COMPUTATIONAL EVALUATION OF PHYTOCHEMICALS TARGETING BACTERIAL NITRITE REDUCTASE FOR NITROGEN REMOVAL IN AQUACULTURE WASTEWATER

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GARIMA KHATRI
24/MSCBIO/50

Under the Supervision of
PROF. JAI GOPAL SHARMA
Professor, Department of Biotechnology

DEPARTMENT OF BIOTECHNOLOGY

DELHI TECHNOLOGICAL UNIVERSITY
(Formerly Delhi College of Engineering)
Shahbad Daulatpur, Main Bawana Road, Delhi-110042. India

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GARIMA KHATRI

24/MSCBIO/50

DELHI TECHNOLOGICAL UNIVERSITY

(Formerly Delhi College of Engineering)
Shahbad Daulatpur, Main Bawana Road, Delhi-42

CANDIDATE'S DECLARATION

I, Garima Khatri hereby certify that the work which is being presented in the thesis entitled "Computational Evaluation of Phytochemicals Targeting Bacterial Nitrite Reductase for Nitrogen Removal in aquaculture wastewater" in partial fulfillment of the requirements for the award of the Degree of Master of Science, submitted in the Department of Biotechnology, Delhi Technological University is an authentic record of my own work carried out during the period from 2024 to 2026 under the supervision of Prof Jai Gopal Sharma.

The matter presented in the thesis has not been submitted by me for the award of any other degree of this or any other Institute.

Candidate's Signature

DELHI TECHNOLOGICAL UNIVERSITY

(Formerly Delhi College of Engineering)
Shahabad Daulatpur, Main Bawana Road, Delhi-42

CERTIFICATE BY THE SUPERVISOR

Certified that Garima Khatri (24/MSCBIO/50) has carried out the research work presented in this thesis entitled "Computational Evaluation of Phytochemicals Targeting Bacterial Nitrite Reductase for Nitrogen Removal in aquaculture wastewater" for the award of Master of Science from Department of Biotechnology, Delhi Technological University, Delhi, under my supervision. The thesis embodies results of original work, and studies are carried out by the student himself and the contents of the thesis do not form the basis for the award of any other degree to the candidate or to anybody else from this or any other University.

Prof. Jai Gopal Sharma
Supervisor
Professor
Department of Biotechnology
Delhi Technological University

Prof. Yasha Hasija

Head of the Department
Department of Biotechnology
Delhi Technological University

Date:

ABSTRACT

Aquaculture wastewater contains nitrogenous compounds such as ammonia, nitrite, nitrate, and dissolved organic nitrogen, which can affect aquatic animal health, microbial stability, and the ecological quality of receiving water bodies. Biological nitrogen removal is largely mediated by bacterial enzymes involved in nitrification and denitrification pathways. Since these enzymes regulate the transformation of toxic nitrogen compounds into less harmful forms, their molecular-level interaction with bioactive compounds can provide useful preliminary insight for environmental biotechnology and aquaculture wastewater management. The present study aimed to perform an in silico molecular docking analysis of selected natural compounds against cd1 nitrite reductase NirS, a bacterial enzyme associated with nitrogen removal from aquaculture wastewater.

The target nitrite reductase enzyme was prepared as the receptor for docking and docked with five selected natural ligands: apigenin, catechin, naringenin, quercetin, and kaempferol. Molecular docking was performed using PyRx with the Auto Dock Vina algorithm, and the resulting docked complexes were analysed on the basis of binding affinity, binding pose, and protein–ligand interaction patterns. The docking results showed that all selected ligands interacted with the target enzyme with measurable binding affinity. Among the screened compounds, apigenin showed the strongest predicted binding affinity with a docking score of −9.2 kcal/mol, followed by catechin at −8.6 kcal/mol, naringenin at −8.4 kcal/mol, quercetin at −8.3 kcal/mol, and kaempferol at −7.1 kcal/mol.

The findings indicate that apigenin demonstrated the most favourable binding compatibility with the selected cd1 nitrite reductase NirS under the applied docking conditions. Catechin, naringenin, and quercetin also showed comparatively strong interactions, suggesting their potential relevance for further computational and experimental investigation. The study supports the use of molecular docking as a preliminary screening method for exploring enzyme–ligand interactions related to nitrogen-removal processes in aquaculture wastewater. However, the docking findings are predictive and require further validation through molecular dynamics simulation, enzyme assays, microbial studies, and wastewater-scale experiments.

