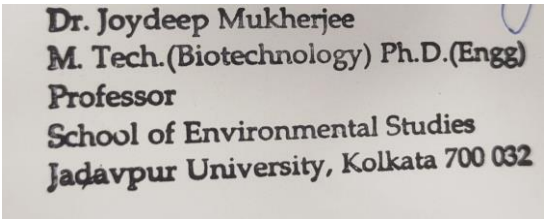
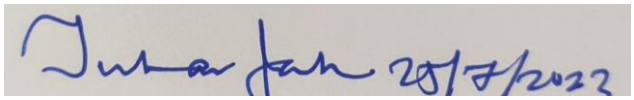


TO WHOM IT MAY CONCERN

It is hereby notified that this thesis titled "**Biochemical and Genomics-based Studies on *Oxynema aestuarii*, a Potent Producer of Bioactive Compounds**", is prepared and submitted for the partial fulfilment of the continuous assessment of Master of Technology in Environmental Biotechnology course of Jadavpur University by Debangshi Das (002130904001), a student of the said course for session 2021-2023. It is also declared that no part of this thesis has been presented or published elsewhere.



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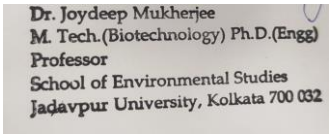

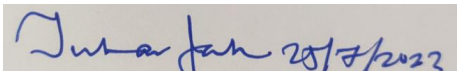

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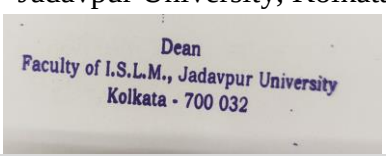

CERTIFICATE OF APPROVAL

This foregoing thesis is hereby approved as a credible study of an engineering subject carried out and presented in a manner satisfactorily to warranty its acceptance as a prerequisite to degree for which it has been submitted. It is understood that by this approval the undersigned do not endorse or approve any statement made or opinion expressed or conclusion drawn therein but approve the thesis only for purpose for which it has been submitted.

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Final examination for evaluation of thesis

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Signature of the Examiner

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Debangshi Das

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List of abbreviations

- % - Percentage
- nm - Nanometre
- gm - Gram
- mg - Milligram
- m - Metre
- cm - Centimetre
- µl - Microlitre
- µmol - Micromole
- µg - Microgram
- ml - Millilitre
- ˚ C - Degree Celsius
- kDa - Kilodalton
- sec - Second
- s - Second
- min - Minute
- v/v - Volume/volume
- rpm - Revolutions per minute
- DNA - Deoxyribonucleic acid
- RNA - Ribonucleic acid
- UVR - Ultraviolet radiation
- ASN III - Artificial seawater nutrients III
- m/z - Mass to charge ratio

- HPLC - High performance liquid chromatography
- LC/MS - Liquid chromatography / mass spectrometry
- MAA - Mycosporine-like amino acid
- λ - Lambda
- M - Molar
- antiSMASH - Antibiotics and secondary metabolite analysis shell
- DNAse - Deoxyribonuclease
- RNAse - Ribonuclease
- rRNA - Ribosomal RNA
- NCBI - National Center for Biotechnology Information
- BLAST - Basic Local Alignment Search Tool
- ITS - Internal Transcribed Spacer
- NRPS - Non ribosomal peptide synthetase

Abstract

Cyanobacteria are a group of prokaryotic gram-negative bacteria which play an important role as primary producers as well as in biogeochemical cycles. The non-toxic secondary metabolites produced by cyanobacteria have promising advantages for their UV absorption activity and therapeutic values. Mycosporine-like amino acid, a secondary metabolite possesses significant antiUV properties and other biological functions. One of the eight obligately halophilic, filamentous mangrove cyanobacteria previously isolated by our workgroup from Indian Sundarbans, was recently established as a novel species of the newly described genus *Oxynema*. The whole genome sequence of this novel cyanobacterium revealed numerous genes corresponding to valuable secondary metabolites of pharmacological importance. Mycosporine-like amino acids are rapidly emerging as important natural photoprotective compounds with a wide range of applications. This study is an attempt to characterize the genes responsible for the production of these compounds, as well as their partial purification and identification by means of liquid chromatography and mass spectrometry.

Keywords: Cyanobacteria, mycosporine-like amino acid, secondary metabolites, Indian Sundarbans, photoprotective compounds, liquid chromatography, mass spectrometry, UV absorption

1. Introduction

1.1 Cyanobacteria: physiology and morphology

Cyanobacteria, the 'Blue-Green Algae' are an ancient group of oxygen-evolving, photosynthetic prokaryotes that possess features similar to both algae & bacteria and widely distributed in marine and terrestrial ecosystems (Kageyama and Waditee-Sirisattha, 2018). Their cellular composition & structure are quite similar to the prokaryotic cells due to the absence of a well-defined nucleus & other cellular organelles (which are present in eukaryotic cells). The cell wall structure & composition of cyanobacteria are quite similar to that of gram negative bacteria. However, unlike the typical prokaryotes they are capable of oxygenic photosynthesis, similar to algae, due to the presence of chlorophyll *a*. Generally cyanobacteria possess both of the photosystems, PSI & PSII and by using water (H_2O) as the electron donor, they produce oxygen during photosynthesis. In the presence of electron donors like hydrogen sulphide are present, certain cyanobacteria may also carry out anoxygenic photosynthesis with only photosystem I (Madigan, Martinko and Parker, 2003). All known cyanobacteria are photoautotrophic in nature by utilizing carbon dioxide (CO_2) as the carbon source (Castenholz *et al.*, 2001).

Cyanobacteria exhibit a wide range of morphological variations. They can either have a unicellular structure or form spherical, flat, regular or irregular colony clusters. They may also form filaments with or without branches. Some of them are able to produce specific cells such as heterocysts & akinetes. In order to adapt to chemical & physical factors, several cyanobacterial species possess gas vesicles which allow them to regulate their buoyancy as well as to maintain a certain vertical position in the water column (Walsby, Hayes and Boje, 1995). Cyanobacteria

reproduce asexually by forming hormogonia or spores; or by fission in unicellular forms; or by fragmentation in filamentous species.

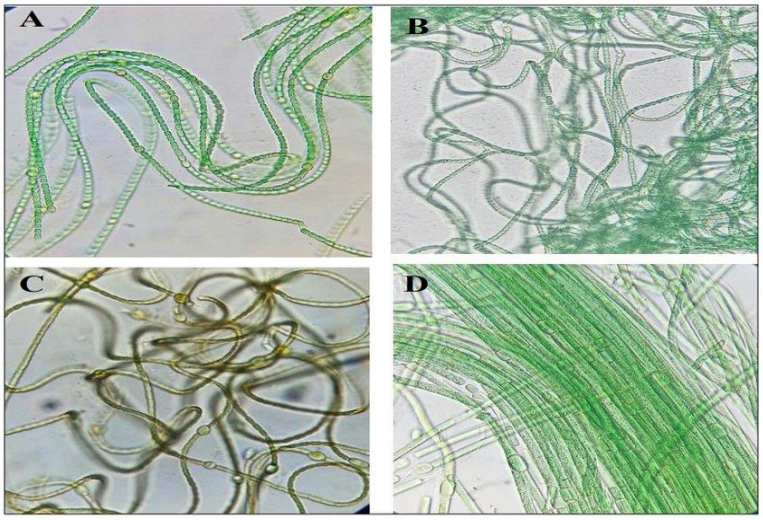


Fig 1: Microphotographs of different cyanobacteria (A) *Nostoc calcicola*, (B) *Nostoc linckia*, (C) *Nostoc punctiforme*, (D) *Anabaena oryzae* (Yadav, Singh and Gupta, 2022)

1.2 Cyanobacteria: unique features

Cyanobacteria are known for their unique features as well as for their contribution to photosynthetic biomass production by converting solar energy & carbon dioxide to chemical energy. They also play an important role in the global biogeochemical cycle owing to their ability to fix atmospheric nitrogen (Sinha *et al.*, 2020). They have evolved certain mechanisms and adopted survival strategies which enable them to thrive under adverse environmental conditions including high salt concentration, desiccation, and highly basic or acidic pH. Moreover, cyanobacteria are highly exposed to ultraviolet radiations during photosynthesis. The increased incidence of solar ultraviolet (UV) radiation on the surface of the earth is thought to be a significant physiological stressor for all living forms (Rastogi, Sonani and Madamwar, 2017). In due course of evolution, these prokaryotes have developed several tolerance and defence

mechanisms to avoid the detrimental effects of UV radiations such as protein re-synthesis, production of antioxidants, DNA repair, and also the synthesis of UV screening/absorbing compounds. These natural substances known as secondary metabolites are not directly essential to an organism's primary metabolism but they are generally unique to a particular organism and absent under optimal environmental conditions. Secondary metabolites are frequently produced by cyanobacteria in response to biotic & abiotic stress conditions in the surrounding environment for protection, thereby providing them certain beneficial advantages over other species. Cyanobacteria are a rich source of secondary metabolites due to their ability to persist in adverse environmental conditions(Sinha *et al.*, 2020). Cyanobacterial secondary metabolites can be toxic or non-toxic. Toxic secondary metabolites are typically classified according to the organs they affect.

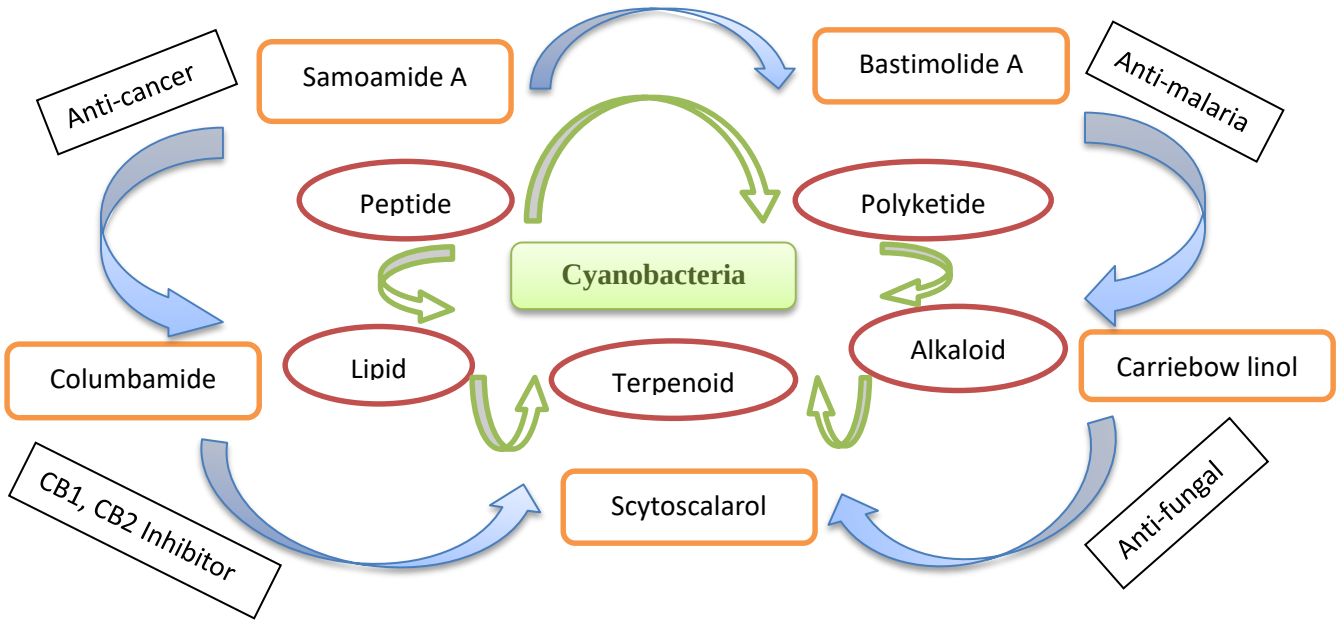


Fig 2: Biologically active cyanobacterial secondary metabolites (Sinha *et al.*, 2020)

Dermatotoxins irritate the skin, hepatotoxins like microcystins damage the liver, neurotoxins like anatoxin-a and saxitoxin affect the neuromuscular transmission, and cardiotoxins like yessotoxin affect the heart. Conversely, phytohormones, siderophores, and different UV-protective substances are examples of non-toxic secondary metabolites. An increase in high-energy solar radiation (280 to 315 nm) has aroused interest in the search for natural photoprotective biomolecules. Numerous UV absorbing/ screening biomolecules have been reported from various species of cyanobacteria such as mycosporine-like amino acids (MAAs), scytonemin (Scy), melanins, carotenoids, flavonoids, parietin, and usnic acid (Rastogi, Sonani and Madamwar, 2017). Biosynthesis or accumulation of photoprotective compounds such as MAAs and scytonemin has been documented as important shields against short wavelength UV-A or UV-B radiation in several micro/ macroalgae and cyanobacteria (Sinha *et al.*, 2020).

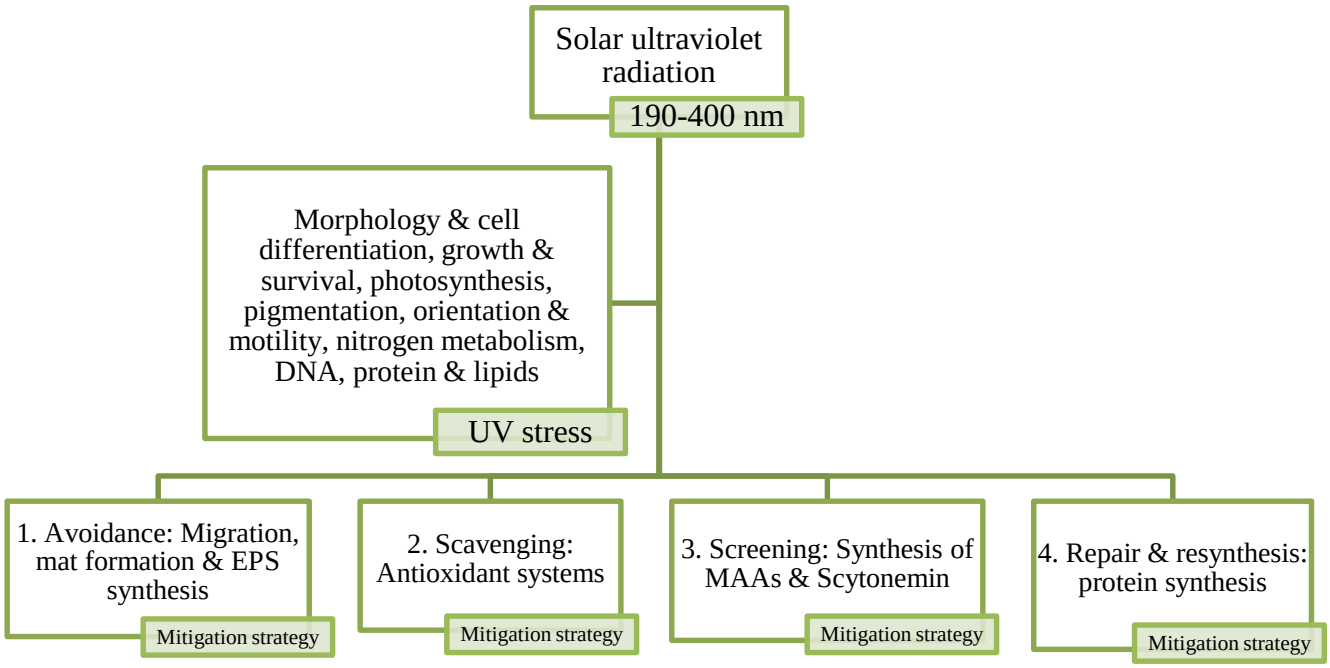


Fig 3: Defense mechanisms adopted by cyanobacteria to overcome the detrimental effects of solar ultraviolet radiation (Rastogi, Sonani and Madamwar, 2017)

Several studies have revealed that cyanobacteria produce various compounds which possess pharmaceutical properties. Most of the biological activity exhibited by cyanobacteria is usually associated with lipopeptides which show anti-tumor, antiviral, antibiotic, anti-malarial or anti-mycotic activities. Over the past few years, several classes of myeloma cell toxins with apoptotic properties have been identified in marine cyanobacteria. Some important compounds isolated from cyanobacteria that target cancers are given as follows: colon cancers are targeted by minutissamides, microcystilide A, laxaphycins, cylindrocyclophanes and bauerines A-C while breast cancers are targeted bycarbamidocyclophanes, dendroamide, hapalosin and tolyporphins; lung cancer is targeted by pahayokolide A; and prostate cancer by tychonamide (Basu *et al.*, 2021).

These properties have prompted the exploration of cyanobacteria as prospective candidates in the field of pharmaceutical research. Focus on the discovery of new taxonomic species may be advantageous regarding the production of novel compounds. Overall, cyanobacteria produce numerous bioactive secondary metabolites, which has inspired researchers to modify these organisms to increase their productivity.

1.3 Mycosporine like amino acids (MAAs): properties, synthesis, features

Cyanobacteria synthesize mycosporine-like amino acids (MAAs) in response to UV radiation. MAAs are one of the strongest UV absorbing compounds (having absorption maxima ranging from 268 to 362 nm) in nature (Sinha *et al.*, 2020). MAAs are small, colourless, hydrophilic, intracellular secondary metabolites that exhibit molecular weights ranging from 188 to 1050 Da and comprised of cyclohexenone or cyclohexenimine chromophores conjugated with the amino acid nitrogen substituent or its imino alcohol (Sinha *et al.*, 2020). Generally, the ring system of

MAAs contains a glycine subunit at the third carbon atom. Some MAAs also contain sulfate esters or glycosidic linkages from the imine substituent. MAAs are extremely hydrophilic because of the zwitter-ionic form derived from amino acid substitution. MAAs have higher molar extinction coefficient with molar attenuation coefficients (ε) between 28,100 and 50,000 M^{-1} cm^{-1} (Sinha *et al.*, 2020). Their photostability in seawater in the presence of photosensitizers, high UV absorption maxima, resistance & stability in the presence of various physicochemical stressors make them potent natural photoprotectants.

Various studies have suggested that the enzymatic machinery for MAA biosynthesis likely originated from cyanobacteria or cyanobacterial ancestors, whereas the endosymbiotic events & prokaryote-to-eukaryote lateral gene transfer events during evolution are thought to be responsible for their prevalence among all other biological taxa. Two novel enzymatic pathways for imine production have been revealed by the identification of an MAA biosynthetic gene cluster in a cyanobacterium, which led to the discovery of related pathways in other sequenced species. Thereafter, the pentose phosphate pathway and the shikimate pathway have been designated as the primary routes for UV induced MAA biosynthesis.

MAA biosynthesis supposedly occurs via the initial part of the shikimate pathway, where 3-dehydroquinate is formed during the preliminary stages of this pathway which serves as a precursor for the synthesis of primary MAAs via 4-deoxygadusols (4-DG) (Kageyama and Waditee-Sirisattha, 2018).

On the other hand, it has also been suggested that the MAAs may derive from the pentose phosphate pathway by forming sedoheptulose-7-phosphate as an intermediate via 4-DG (Rastogi, Sonani and Madamwar, 2017).

It was found that in *Anabaena variabilis*, a cluster of four genes dehydroquinate synthase (DHQS) homolog Ava_3858, O-methyltransferase (O-MT) Ava_3857, ATP-grasp Ava_3856, and nonribosomal peptide synthetase (NRPS) homolog Ava_3855 was found, responsible for MAA (shinorine) biosynthesis (Rastogi, Sonani and Madamwar, 2017) (Pope *et al.*, 2014).

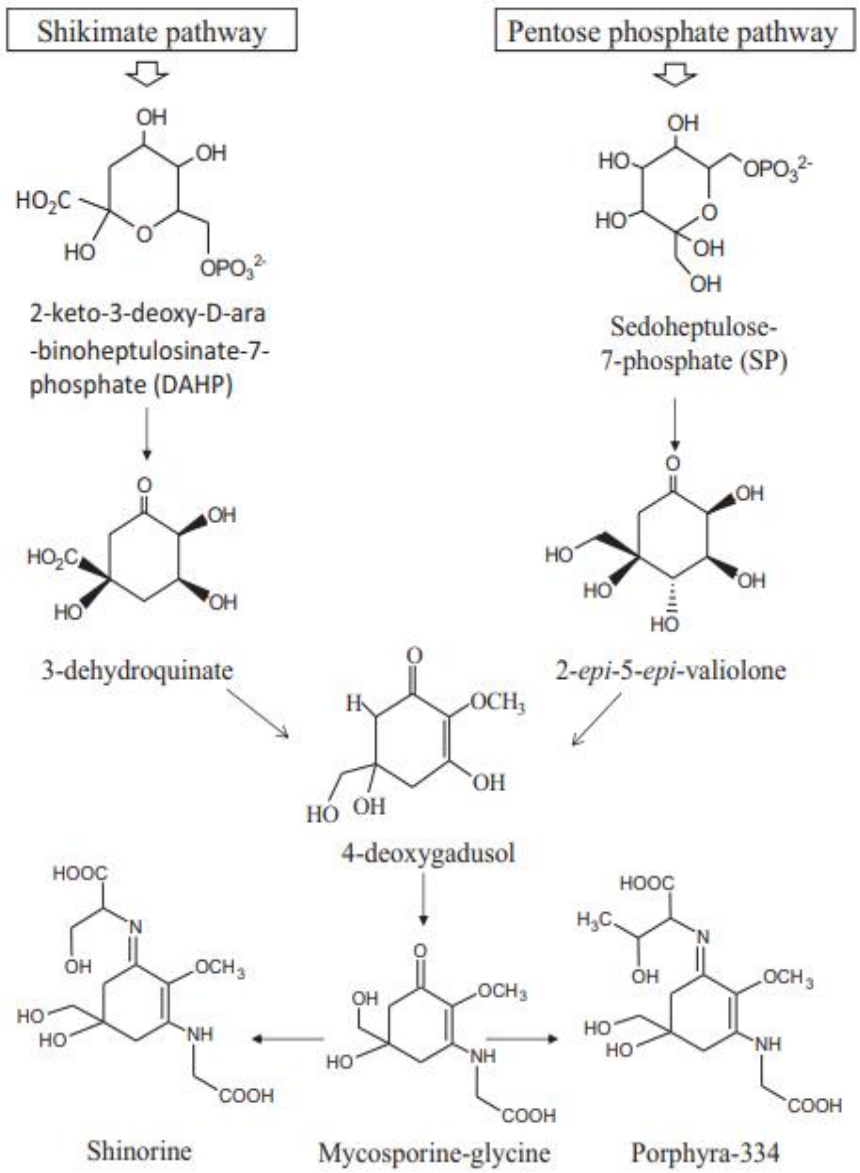


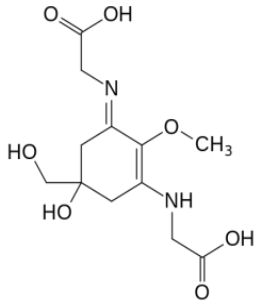
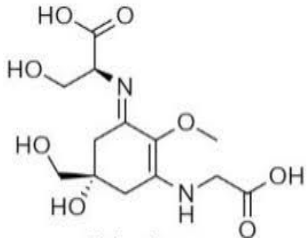
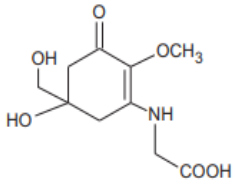
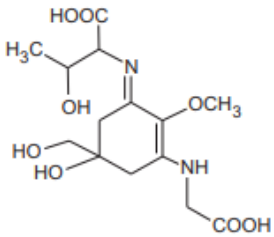
Fig 4: A proposed pathway of MAAs biosynthesis (Rastogi, Sonani and Madamwar, 2017)

The precursor is converted into 4-DG by the enzymes DHQS & O-MT, whereas the enzyme ATP-grasp catalyzes the step of glycine addition to 4-DG to form mycosporine-glycine (M-Gly) and NRPS catalyzes the addition of serine to M-Gly to form shinorine. It has also been reported that, in the cyanobacterium *Nostoc punctiforme* ATCC 29133, a cluster of four genes (NpR5600, NpR5599, NpR5598, and NpF5597) is responsible for MAA synthesis (Rastogi, Sonani and Madamwar, 2017) (Hickman, 2012). However, *Anabaenopsis elenkinii* CCIBt3563 is likely to possess non-clustered low identity genes which are scattered throughout their genomes related to MAA biosynthesis (Dextro *et al.*, 2023).

As mentioned earlier, photoprotective compounds MAAs exhibit strong UV absorption maxima, high molar extinction coefficients, photostability, and resistance to several abiotic stressors and play an important role in photoprotection of organisms residing under intense solar light. Biosynthesis or accumulation of MAAs in diverse organisms in response to UV-A/B radiations strongly favours their role in diminishing the harmful effects of short wavelength UV radiation. MAAs can dissipate absorbed radiation efficiently as heat without producing ROS. Additionally, MAAs possess strong antioxidant properties and play a major role in osmotic equilibrium of various marine organisms, especially in the photosymbiotic relationship (La Barre, Roullier and Boustie, 2014). It has been proposed that MAAs provide protection to their producers as well as to the primary and secondary consumers through their food chain. MAAs have become promising candidates for their use in pharmaceutical and cosmetic applications and have been commercialized due to their high-UV absorption coefficients and their ability to protect the skin from UV-mediated damage. Moreover, MAAs have other biological functions along with their UV absorbing properties. For example, mycosporine-2-glycine (M2G) may exhibit free radical scavenging activity, osmoprotectant activity, protection against oxidative stress-induced cell

death, the inhibition of protein glycation and the inhibition of collagenase activity (Sinha *et al.*, 2020).

Currently, more than 25 MAAs have been reported from various organisms including several strains of cyanobacteria.

Mycosporine-like Amino Acid	**Molecular Structure**	**Absorbance λ_{max} (nm)**
Mycosporine-2-glycine		334
Shinorine		334
Mycosporine-glycine		310
Porphyra-334		334

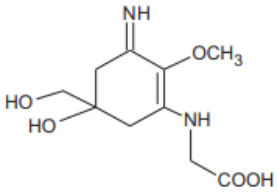
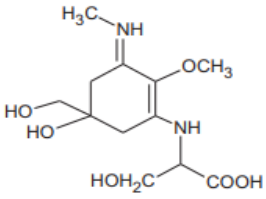
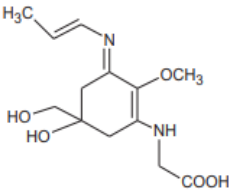
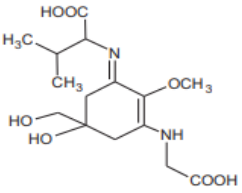
Palythine		320
Mycosporine-methylamine-serine		327
Palythene		360
Mycosporine-glycine-valine		335

Table 1: Some common MAAs isolated from cyanobacteria with their molecular structure and absorbance

MAAs can be characterised by using several techniques such as HPLC analysis, electrospray ionization-mass spectrometry, nuclear magnetic resonance spectroscopy.

2. Literature Review

Microbes from the understudied ecosystems are currently the focus of various scientific researches, based on the notion that they can offer attractive prospects for the discovery of new natural products. The estuarine mangrove ecosystem is one such unexplored habitat. Mangrove forests are large ecosystems found in 112 countries and territories, covering an area of 181,000 km^2 and occupying more than a quarter of the world's coastline. Located at the confluence of land and sea, mangrove forests have the potential of becoming a significant resource for biodiversity & chemodiversity, as the mangrove forests harbour a rich microflora. Furthermore, the existence of cyanobacteria in the microbial communities of mangrove estuaries and their involvement in nitrogen fixation have been one of the major topics of research around the world.

2.1 Study area & field sample collection: The study area for this research lies in the Sundarbans mangrove forest, one of the largest tidal mangrove forests in the world (140,000 ha). The forest is located on the delta formed by the Ganges, Brahmaputra and Meghna rivers on the Bay of Bengal. This ecosystem harbours several novel halophilic and halotolerant cyanobacteria. In this background, a systemic investigation has been conducted on the marine cyanobacteria of the Indian Sundarbans in the search novel bioactive compounds (Chauhan, 2006).

The sampling was done by aseptic collection of the cyanobacterial soil surface biofilms in the post-monsoon period from 14 locations on Sagar island (21°44′7″ N, 88°7′2″ E) in November 2006 and 12 locations on Lothian island (21°39′1″ N, 88°19′37″ E) in December 2007 (Pramanik *et al.*, 2011)

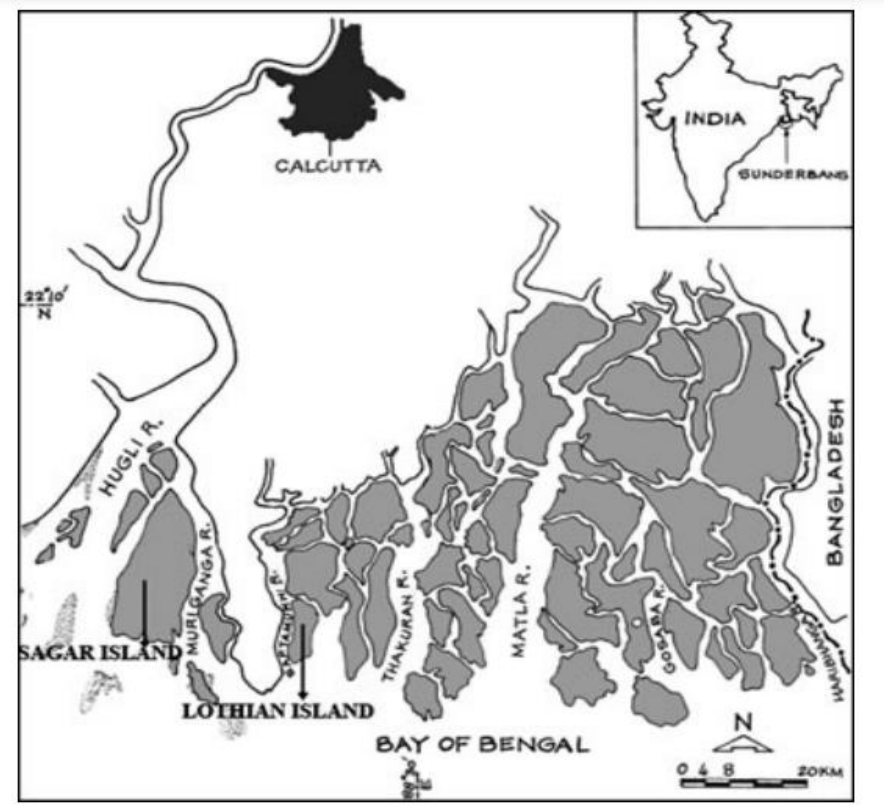


Fig 5: Sampling stations of the area under study. Gray shade represents the islands of the Indian Sundarbans. Samples were collected from the southern coast of the Sagar Island and the entire coast of the Lothian Island at near equidistant locations (Pramanik *et al.*, 2011)

2.2 Sample identification & processing: Eight obligately halophilic, euryhaline cyanobacteria from intertidal soil from the Sagar & Lothian islands were isolated in Artificial Seawater Nutrient III (ASN-III) medium (pH 7.5±1) and labelled as AP3, AP9F, AP17, AP20, AP24, AP25, AP3b, AP3U (Pramanik *et al.*, 2011). The isolated cyanobacterial strains were grown in culture rooms having fluorescent irradiance (50 µmol photons. m^{-2}. s^{-1}) in 12:12 hours light: dark cycle at 25±1° C as well as periodically checked for purity (Chakraborty *et al.*, 2018). Based on their morphological characteristics, six out of the eight types of isolated cyanobacteria were formerly categorized under the *Lyngbya Phormidium-Plectonema* (LPP) group B, and one each was assigned to *Oscillatoria* and *Synechocystis* genera (Chatchawan *et al.*, 2012).

Four novel strains were discovered via polyphasic approach based taxonomic characterisation. Out of these four, the strains AP17 & AP24 which were isolated from the biofilm obtained from Lothian Island & Sagar Island respectively, are novel species, named as *Oxynema aestuarii* belonging to *Oxynema* genus. The suitable range of salinity for the growth of *Oxynema aestuarii* was in between 5% to 8% NaCl. The growth was inhibited at salinity greater than 14%.

Antimicrobial activity, 16S rRNA gene sequences, phenotypic characters as well as growth and antibiosis in response to variable salinity, temperature, phosphate concentration, and pH were also studied for these strains.