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CHAPTER 1: INTRODUCTION

1.1 Background of Aquaculture Wastewater Pollution

Aquaculture is one of the world's most significant food production industries, supplying significant numbers of fish, shellfish and crustaceans for human food. This growth in aquaculture has resulted in a significant decrease in the pressure that capture fisheries had on fish supplies, but also a higher environmental responsibility for intensive aquaculture. In 2022, the Food and Agriculture Organization (FAO) revealed that production of aquaculture surpassed that of capture fisheries for the first time, highlighting the increasing reliance of global food systems on farmed aquatic animals (FAO, 2024). This growth has made the disposal of nutrient-laden wastewater a serious environmental problem, especially in intensive and semi-intensive aquaculture systems where high stocking rates cause a buildup of nitrogenous compounds in wastewater.

Aquaculture effluents include uneaten feed, faecal waste, suspended solids, microbial biomass, ammonia, nitrite, nitrate and dissolved organic material. Of these, nitrogenous compounds are particularly significant, as they directly impact water quality, health of aquatic animals, microbial balance and receiving-water ecosystems. The main source of nitrogen input into aquaculture ponds and recirculating aquaculture systems is protein-rich feeds. A small proportion of the food nitrogen is incorporated into the fish or shrimp mass, while the rest is released in the water as dissolved ammonia, particulate organic nitrogen, urea, faecal nitrogen and degraded organic wastes. If these compounds are not removed effectively, they build up and adversely affect the biological stability of the culture system.

In aquaculture effluents, ammonia is one of the most poisonous nitrogenous waste products. It exists in two major forms: ionised ammonium and unionised ammonia. The unionised form is more toxic and can penetrate into a biological membrane and disrupt cellular metabolism, respiration and osmoregulation in aquatic animals. Nitrogen control is especially critical in warm-water aquaculture systems as ammonia toxicity is dependent on pH and temperature. Nitrite also has an adverse effect because it reacts with haemoglobin in fish and forms methaemoglobin which is responsible for brown blood disease, a condition that affects the oxygen transport ability. Nitrogen (N) as nitrate is relatively less toxic, although the N buildup can still cause physiological stress in aquatic organisms, and can promote eutrophication when released into natural water bodies.

Aquaculture wastewater can potentially pose environmental risks beyond the culture unit itself. Untreated or partially treated wastewater, discharged into rivers, lakes, estuaries or coastal areas, can cause an excess of nitrogen that can lead to algal blooms, lower dissolved oxygen levels, change microbial populations, and upset aquatic biodiversity. Enrichment with nitrogen also may be a factor in the development of hypoxic areas in receiving waters. Nitrogen removal is thus both a production requirement and an environmental-management requirement. Sustainable aquatic farming relies on the strategies used to treat the water inside the system to ensure it is kept in good condition, whilst minimising nutrient discharge from the system.

1.2 Biological Nitrogen Removal in Aquaculture Systems

Overall, biological nitrogen removal is considered as the most feasible method for the control of nitrogenous wastes in an aquaculture system. Solids are removed using either physical or chemical treatment, water chemistry is changed using physical or chemical treatment; microbial processes transform the dissolved inorganic nitrogen into less harmful or removable forms. In recirculating systems, biofilters are used to maintain a microbial community that will convert ammonia to nitrite and then to nitrate through nitrification. Nitrogen can then be removed from the water column as a gas by denitrifying systems (van Rijn et al., 2006).

The typical biological nitrogen-removal process consists of: nitrification and denitrification. Nitrification is an aerobic process and ammonia-oxidising microorganisms converts ammonia into nitrite and nitrite-oxidising microorganisms converts nitrite to nitrate. In aquaculture this is necessary for the rapid removal of ammonia toxicity. In general, denitrification is the reduction of nitrate to nitrite, nitric oxide, nitrous oxide and finally dinitrogen gas, which is accomplished in anoxic conditions. Nitrification and denitrification are coupled processes that offer a full pathway for nitrogen removal, particularly in systems where accumulation of nitrate is a potential issue.

The most important parameters affecting the nitrification efficiency in aquaculture biofilters are dissolved oxygen, temperature, pH, alkalinity, salinity, organic loading, hydraulic retention time and biofilm stability. Nitrifying bacteria are slow growing and can be inhibited by sudden changes in the environment. Another way in which organic matter can influence nitrification efficiency is by promoting the growth of heterotrophic bacteria which compete with the nitrifiers for oxygen and space. Because of these limitations, the biochemical

knowledge of the nitrogen-removal enzymes is important since it dictates nitrogen transformation rates and stability at the enzyme level.

Heterotrophic nitrification and aerobic denitrification are also recently identified processes for nitrogen removal in wastewater and aquaculture studies. It is possible to aerobically and/or under mixed oxygen conditions to perform ammonia oxidation and denitrification by some bacterial strains, thus reducing the need for strictly separated aerobic and anoxic treatment units. In aquaculture systems, continuous circulation and oxygenation of the water is essential for the survival of animals, and this microbial flexibility can be helpful. But the effectiveness of these pathways is related to the activity of certain enzymes, which catalyse the conversion of nitrogen. Thus, knowledge of the structure and functional dynamics of these enzymes is crucial for the development of more effective nitrogen removal measures.