2.3 Morphological characterization of *Oxynema aestuarii*: Both of the strains AP17 & AP24 showed benthic attachment to the surface of the flasks used for subculturing. The morphology of these two strains were similar belonging to the *Oxynema* genus which was separated from the Phormidium-Group I based on certain morphological features such as attenuated trichomes, thylakoidal morphology as well as different growth patterns. Light microscopy showed that the strain *O. aestuarii* was comprised of long, straight trichomes with unbranched simple filaments and none of the trichomes included akinetes, heterocytes, or gas vacuoles (Chakraborty *et al.*, 2018). By using scanning electron microscopy, the presence of well-defined mucilaginous sheath around the filaments was confirmed. The longitudinal axis of the cell consisted thylakoids along its whole volume (Chakraborty *et al.*, 2018). Apart from the general arrangement, the thylakoid membranes were parted due to the presence of double row of phycobilisomes, the light harvesting antenna complex (Chakraborty *et al.*, 2018). Transmission electron microscopy revealed the presence of a prominent cell wall, cellular inclusions such as cyanophycin granules and carboxysomes and absence of gas vacuoles (Chakraborty *et al.*, 2018)

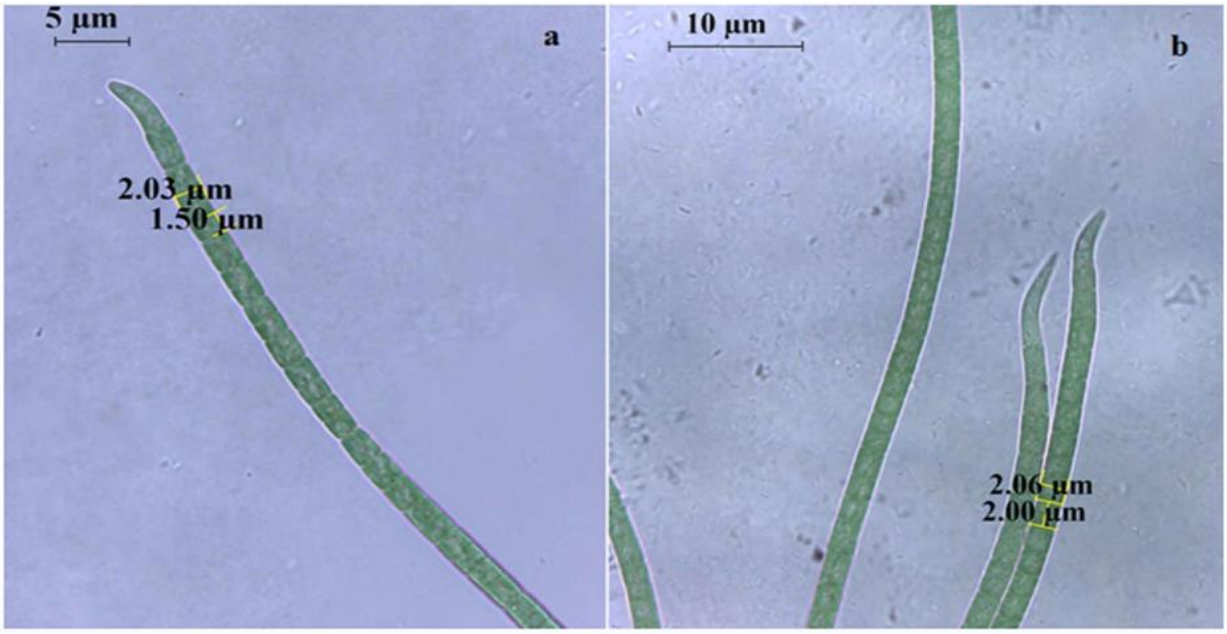


Fig 6: Light microscopy of *Oxynema aestuarii* a. Microphotograph showing the filaments of strain AP24 with cellular dimensions. b. Microphotograph of strain AP17 showing filaments with cellular dimensions (Chakraborty *et al.*, 2018)

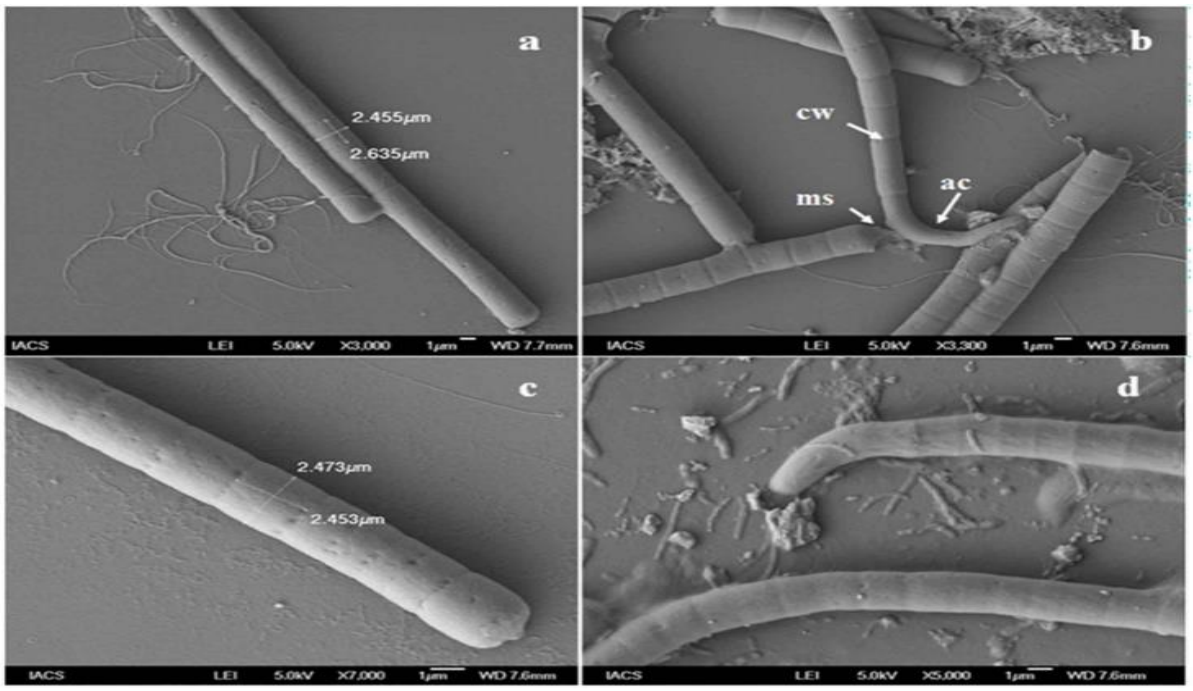


Fig 7: Scanning electron microscopy of *Oxynema aestuarii* (non-axenic culture) a and b Part of filament of AP17 showing cellular dimensions along with apical cell morphology and mucilage sheath; c and d Part of filament of strain AP24 showing cellular dimensions. (ac= apical cell, cw= cross wall, ms= mucilaginous sheath) (Chakraborty *et al.*, 2018)

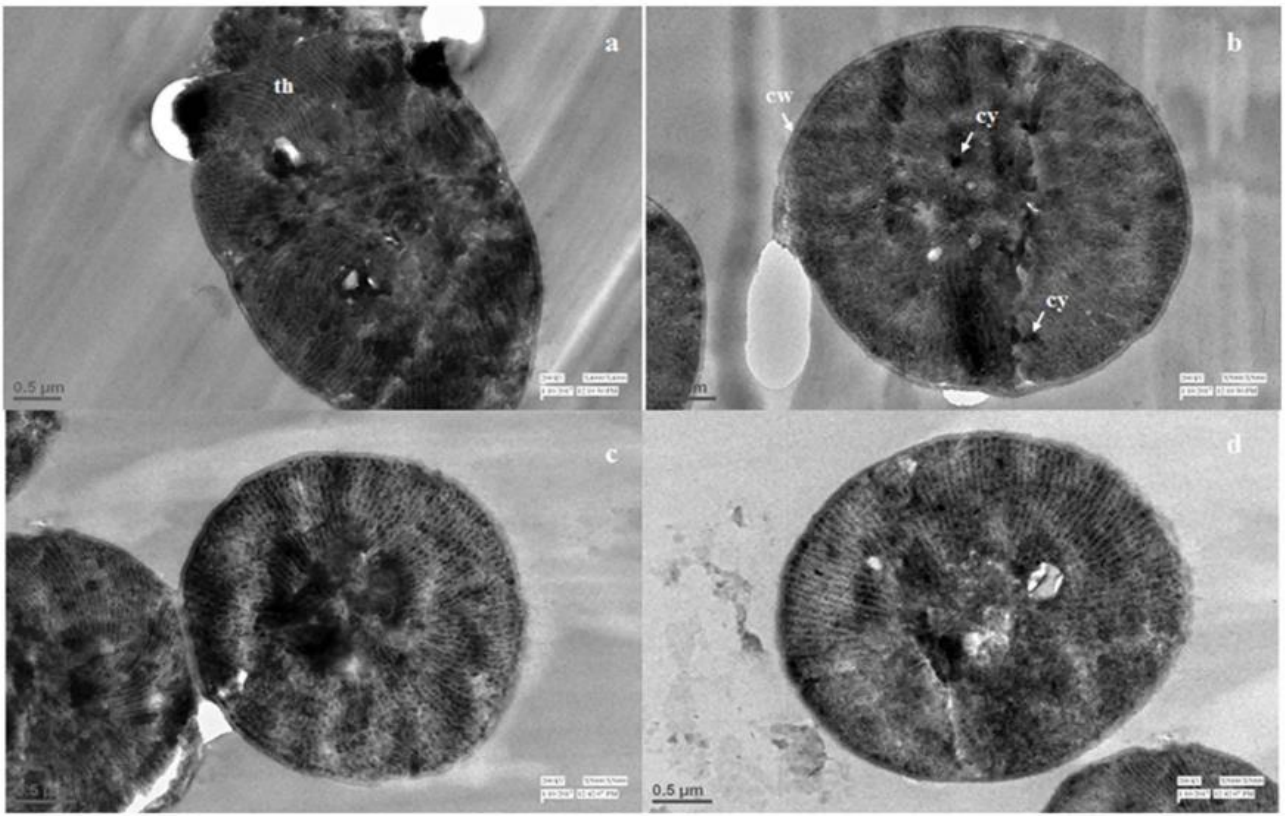


Fig 8: Transmission electron microscopy of Oxynema aestuarii. a and b Cross sections of the filaments of strain AP17; c and d Cross sections of the filament of strain AP24. Characteristics show prominent radial arrangement of thylakoids and presence of few cynophycin granules. (cw= cell wall, cy= cynophycin, th= thylakoids) (Chakraborty *et al.*, 2018)

2.4 Analysis of 16s rRNA gene sequences of *Oxynema aestuarii*: By using Basic Local Alignment Search Tool (BLAST) the 16S rRNA gene sequences of AP17 & AP24 as well as the reference strain were searched within the National Center of Biotechnology Information (NCBI) database and pairwise similarity was determined with the other available species. In order to establish a valid evolutionary relationship with the isolated strains, a consensus phylogenetic tree was created using the partial 16S rRNA gene sequences (length >1100 bp) of the closest relatives of the strains (AP17 & AP24) as well as the sequences of the representative

members (reference strains) of each cyanobacterial group from the NCBI database (Chakraborty *et al.,* 2018).

According to the phylogenetic analysis, AP17 (1387 bp) & AP24 (1369 bp) showed 99% to 100% sequence similarity with *Oxynema thaianum* CCALA960 with a query coverage of 81% and referred as the sister taxon (Chakraborty *et al.*, 2018). The phylogenetic tree has revealed that clade of AP17 & AP24 was separated from the clade of *Oxynema thaianum* CCALA960, the closest related taxa and their relationship was supported by the high bootstrap value (Chakraborty *et al.*, 2018). On the other hand, the constructed phylogenetic tree showed low genetic similarity between AP17 & AP24 with *Oxynema acuminatum* (available as *Oscillatoria acuminata* PCC6304, accession no. NR_102463.1 in NCBI database) although the comparison 0f 16s rRNA gene sequence of AP17 & AP24 with *Oxynema acuminatum* showed 91% sequence similarity and query coverage of 100% (Chakraborty *et al.*, 2018). The occurance of both the newly isolated strains and *Oxynema acuminatum* in separate clusters of the phylogenetic tree has suggested that *Oxynema aestuarii* & *Oxynema acuminatum* are not same species and distantly related to reach other (Chakraborty *et al.*, 2018). Moreover, sequence similarity of these two isolates with other genera was lower than 95%.

2.5 Analysis of the 16S-23S ITS gene sequences of *Oxynema aestuarii*: The internal transcribed spacer (ITS) sequences of AP17 and AP24 strains showed differences with the reference strain *Oxynema thaianum* CCALA960 which included insertion of 2 nucleotides in the pre Box B spacer region, insertion of 9 nucleotides in the D2 with spacer region, deletion of 8 nucleotides in the D4 region, deletion of 2 nucleotides in the post Box B spacer region, deletion of 8 nucleotides in V3 region and insertion of 2 nucleotides in the D5 region of the ITS

sequences of AP17 and AP24 compared to CCALA960 (Chakraborty *et al.*, 2018). After analyzing the structural details of Box B helices of those two isolates (AP17 & AP24), it was revealed that even though there lengths were identical but their sequences were different from the reference strain CCALA960 (Chakraborty *et al.*, 2018). The secondary structure of V3 regions of AP17 & AP24 (comprising of 51 nucleotides) a bigger bilateral bulge and a small terminal bulge whereas the analogous structure of *Oxynema thaianum* CCALA960 (containing 59 nucleotides) showed one additional bilateral bulge compared to the two isolates (Chakraborty *et al.*, 2018).

Hence, based on the ecological, morphological and molecular differences compared to other species, these two new strains isolated from the Indian Sundarbans, AP17 & AP24 were proposed to be categorized under *Oxynema* genus as a second novel species named as *Oxynema aestuarii*.

2.6 Background of anti-tumor & anti-angiogenic activity of secondary metabolites produced by AP24 isolate (Basu *et al.*, 2021): The isolated cyanobacterial strain AP24 collected from Sagar island of Sundarbans, was grown in ASN III medium at optimum pH level and temperature as mentioned. After that, mass cultivation of the established mono-culture strain of cyanobacteria was individually grown in 20 litre volume plastic jar (washed and disinfected with benzalkonium chloride), followed by adding 12 litre of sterile ASN III medium along with approximately 5 grams (wet mass) cyanobacterial cultures as inoculum. Aeration was achieved by using pumps to mix the media components as well as maintaining the light and temperature conditions for growth. To test the anti-inflammatory activity, human monocytic leukemia THP-1 cells were used which were previously treated with

Dexamethasone (1 μM) for 12 hours. Later the pretreated cells were stimulated for 4 hours with LPS-50ng/ml. After that, ELISA was done for TNF measurement of the collected cell supernatant. For anti-angiogenic activity measurement, the EA.hy926 endothelial cell line obtained from hybridization of human umbilical vein endothelial cell line with A549/8 human lung cancer cell line was used for in-vitro assay. Secondary metabolites obtained from the cyanobacterial biomass extracts (with ethyl acetate) have shown promising anti-inflammatory activity by ≥75% inhibition of inflammation compared to Dexamethasone at the initial 100 μg/ml test dose and anti-angiogenic activity by reducing the capillary structures (>25% inhibition compared to standard compound, Sunitinib) at the initial 100 μg/ml test dose (tested at CDRI, Lucknow).

3. Aims & Objectives

MAAs may be considered as broad-spectrum UV absorbers/protectors and can be used as potential ingredients in cosmetics and other cosmeceutical industries as MAAs can prevent the occurrence of UV induced skin damage. Moreover, identification of UV screening pigments in aquatic ecosystem is difficult since there is no proper information and guidelines. Additionally, dedicated study of photoprotective compound in mangrove cyanobacteria is extremely limited.

- This study work is aimed at the partial purification of the anti-UV compounds (MAAs) produced by *Oxynema aestuarii* isolated from a biofilm collected from Sagar island.
- In silico "antibiotics and secondary metabolite analysis shell—antiSMASH" analysis of the secondary metabolite biosynthetic gene clusters in the genome of *Oxynema aestuarii* entails the second objective of this study.
- Bioinformatics based mapping of genes responsible for MAA biosynthesis comprises the final objective of this study.

4. Materials & Methodology

4.1 Materials required for high performance liquid chromatography (HPLC) analysis:

For the growth of the cyanobacterial biomass:

- Artificial Seawater Nutrients (ASN-III) medium, (pH 7.5±0.1)

  Chemicals required for preparing 1lit of ASN-III media:

  i. Sodium chloride (NaCl) - 25 gm
  ii. Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) - 3.5 gm
  iii. Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) - 2.0 gm
  iv. Sodium nitrate ($NaNO_3$) - 0.75 gm
  v. Calcium chloride dihydrate ($CaCl_2.2H_2O$) - 0.5 gm
  vi. Potassium chloride (KCl) - 0.5 gm
  vii. Citric acid - 3 mg
  viii. Mg EDTA - 0.5 mg
  ix. A5 trace element mixture - 1 ml
  x. Ferric ammonium Citrate - 3 mg
  xi. Distilled water - 1 lit
  xii. Di-potassium hydrogen phosphate trihydrate ($K_2HPO_4.3H_2O$) - 0.02 gm
  xiii. Sodium carbonate (Na_2CO_3) - 0.02 gm

  For preparing 1 lit of A-5 trace element mixture:

  - Boric acid (H_3BO_3) - 2.86 gm

- Manganese(II) chloride tetrahydrate ($MnCl_2.4H_2O$) - 1.81 gm
- Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) - 0.222 gm
- Sodium molybdate dihydrate ($NaMoO_4.2H_2O$) - 0.39 gm
- Copper sulfate pentahydrate ($CuSO_4.5H_2O$) - 0.079 gm
- Cobalt (II) nitrate hexahydrate ($Co(NO_3)_2.6H_2O$) - 49.4 mg
- Distilled water- 1 lit

For extraction of MAAs:

- Cyanobacterial biomass (*Oxynema aestuarii*, strain collected from the intertidal soil of the mangroves of Sagar island, Sunderbans and isolated & grown in artificial seawater nutrients III (ASN-III) medium (pH 7.5±0.1))
- High performance liquid chromatography (HPLC) grade methanol
- HPLC grade water

For purification of MAAs:

- HPLC grade acetic acid
- HPLC grade methanol
- HPLC grade water
- Crude MAAs (extracted from the cyanobacterial biomass)

- To find the genes responsible for MAA biosynthesis and to analyse the biosynthetic gene clusters of *Oxynema aestuarii*, several softwares were used listed below.

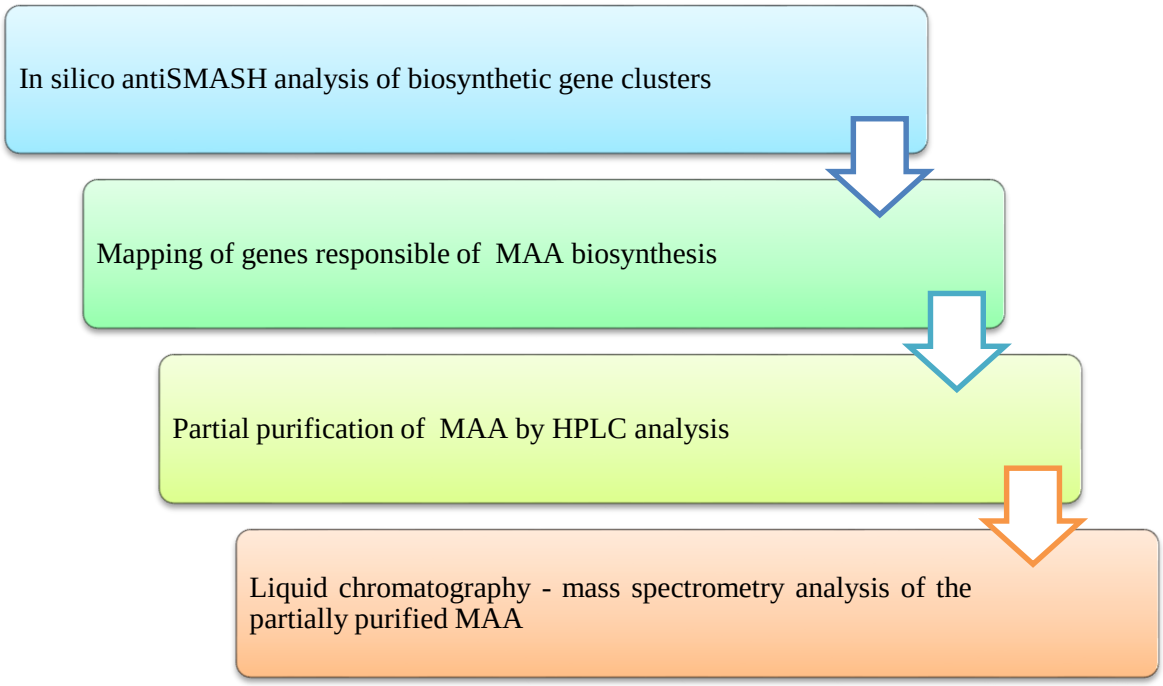


Fig 9: Schematic representation of methodologies

4.2 Software analysis:

4.2.1 In silico antiSMASH analysis:

The "antibiotics and secondary metabolite analysis shell—antiSMASH" (https://antismash.secondarymetabolites.org/) is a free-to-use web server and supports researchers in the field of microbial genome mining. It is currently the most widely used tool for detecting and characterising biosynthetic gene clusters (BGCs) in bacteria and fungi.

- The *Oxynema aestuarii* genome (accession number - NZ_CP051167.1) was retrieved from Genbank of NCBI database.

- Web-access to the antiSMASH database - the antiSMASH database is located at https://antismash.secondarymetabolites.org/#!/start .
- Data input: the accession number of *O. aestuarii* (NZ_CP051167.1) was uploaded and the detection strictness was set as 'relaxed' and the desired features were selected by clicking the check boxes. After that the submit button was clicked.

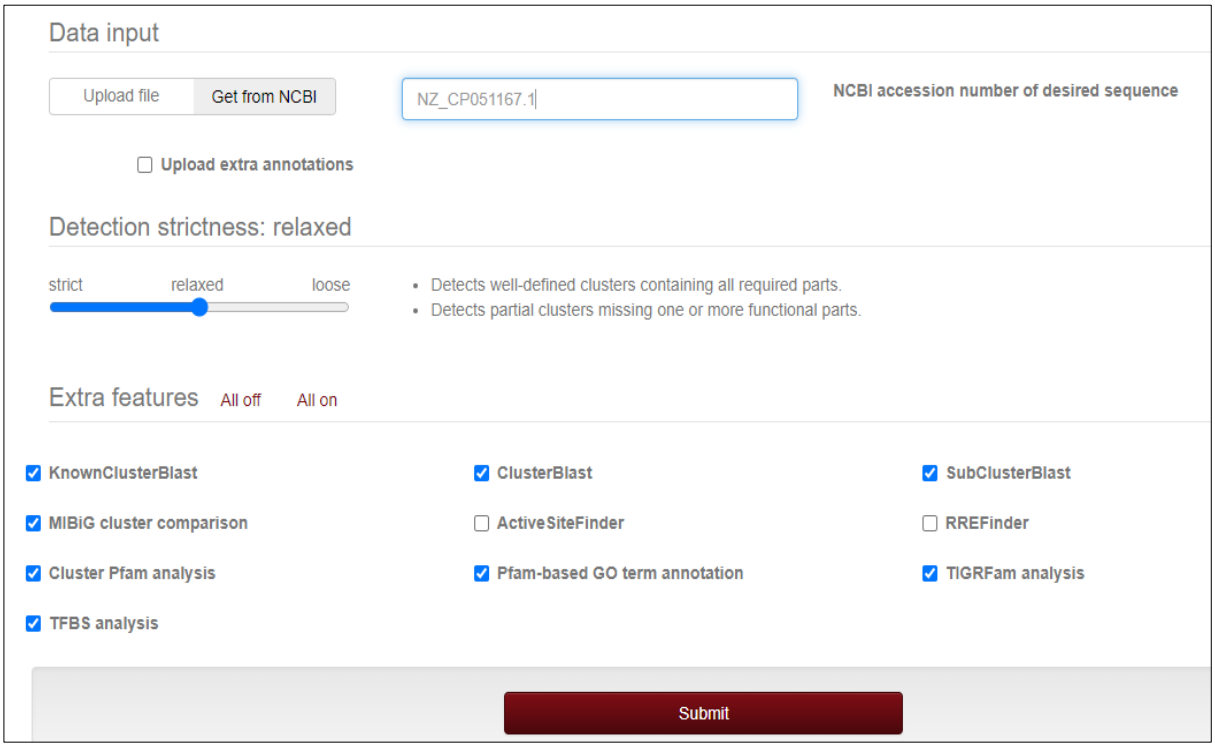


Fig 10: Data entry on the antiSMASH web server

- The result was obtained via email through hyperlink.
- The data was downloaded from the antiSMASH portal.
- The downloaded GenBank data format files (.gbk) of the three identified secondary metabolite regions were processed by uploading the files on https://proksee.ca/ .
- The protein family (pfam) domains were analysed of each region and later the function of the proteins were studied

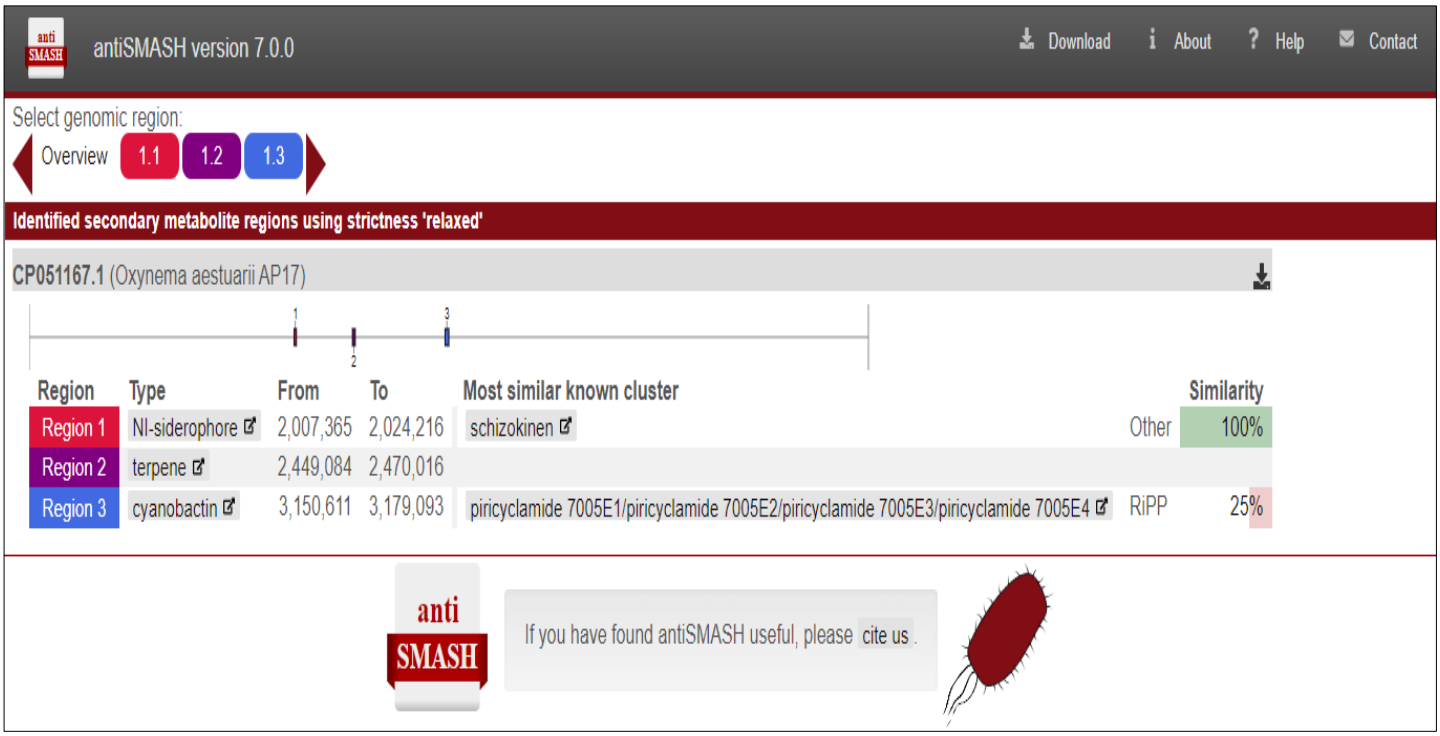


Fig 11: Obtained data from antiSMASH analysis

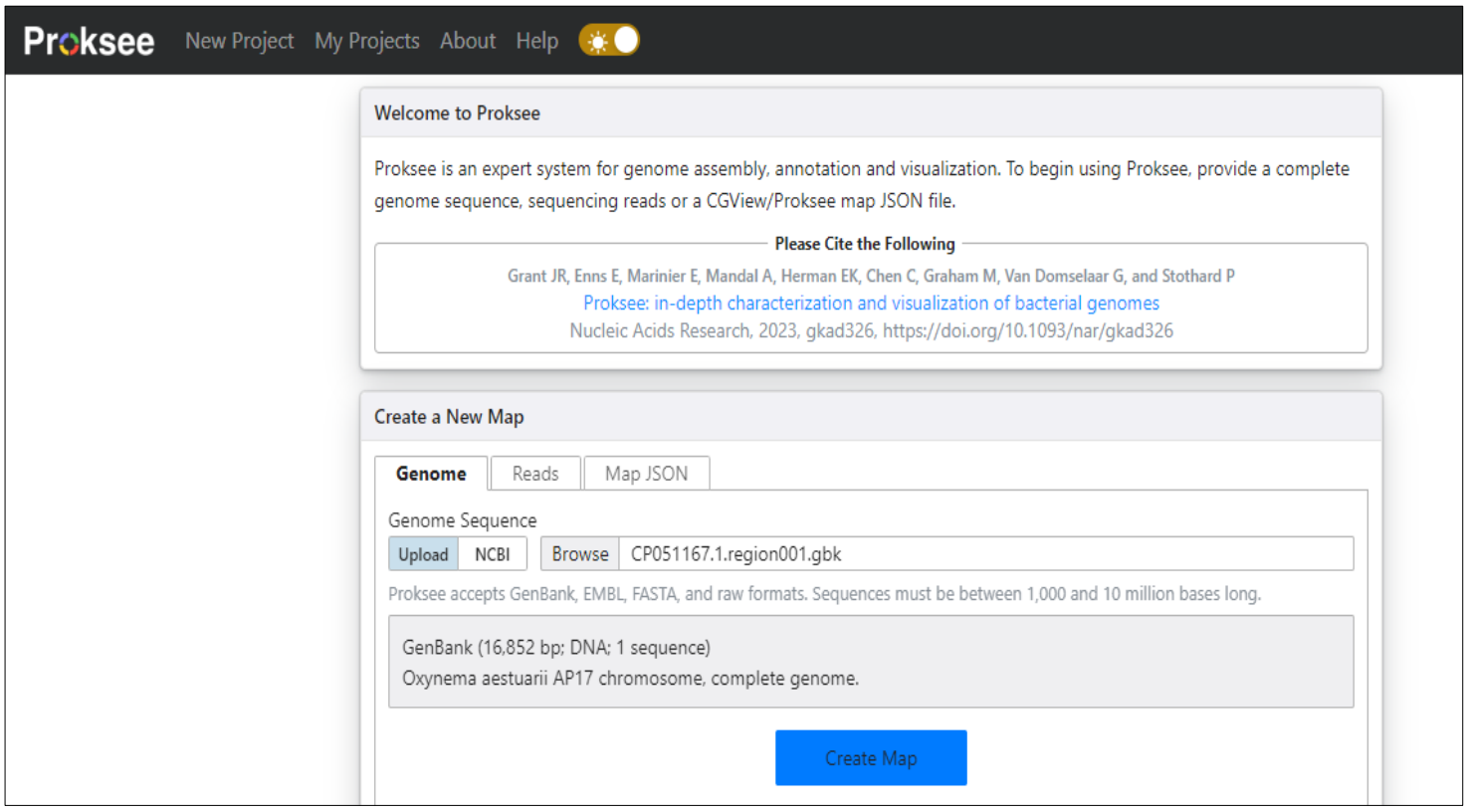


Fig 12: Data processing on Proksee web server

4.2.2 Mapping of genes responsible for MAA biosynthesis:

- The proteins encoded by the genes responsible for MAA biosynthesis given in the Dextro *et al.*, (2023) (accession numbers- QKX45747, QKX45748, QKX45749, QKX45750, WP_063871917.1, WP_063871918.1, WP_063871919.1) were used as reference to find their analogs in *Oxynema aestuarii* proteome by using protein BLAST (blastp) against the protein sequences of the *O. aestuarii* (NCBI Reference Sequence: NZ_CP051167.1).
- After the blastp search with default parameters, it was found that four genes are having query coverage more than 50% against the subject sequence of *O. aestuarii*.
- The genes are mysA, mysB, mysD, mysE encodings 3-dehydroquinate synthase, SAM-dependent methyltransferase, d-Alanine d-Alanine ligase, non-ribosomal peptide synthetase (NRPS-like) respectively.
- Later, the mapping of the genes (obtained by BLAST search from available literature) was done by using Artemis software which is a free genome browser and annotation tool that allows visualisation of sequence features, next generation data and the results of analyses within the context of the sequence, and also its six-frame translation. Artemis is written in Java, and is available for UNIX, Macintosh and Windows systems. It can read EMBL and GENBANK database entries or sequence in FASTA, indexed FASTA or raw format. Other sequence features can be in EMBL, GENBANK or GFF format.
- The FASTA file of the genome of *O. aestuarii* was uploaded on the Artemis software to find the position of the genes.

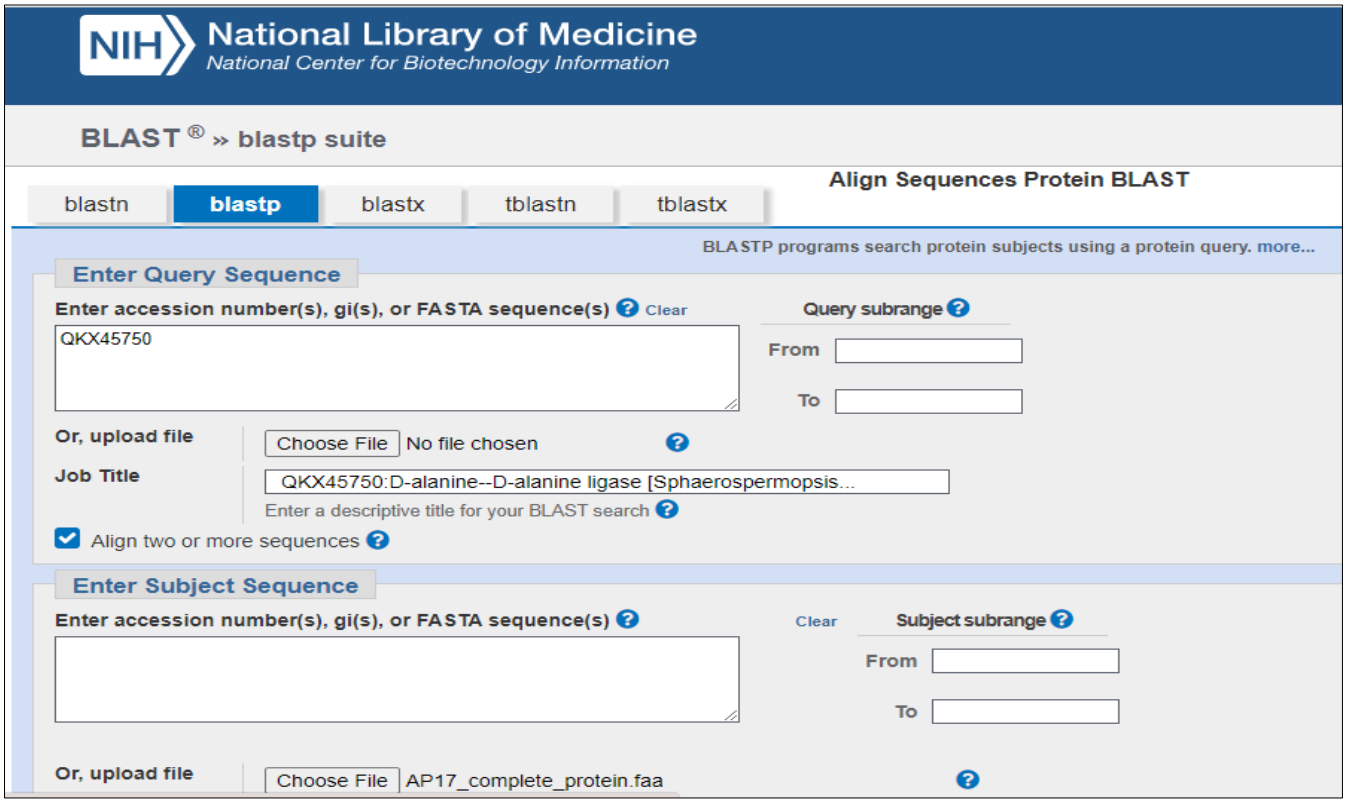


Fig 13: Example of blastp search

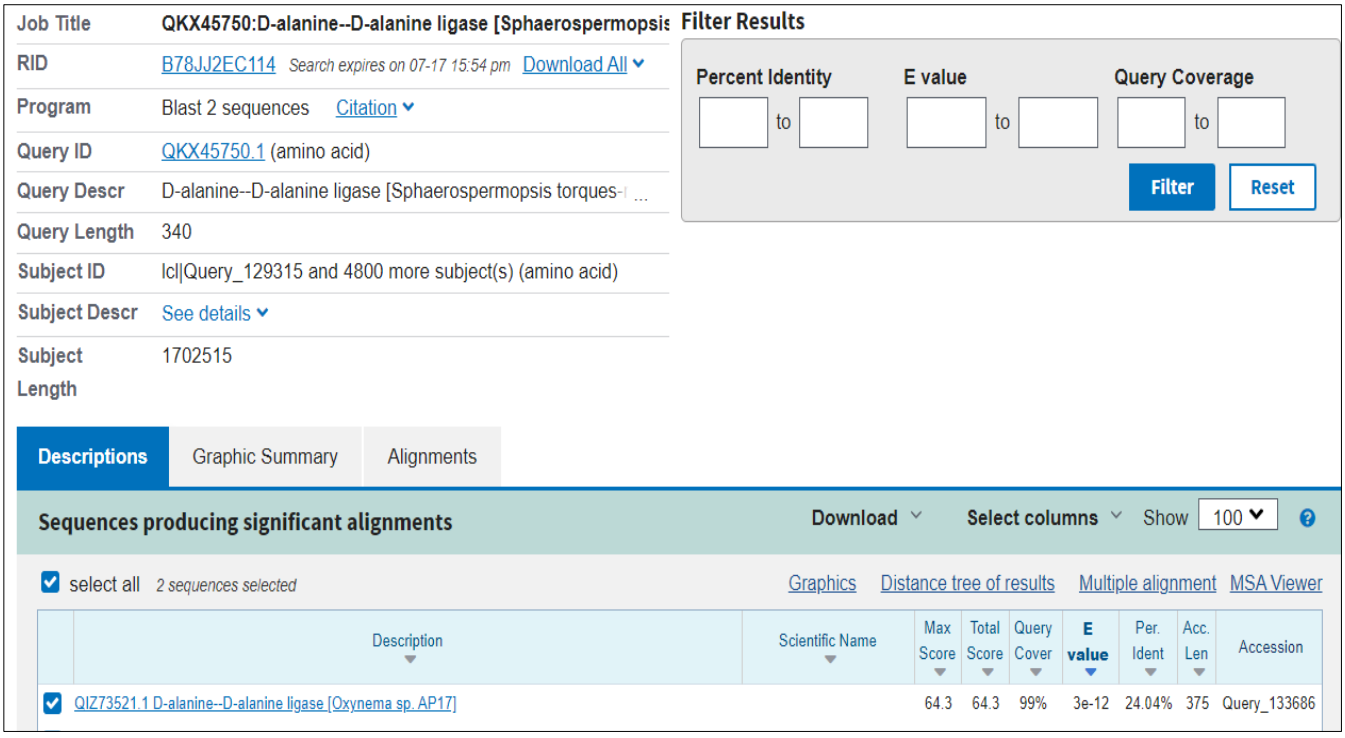


Fig 14: Example of blastp search result

4.3 Procedure of partial purification of MAA by HPLC analysis:

The HPLC analysis was done by following a three step separation technique mentioned in (Ngoennet *et al.*, 2018)

1. For the induction of MAA bioproduction, *Oxynema aestuarii* cells were grown in the ASN III culture media in Hopkins flask for around 60 days and collected from the liquid culture. Typically, 7.6 gm fresh weight cells were obtained from a 1 lit culture.
2. About 80 ml of HPLC grade methanol was added into the cell biomass and mixed by using a blender to make it a homogenous solution.
3. The cells suspension was disrupted with the help of the sonicator (7 cycles of sonication were performed, each cycle of 60 seconds). The suspension should be cooled on ice during sonication (30 seconds sonication followed by 30 seconds ice shock).
4. The sample was refrigerated at 4˚ C overnight.
5. The cell suspension was centrifuged at 22˚ C & 8500 rpm for 8 minutes. The supernatant was transferred into separate new tubes.
6. The sample was desiccated into rotary evaporator at 35˚ C for methanol separation and drying (20-25 minutes per batch). This step took approximately 2-3 hours.
7. After separation the dried residual was dissolved in ultrapure water and collected into a centrifuge tube of volume 50 ml.

8. The sample was lyophilised for 5 days for volume reduction to obtain sharp peaks during HPLC.
9. After lyophilisation the sample was divided into two centrifuge tubes of volume 15 ml each. Each sample was dissolved in 3 ml of HPLC grade water and mixed thoroughly by using vortex.
10. The samples were centrifuged at 25˚C & 8000 rpm for 10 minutes. After centrifugation the supernatant were collected into separate centrifuge tubes. The sample volume was 3.5 ml per tube.
11. Approximately 280 µl of HPLC grade chloroform was added to the supernatant into each tube and later centrifugation was done at 25˚C & 8000 rpm for 10 minutes.
12. The supernatant (aqueous phase) from each tube was collected into centrifuge filter tubes.
13. Ultracentrifugation was done with 3 kDa centrifuge filter (Amicon) at 25˚C & 8000 rpm for 10 minutes for 5 times.
14. The filtered samples (crude MAA) were collected into a separate centrifugation tube.
15. Absorbance of the crude MAA was measured by means of a scan ranging from 400 nm to 190 nm with a spectrophotometer. The absorbance of the crude MAA was 0.0517Å.
16. For partial purification of the crude MAA, HPLC analysis was done by using 0.1% acetic acid as mobile phase.

17. HPLC analysis:

    Columns: Shim-pack solar C18 reversed-phase column (5 µm particle size; 4.6 mm × 150 mm long; Shimadzu Corporation, Kyoto, Japan) connected to a guard column with Shimadzu Photodiode array detector

    Detector: SPD-M20A (Shimadzu Corporation, Kyoto, Japan)

    Chromatograph: LC-20AT (Shimadzu Corporation, Kyoto, Japan)

    Injection volume: 100 µl

    Mobile phase: 0.1% acetic acid (v/v) in water

    Flow rate: 1 ml/min

    Pressure: 400 Kgf/cm^2

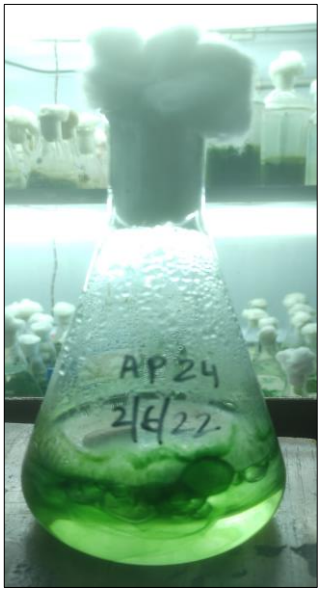
    Separation temperature: room temperature

    Sample acquisition time: 20 min

    Detection wavelengths: 280 nm, 310 nm, 320 nm, 334 nm using a UV-visible detector

Total 20 runs were conducted and the partially purified MAAs were collected in six fractions according to the sharp peaks with respect to the retention time.

18. The partially purified MAAs were analysed via liquid chromatography - mass spectrometry (LC-MS).



Subculture of *O. aestuarii*

Homogenous solution

Ultrasonication (7 cycles; 60 seconds per cycle)

Centrifugation (22˚ C, 8500 rpm for 8 minutes)

Evaporation by rotary evaporator for methanol separation & drying (35˚ C, for 2 to 3 hours)

Lyophilization

Centrifugation (25˚ C, 8000 rpm for 10 minutes); collection of supernatant

Chloroform extraction; collection of the aqueous layer

Ultracentrifugation with 3 kDa centrifuge filter (25˚ C, 9000 rpm for 10 minutes) for 5 times

Partial purification of crude MAA by HPLC analysis

Fig 15: Schematic diagram of partial purification of MAA

5. Result & Data Analysis

5.1 In silico antiSMASH analysis of biosynthetic gene clusters (BCGs) of *Oxynema aestuarii:*

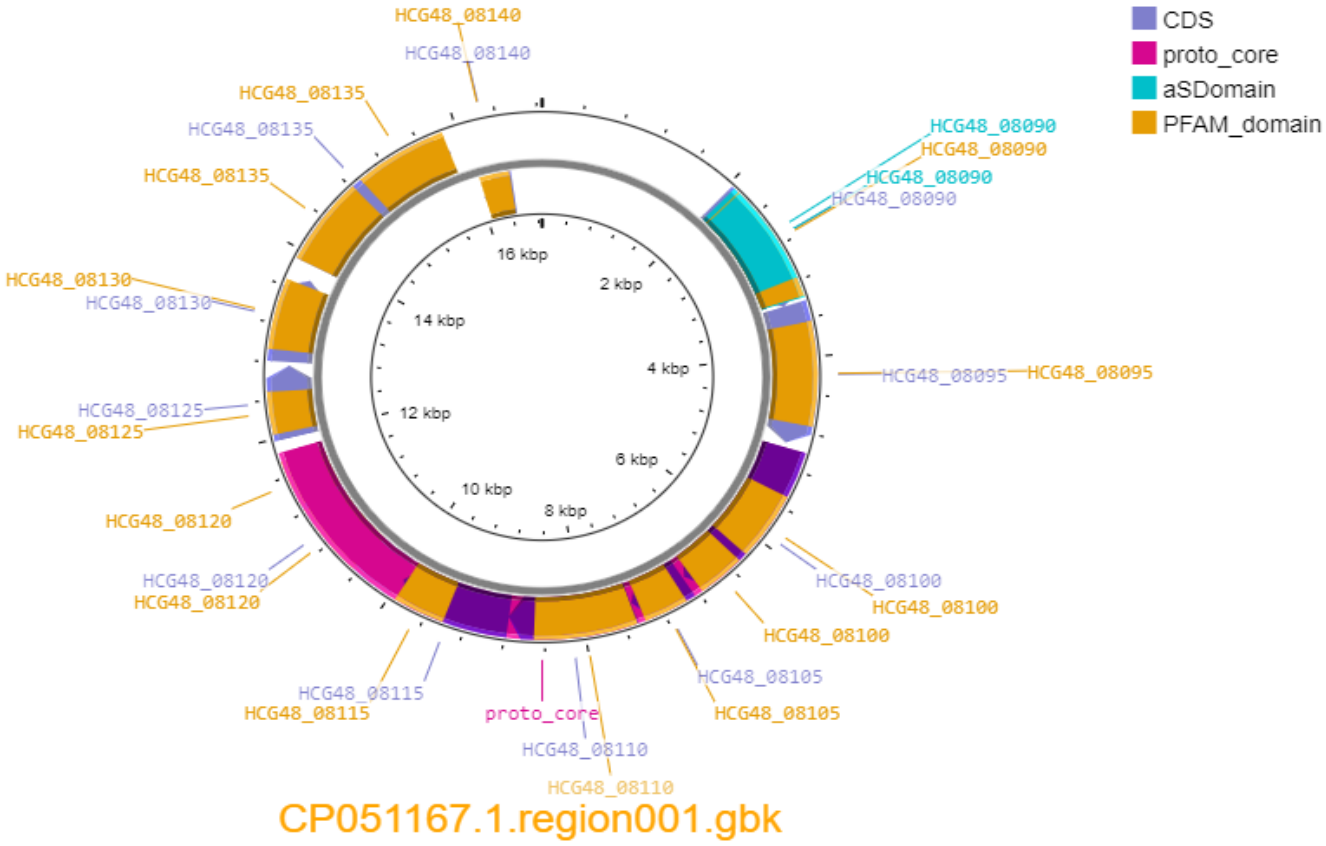


Fig 16: antiSMASH analysis of BCG of region 1 of *Oxynema aestuarii*

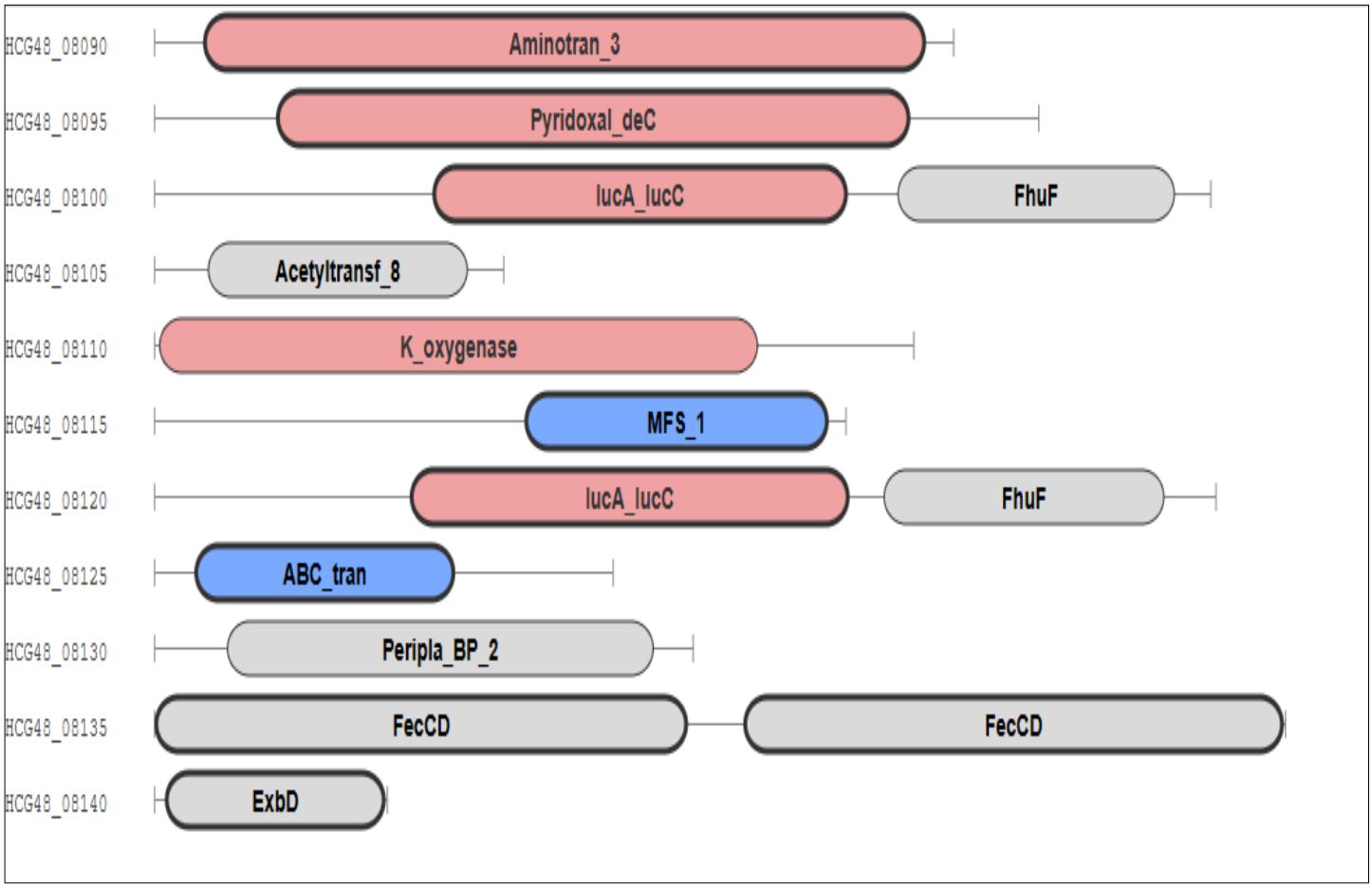


Fig 17: Region1, Pfam domains of *Oxynema aestuarii*

Compound Name	Functions	Reference
Aminotran-3 (Aminotransferase-3)	Aminotransferase-3 may function in signal transduction, gene transcription, and molecular transit	(Zhou *et al.*, 2012)
Pyridoxal-deC (Pyridoxal-dependent decarboxylase)	This conserved domain possess carbon-carbon lyase activity, pyridoxal phosphate binding activity.	https://www.ebi.ac.uk/interpro/entry/pfam/PF00282/
IucA-IucC (members of a family of nonribosomal peptide synthetase-independent siderophore (NIS) synthetases)	IucA and IucC are involved in the biosynthesis of siderophores. In this protein domain is used for iron metabolism or transport.	(Mydy *et al.*, 2020)

FhuF (Ferric iron reductase FhuF-like transporter)	This family consists of several bacterial ferric iron reductase protein (FhuF) sequences. FhuF reduces ferrioxamine B and other hydroxamate siderophores in the cytoplasm	(Cain and Smith, 2021)
Acetyltransf-8 (Acetyltransferase (GNAT) domain)	GNAT enzymes may catalyse the transfer of an acetyl group from acetyl coenzyme A to substrates i.e. amino acids, polyamines, peptides, vitamins, catecholamines, and proteins.	(Shirmast *et al.*, 2021)
K-oxygenase (L-lysine 6-monooxygenase (NADPH-requiring)	It is a flavin-dependent enzyme that catalyzes the hydroxylation of l-Lys in the presence of oxygen and NAD(P)H in the biosynthetic pathway of the siderophores.	(Abdelwahab *et al.*, 2016)
MFS-1 (Major Facilitator Superfamily (family1))	It has transmembrane transporter activity. It can function by solute uniport, solute/cation symport, solute/cation antiport and/or solute/solute antiport depending on the condition/system.	(Pao, Paulsen and Saier, 1998)
ABC-tran (ATP-binding cassette (ABC) transporters)	ABC-tran utilize the energy of ATP hydrolysis to deliver substrate across the cytoplasmic membrane.	(Zeng and Charkowski, 2021)
Peripla-BP-2 (Periplasmic binding proteins)	Peripla-BP-2 are involved in active transport of nutrients, chemotaxis, and quorum sensing.	(Borrok *et al.*, 2009)
FecCD	FecCD transporter proteins are involved in iron compound ABC transporters.	(Ludwig and Bryant, 2011)
ExbD (Biopolymer transport protein ExbD/TolR)	It stabilizes the outer membrane structure and is involved in the septum formation during cell division.	(Fujita *et al.*, 2021)

Table 2: Results of Pfam analysis of region 1 (antiSMASH)

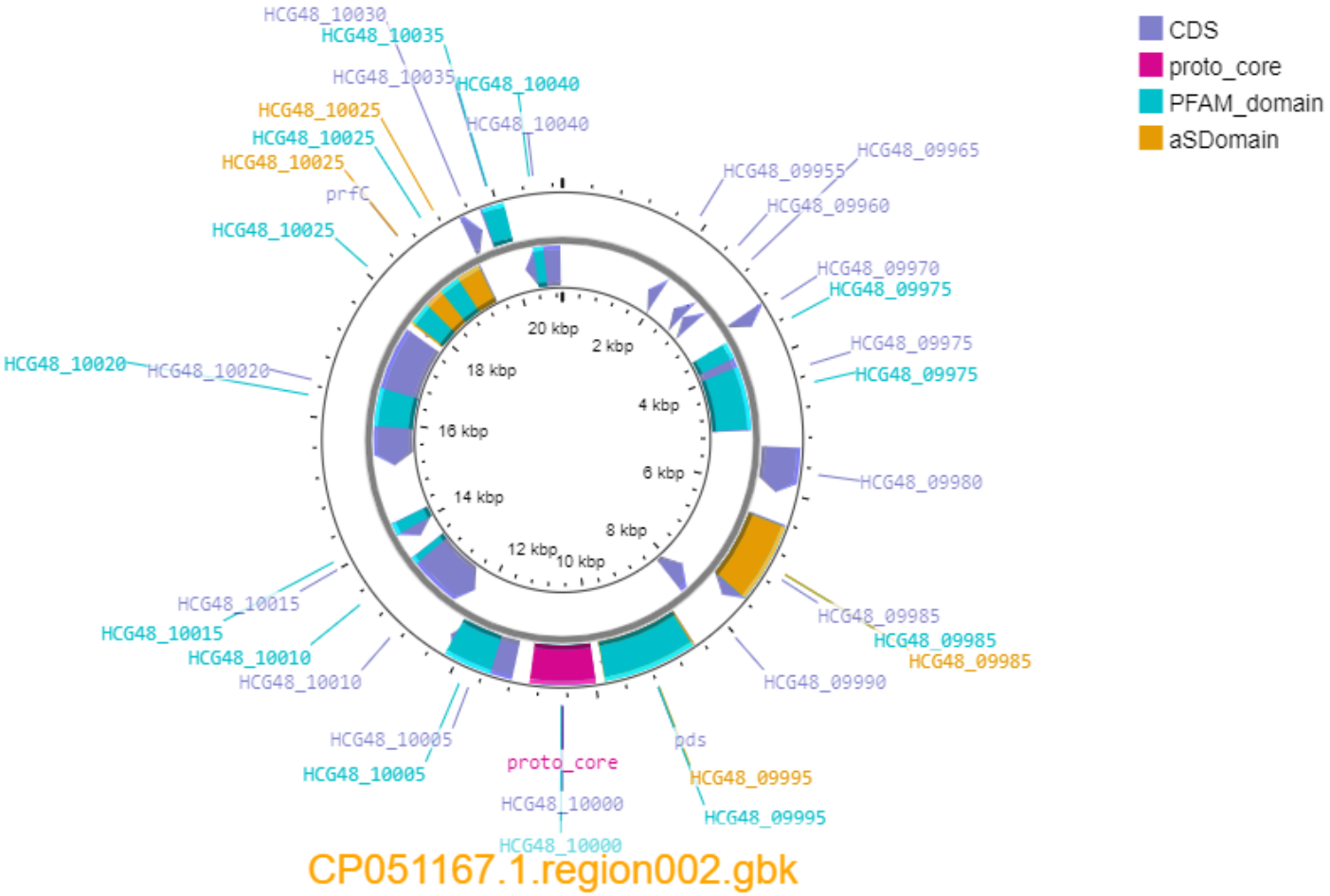


Fig 18: antiSMASH analysis of BCG of region 2 of *Oxynema aestuarii*

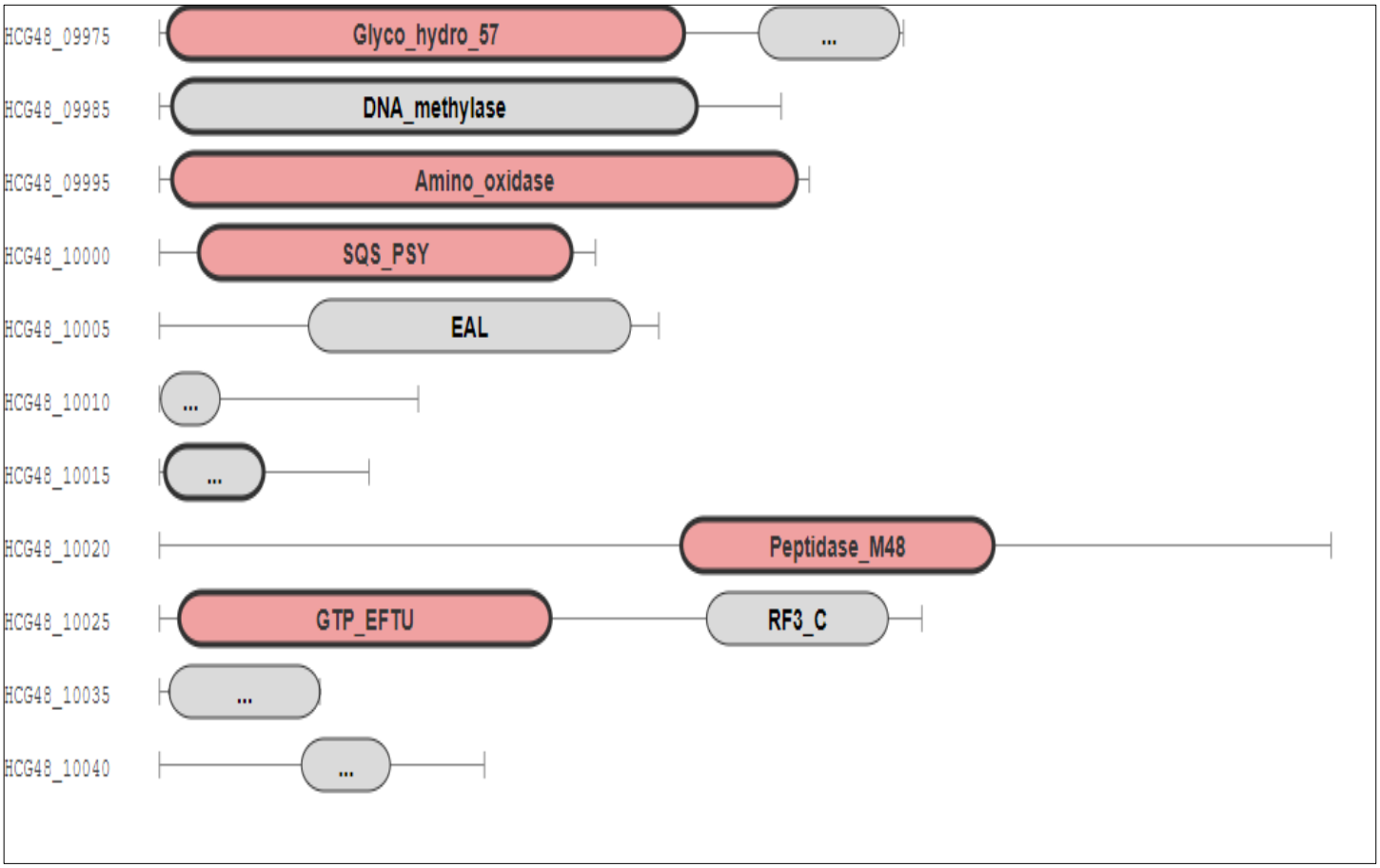


Fig 19: Region2, Pfam domains of *Oxynema aestuarii*

Compound Name	Functions	Reference
Glyco-hydro-57	Glycoside hydrolase family 57 is involved in catalytic activity and carbohydrate metabolic process	(Santos *et al.*, 2011)
DNA-methylase	DNA- methylase plays an important role in DNA replication, DNA repair, and the regulation of gene expression	(Watanabe, 2020)
Amino-oxidase (Flavin containing amine oxidoreductase)	Flavoprotein amine oxidases show oxidoreductase activity by catalyzing the oxidation of various primary, secondary, or tertiary amines via reduction of their flavin cofactor.	(Gaweska and Fitzpatrick, 2011)
SQS-PSY (Squalene/phytoene synthase)	It represents proteins that catalyze the head-to-head condensation of C15 and C20 prenyl units (i.e. farnesyl diphosphate and genranylgeranyl	https://www.ebi.ac.uk/interpro/entry/pfam/PF00494/

	diphosphate). This enzymatic step constitutes part of steroid and carotenoid biosynthesis pathway.	
EAL	EAL domain is a conserved protein domain and found in various bacterial signalling pathways. It may function as a diguanylate phosphodiesterase	(Galperin, Nikolskaya and Koonin, 2001)
Peptidase-M48	It shows metalloendopeptidase and proteolytic activity.	https://www.ebi.ac.uk/interpro/entry/pfam/PF01435/
GTP-EFTu (Elongation factor Tu GTP binding domain)	Elongation factor Tu (EF-Tu) is a GTP-binding protein that is crucial for protein biosynthesis. In the GTP form of the molecule, EF-Tu binds tightly to aminoacyl-tRNA, forming a ternary complex that interacts with the ribosomal acceptor site. During this interaction, GTP is hydrolyzed, and EF-Tu.GDP is ejected.	(Kjeldgaard *et al.*, 1993)
RF3_C (Class II release factor RF3)	The class II release factor 3 (RF3) is a GTPase and functions downstream of peptide release where it accelerates the dissociation of RF1/RF2 prior to ribosome recycling during termination.	(Petropoulos *et al.*, 2014)

Table 3: Results of Pfam analysis of region 2 (antiSMASH)

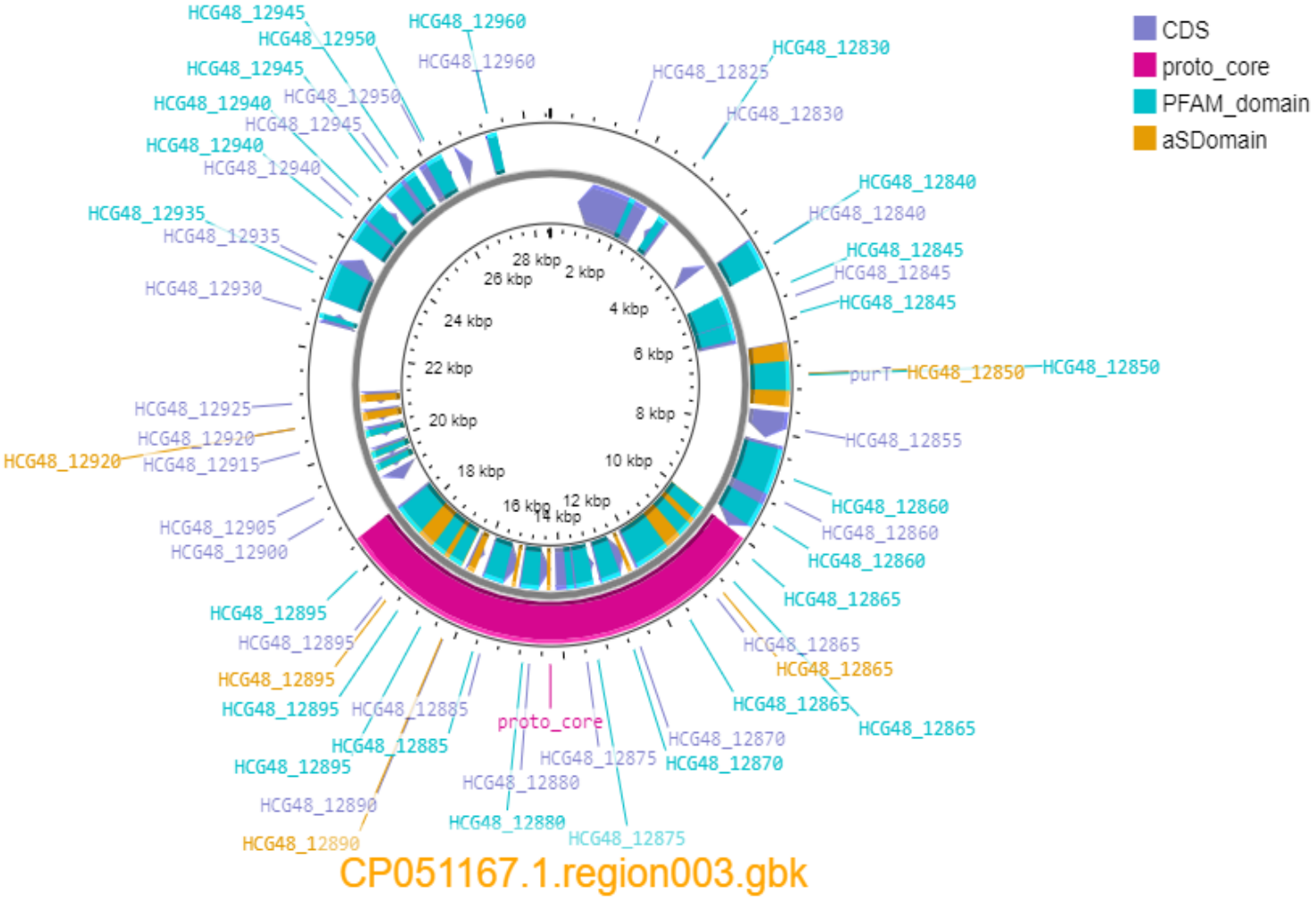


Fig 20: antiSMASH analysis of BCG of region 3 of *Oxynema aestuarii*

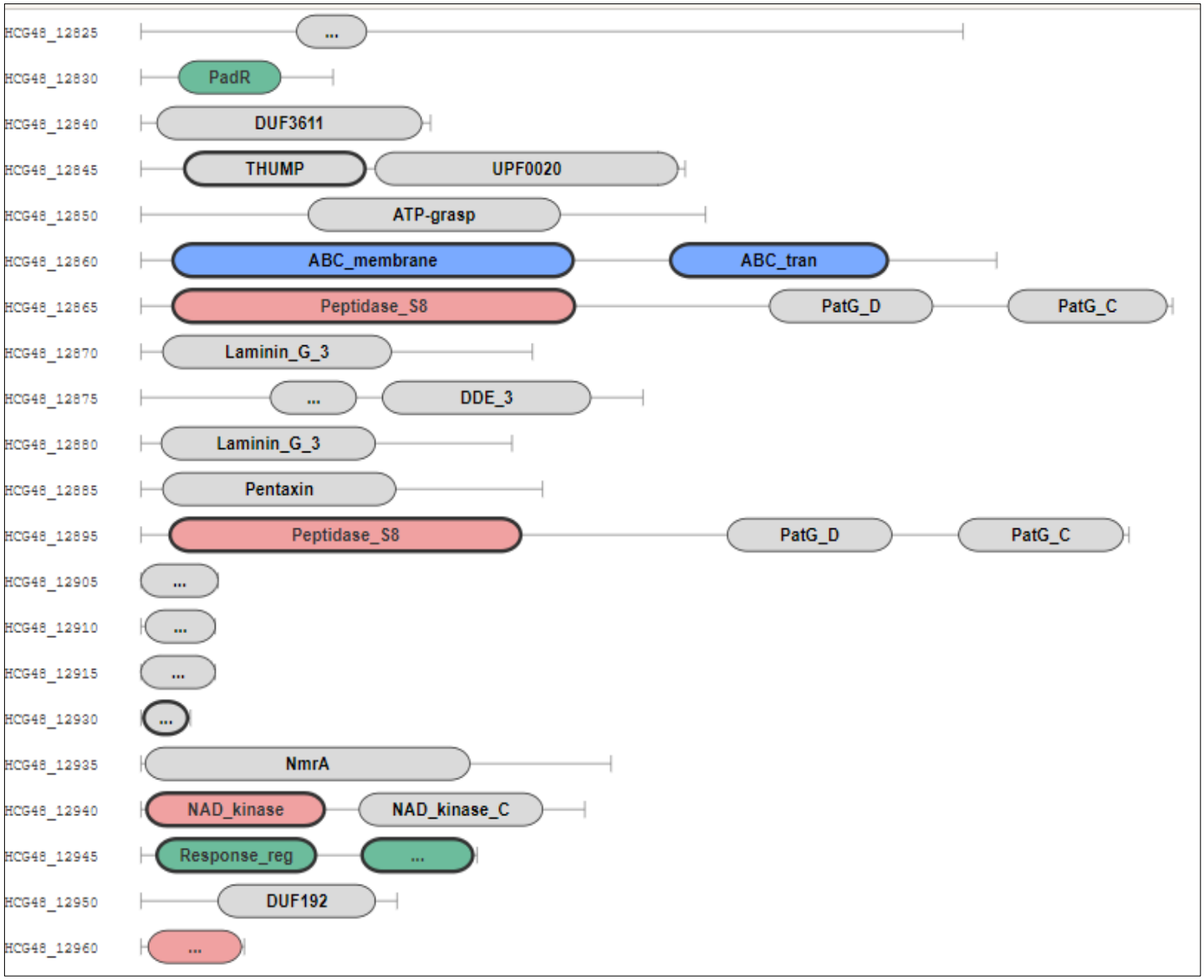


Fig 21: Region 3 Pfam domains of *Oxynema aestuarii*

Compound Name	Functions	Reference
PadR	This protein family acts as a transcriptional regulator.	https://www.ebi.ac.uk/interpro/entry/pfam/PF03551/
DUF3611	It represents a large group of uncharacterised conserved proteins. Their function is not clear.	
THUMP (Thiouridine synthases, RNA methyltransferases, and pseudouridine synthases)	THUMP is an RNA-binding domain that mediates the delivery of various RNA-modifying activities to their target RNAs.	(Aravind and Koonin, 2001)
UPF0020	This domain is probably a methylase. It is associated with the THUMP domain that also occurs with RNA modification domains.	https://www.ebi.ac.uk/interpro/entry/pfam/PF01170/
ATP-grasp (Formate-dependent phosphoribosylglycinamide formyltransferase)	It catalyses the third step in the de novo purine biosynthesis pathway, the formylation of glycinamide ribonucleotide that requires Mg2, ATP and formate.	(Thoden *et al.*, 2002)
ABC_membrane (ABC transporter transmembrane region)	These transporters use the binding and hydrolysis of ATP to power the translocation of a diverse assortment of substrates, ranging from ions to macromolecules, across membranes. ABC transporters function as either importers, bringing nutrients and other molecules into cells, or as exporters that pump toxins, drugs and lipids across membranes.	(Rees, Johnson and Lewinson, 2009)
ABC_tran (ATP-binding cassette (ABC) transporters)	ABC-tran utilize the energy of ATP hydrolysis to deliver substrate across the cytoplasmic membrane.	(Zeng and Charkowski, 2021)
Peptidase_S8 (Subtilase family)	This protein family belongs to serine proteases and shows serine type peptidase activity & proteolysis	(Mahmoud *et al.*, 2021)

	activity.	
PatG_D	These proteins are involved in prfocessing the precursor peptide to yield the cyclic Patellamide.	https://www.ebi.ac.uk/interpro/entry/pfam/PF18047/
PatG_C	This C terminal domain process the precursor peptide to yield the cyclic Patellamide.	(Mann *et al.*, 2014)
Laminin_G_3	LG3 are required for the binding of agrin to its receptor at the neuromuscular junction, which initiates acetylcholine receptor clustering; agrin isoforms with an insert at z(B) also display diminished binding to α-DG.	(Hohenester *et al.*, 1999)
DDE_3	DDE-3 domain characteristic from DDE transposase displaying the carboxylate residues responsible for the endonuclease activity of the protein.	(Parisot *et al.*, 2014)
Pentaxin (Pentraxin)	Several receptors and pathways have been proposed that could be associated with the mechanisms employed by the pentraxin family in mediating tumor progression. Most members of the pentraxin family can activate the PI3K/AKT/mTOR pathways, thereby interfering with the normal cell- cycle.	(Wang *et al.*, 2020)
NmrA	It is a negative transcriptional regulator involved in the post-translational modification of the transcription factor AreA.	(Stammers *et al.*, 2001)
NAD_kinase (Nicotinamide adenine dinucleotide kinases)	These are essential and ubiquitous enzymes involved in the production of NADP(H) which is an essential cofactor in many metabolic pathways. Targeting	(Oka *et al.*, 2023)

	NAD kinase (NADK), a rate limiting enzyme of NADP biosynthesis pathway , represents a new promising approach to treat bacterial infections. NADK is considered as an attractive target for the development of novel antibiotics with an original mode of action.	
NAD_kinase_C (ATP-NAD kinase C-terminal domain)	Members of this family include ATP-NAD kinases, which catalyses the phosphorylation of NAD to NADP utilising ATP and other nucleoside triphosphates as well as inorganic polyphosphate as a source of phosphorus.	https://www.ebi.ac.uk/interpro/entry/pfam/PF20143/
Response_reg (Response regulator receiver domain)	It may play an important role in phosphorelay signal transduction system.	(Pao and Saier, 1995)
DUF192	It represents a large group of uncharacterised conserved proteins. Their function is not clear.	

Table 4: Results of Pfam analysis of region 3 (antiSMASH)

5.2 Mapping of the genes responsible of MAAs synthesis by Artemis:

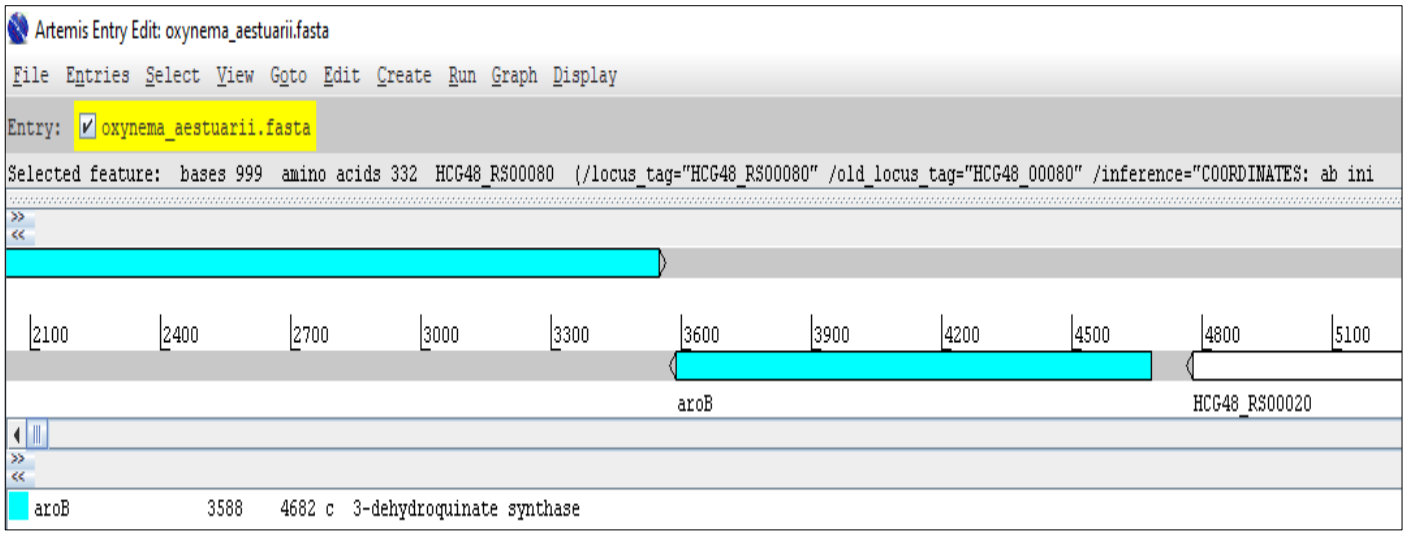


Fig 22: Position of 3-dehydroquinate synthase on gene mapped by Artemis; locus tag: 'aroB' from 3588 bp to 4682 bp

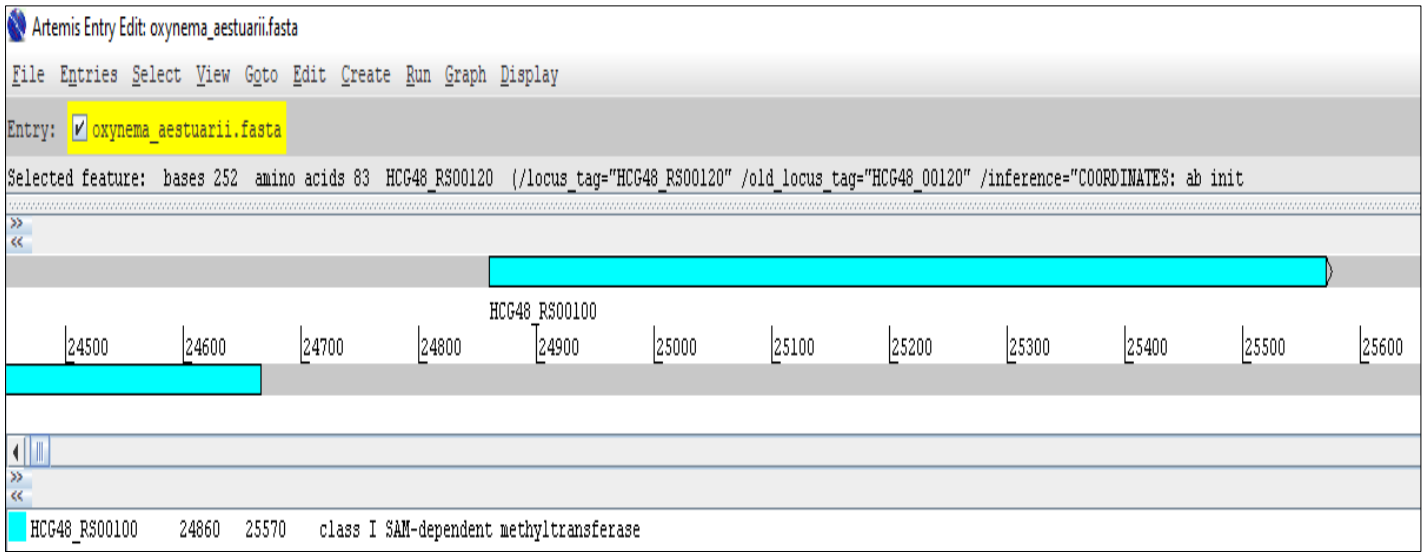


Fig 23: Position of SAM-dependent methyltranferase on gene mapped by Artemis; locus tag: 'HCG48_RS00100' from 24860 bp to 25570 bp

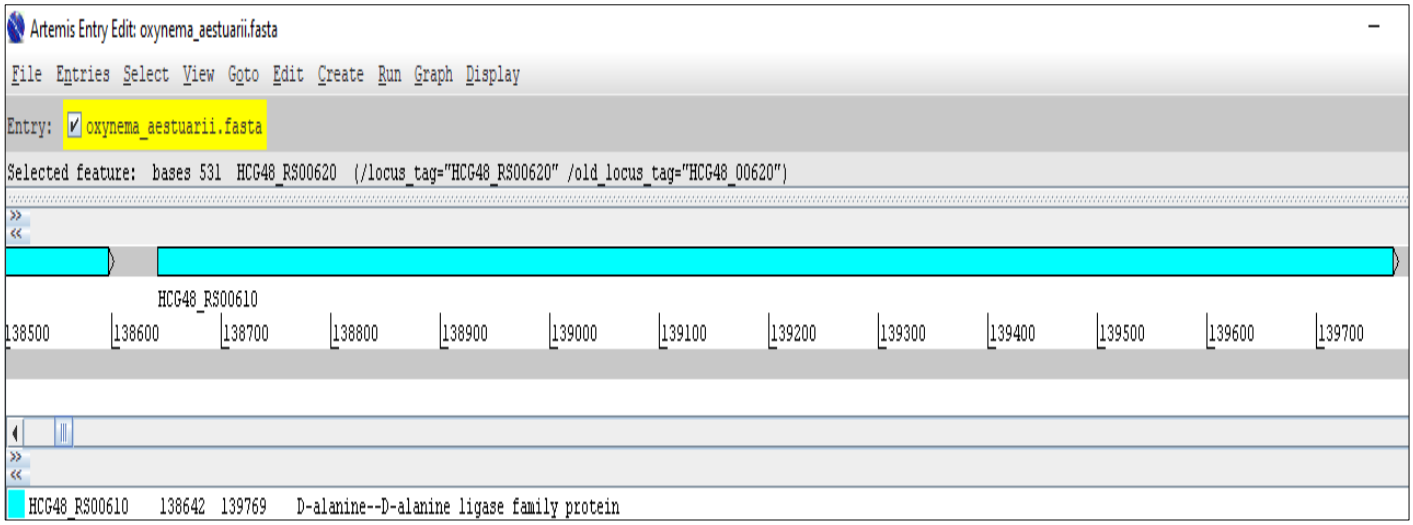


Fig 24: Position of D-alanine--D-alanine ligase on gene mapped by Artemis; locus tag: 'HCG48_RS00610' from 138642 bp to 139769 bp

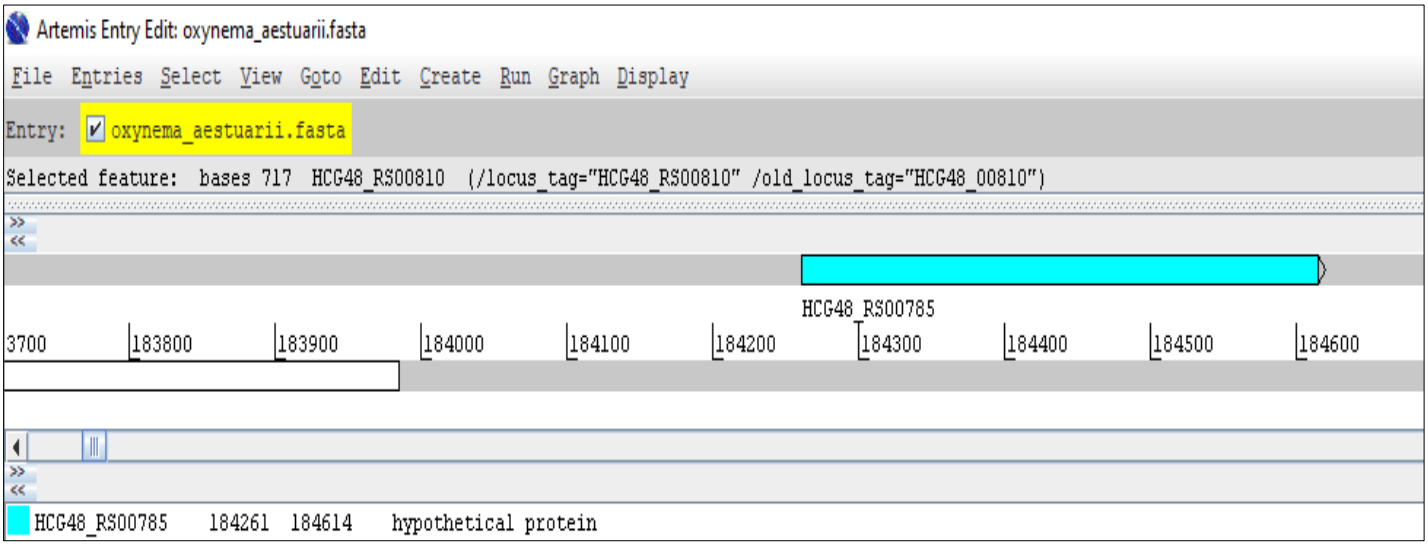


Fig 25: Position of hypothetical protein on gene mapped by Artemis; locus tag: 'HCG48_RS00785' from 184261 bp to 184614 bp

5.3 Result of HPLC analysis:

Serial no.	Retention time w.r.t sharp peaks obtained (seconds)	Retention time period of collectable fraction (seconds)	Weight of the fraction obtained (mg)
Fraction 1	2.547	2.2 to 2.8	3.5
Fraction 2	3.00	2.8 to 3.5	1.2
Fraction 3	4.855	4.5 to 5.4	0.6
Fraction 4	10.7	10.00 to 11.2	1.2
Fraction 5	11.6	11.2 to 12.5	0.2
Fraction 6	13.06	12.5 to 13.8	0.2

Table 5: Analysis of six fractions of MAAs obtained by HPLC

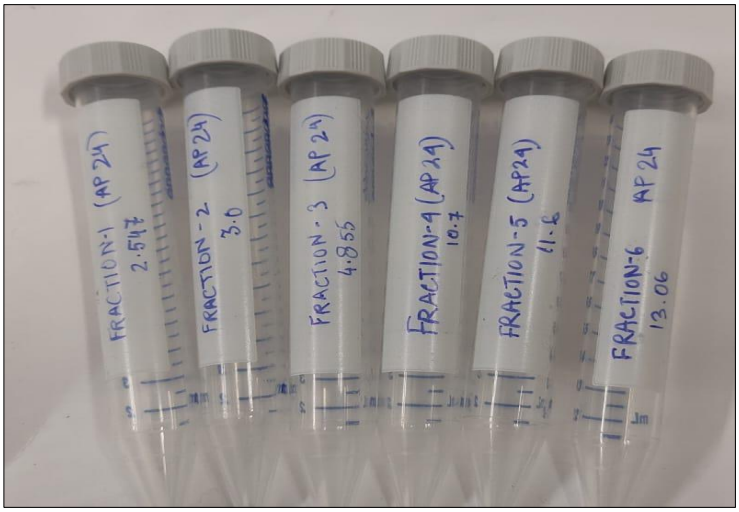


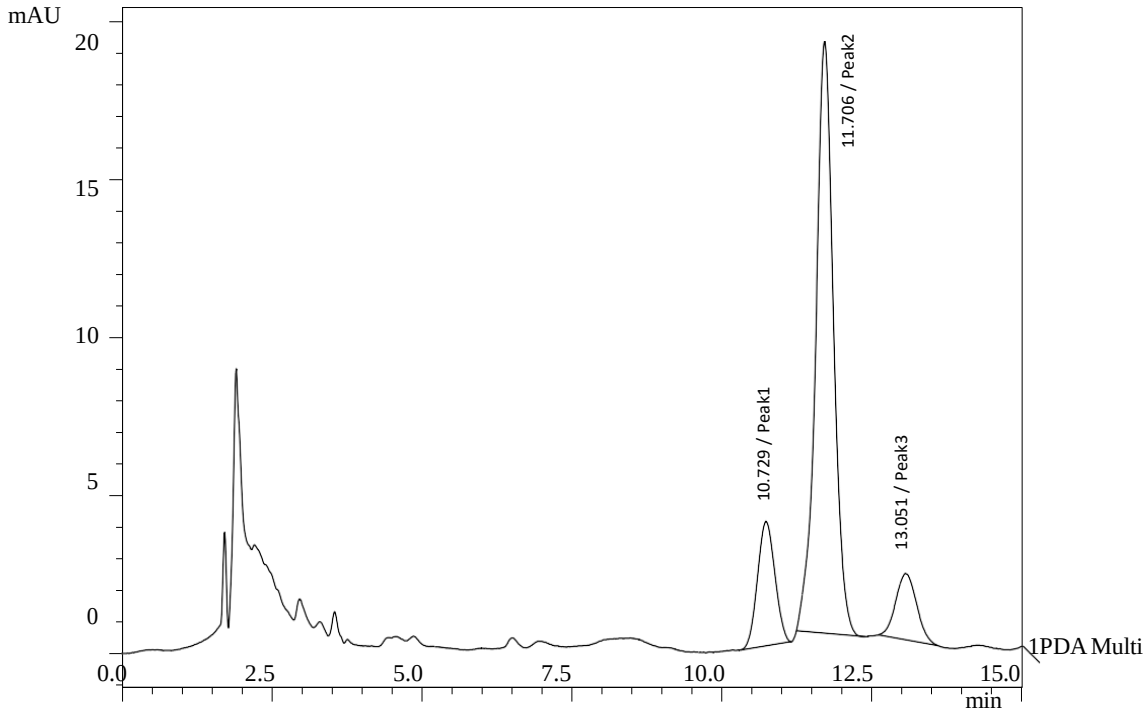
Fig 26: Six fractions of MAAs

- The main sharp peaks were obtained at 280 nm and 334 nm. Later, the chromatograms were obtained from the post-run application of the LabSolutions software (Shimadzu Corporation, Kyoto, Japan).

Sample Information

Acquired by	: Admin
Sample Name	: AP24_MAA_12
Sample ID	: AP24_MAA_12
Tray#	: 1
Vail#	: 1
Injection Volume	: 100 uL
Data Filename	: AP24_MAA_12_ed.lcd
Method Filename	: AP24_MAA.lcm
Batch Filename	:
Report Filename	: Default.lcr
Date Acquired	: 13/12/2022 2:59:16 PM
Data Processed	: 30/5/2023 12:44:20 PM

Chromatogram
AP24_MAA_12 E:\AP24_MAA\AP24_MAA_12_ed.lcd



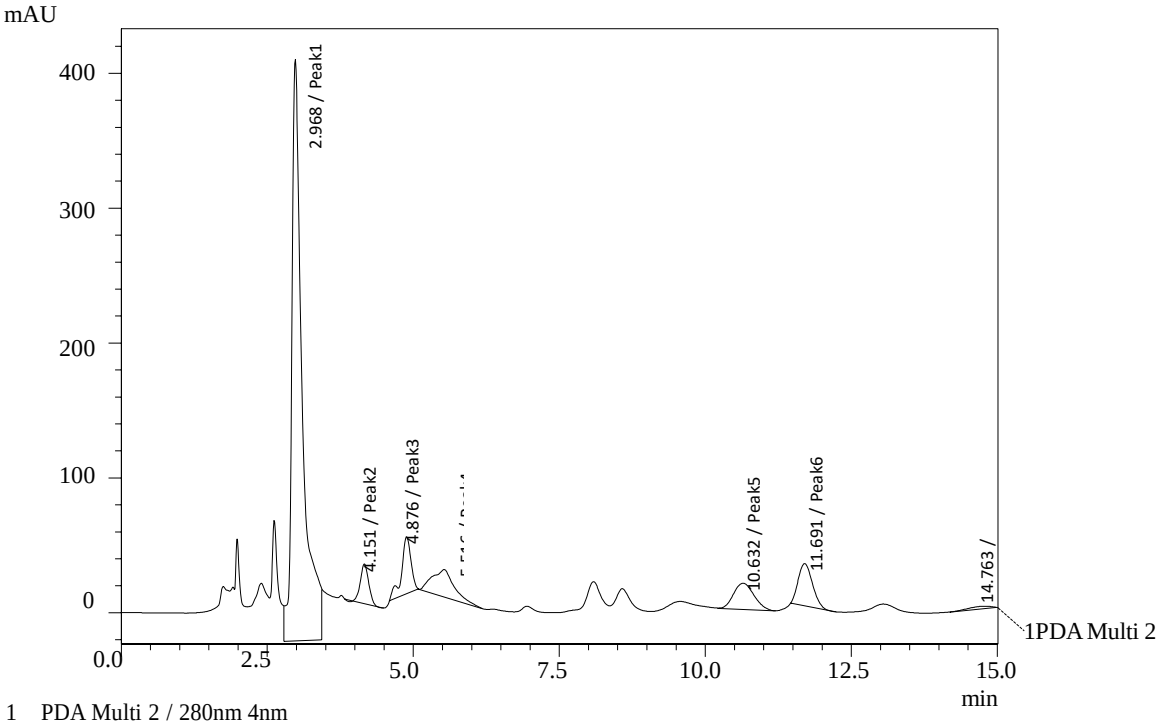
1 PDA Multi 1 / 334nm 4nm

Sample Information

Acquired by	: Admin
Sample Name	: AP24_MAA_13
Sample ID	: AP24_MAA_13
Tray#	: 1
Vail#	: 1
Injection Volume	: 100 uL
Data Filename	: AP24_MAA_13_ed.lcd
Method Filename	: AP24_MAA.lcm
Batch Filename	:
Report Filename	: Default.lcr
Date Acquired	: 12/13/2022 3:15:15 PM
Data Processed	: 30/05/2023 12:40:24 PM

Chromatogram

AP24_MAA_13 E:\AP24_MAA\AP24_MAA_13_ed.lcd



1 PDA Multi 2 / 280nm 4nm

5.4 Liquid chromatography - mass spectrometry (LC-MS) analysis:

The collected fractions from HPLC analysis were analysed by LC-MS. After that, the data was processed through MZmine v.2.53 which is an open source software toolbox for LC-MS data processing.

As per the processed data, presence of some other biologically active non-toxic secondary metabolites were also detected along with the presence of MAAs.

MAAs i.e. Mycosporine-threonine, Hexose-shinorine, Mycosporine-lysine, Palythine-threonine, Hexose-palythine-serine, Mycosporine-GABA have been found from the processed data via MZmine.

The secondary metabolites were analysed with respect to the obtained m/z value (often considered to be the mass) through the CyanoMetDB which is a comprehensive public database of secondary metabolites from cyanobacteria (Jones *et al.*, 2021). Compounds like Cusperin A, Trichophycin E, Floridamide, Spumigin H, Ambiguine N isonitrile have been found which may possess pharmacological properties. The data was also matched with the MYCAS database which is a free online database for mycosporine and MAAs (Geraldes and Pinto, 2021).

ID:		m/z:		RT:		Identity:		Comment:						
ID	Average		Identity	Comment	Peak shape	102 SB_SSS scan mode_001.mzXML			3 SB_SSS scan mode_003.mzXML					
	m/z	RT				Status	Height	Area	Status	Height	Area			
1	228.2583	6.65	Lyngbyacarbonate				2.0E7	6.9E7						
2	272.2500	6.55	Mycosporine-GABA				2.0E7	6.3E7						
5	584.3938	10.37	586-Da MAA				6.4E6	4.1E7						
6	297.0322	4.15	Mycosporine-taurine							4.8E6	2.8E8			
8	926.8065	9.77	Prenylagaramide B				3.2E6	4.4E8						
9	130.7215	10.82	1-ethenoxy-2-methylb..							7.4E6	2.9E8			
10	610.1788	9.69	Glycosylated Palythin..				2.8E6	3.6E8						
11	223.0176	0.36	Anaephene A				2.1E6	3.3E8		3.0E6	4.0E8			
14	110.3106	10.73								DETECTED	1.3E9			
15	182.9607	10.84	cis-Dihydrohomoanat...							2.0E7	8.6E8			
18	89.6363	10.84								1.8E7	6.1E8			
19	633.2453	8.16	Anabaenolysin C							1.5E7	3.0E8			
20	781.3523	7.98	Nostamide C							1.0E7	4.7E8			
21	945.6115	10.52	Tutuilamide C							1.0E7	1.4E9			
22	924.4060	7.17	Microphycin KB921							7.3E6	7.1E8			
23	929.2142	8.89	Brintonamide C							7.1E6	1.0E9			
24	557.9933	6.19	Anabaenolysin var 4							7.0E6	2.3E8			
26	1020.1404	9.23	Tutuilamide A							5.4E6	6.7E8			

Fig 27: Example of LC-MS processed data through MZmine v.2.53 with custom database search (MYCAS and CyanoMetDB)

6. Conclusion

From the above study, it is evident that *Oxynema aestuarii*, a novel species of cyanobacterium isolated from the mangroves of Sundarbans is a potential candidate for production of photoprotective compounds such as mycosporine-like amino acids. It is also a resource of several biologically active secondary metabolites which has been revealed by the whole genome sequencing by in silico antiSMASH analysis of the biosynthetic gene clusters and the Pfam domains analysis. Additionally, Gene mapping by Artemis software has revealed the presence of the genes responsible for MAA biosynthesis in the organism. Genetic studies supplemented by HPLC analysis and LC-MS analysis have revealed that this organism is capable of producing several secondary metabolites having pharmacological properties. Furthermore, it has been shown that these secondary metabolites obtained from this particular species of cyanobacterium may possess anti-cancer & anti-angiogenic activity (Basu *et al.*, 2021). In a nutshell, these secondary metabolites having pharmaceutical properties may exhibit therapeutic effects. So, the anti-angiogenic & anti-tumor activity of these partially purified MAAs can be studied as well which may be beneficial for the pharmaceutical industries in the long run.

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