1.3 Bacterial Enzymes Involved in Nitrogen Removal

Nitrogen removal is in the end an enzyme-mediated process. The bacterial enzymes catalyse the conversion of nitrogen between different chemical forms, with each step in the transformation involving a specific architecture of the enzyme active site, cofactors, electron-transport mechanisms, and substrate recognition. The oxidation of ammonia to hydroxylamine is catalysed by ammonia monooxygenase in nitrification. Further oxidation of hydroxylamine follows with the hydroxylamine oxidoreductase as the key enzyme of ammonia-oxidising bacteria. Nitrite oxidoreductase is an enzyme that is present in bacteria known as nitrite-oxidising bacteria, which are responsible for the oxidation of nitrite to nitrate. These reactions help to convert toxic ammonia and nitrite into nitrate, which is less toxic, in aquaculture systems.

There are other enzymes involved in denitrification. The nitrate is reduced to nitrite by nitrate reductase, the nitrite is reduced to nitric oxide by nitrite reductase, nitric oxide is reduced to nitrous oxide by nitric oxide reductase, and nitrous oxide is reduced to dinitrogen gas by nitrous oxide reductase. These enzymes are necessary as they help the release of oxidised nitrogen in the form of a gas from the wastewater. The pathway is of particular environmental importance as it may result in the building up of a potent greenhouse gas, nitrous oxide, if denitrification is incomplete. So, the removal of nitrogen research should not only include consideration of the transformation of nitrogen but also the completion and efficiency of the transformation process.

The enzymology of denitrification and nitrification are directly related to aquaculture wastewater treatment. The enzymes which oxidize ammonia, nitrite, nitrate and nitrite are inhibited, making nitrogen removal inefficient and toxic intermediates can build up. On the other hand, if enzyme activity is promoted or understood, treatment systems can be better designed. The following factors affect enzyme activity indirectly: biofilter design, microbial inoculation, pH control, carbon supplementation and oxygen management. Computational approach, however, provides for a more direct examination of enzyme–ligand interactions at the molecular level.

Structural studies of enzymes involved in nitrogen cycles have revealed the presence of catalytic function, depending on particular amino acid residues, metal centres and binding pockets. They have structural features that make them good candidates for molecular docking if there are known three-dimensional protein structures. Small molecule docking can be used to predict the way in which small molecules bind to the active site of an enzyme or to nearby regulatory pockets. With aquaculture effluents, this method could be used to investigate if there is strong binding of natural compounds to bacterial nitrogen-removal enzymes, and if such binding can be used to help predict the ability of the natural compound to alter the activity of the enzyme.

1.4 Role of Molecular Docking in Environmental Biotechnology

Molecular docking is a computational technique that can be used to predict the preferred orientation and binding affinity of a ligand in the binding of the target macromolecule. While docking is a technique commonly applied to drug discovery, it can be applied to other areas. It is very applicable to environmental biotechnology where the interactions between enzyme and substrate, enzyme and inhibitor, and enzyme and pollutant are studied to elucidate biodegradation, biotransformation and microbial metabolism. The structure obtained by docking can serve as the basis for understanding how molecules might bind to the active site of the receptor and form hydrogen bonds, hydrophobic contacts, or interactions with catalytic residues.

Docking Workflow includes preparation of protein structure by removing unwanted molecules, adding hydrogens, assigning charges and converting the receptor into suitable computational format. Ligands are created through energy minimisation and conversion to docking ready format. The docking program then explores the various conformations and

orientations that the ligand may assume in a defined grid box and scores the probable energies of the docked structures. The most frequently used method for this is Auto Dock Vina, which has been found to be efficient in conformational searching and binding-energy estimation for virtual screening (Trott & Olson, 2010). PyRx is a graphical user interface that combines Auto Dock Vina and Open Babel for use in structured ligand-screening workflows (Dallakyan & Olson, 2015).

Binding energy from docking is in kcal/mol. The more negative the binding energy, the stronger the predicted binding between the ligand and receptor. But the binding energy is not enough for biological interpretation. In addition to calculating a favourable docking score, the ligand should be assessed for how it docks, the contacts it makes with the protein by individual residues, the orientation of the docked molecule and if the molecule docks in a region that is structurally consistent with existing functional binding sites of the protein. Directional stabilisation can be achieved by hydrogen bonds, hydrophobic interactions can help to occupy pockets, and π–π or π–alkyl interactions can stabilize aromatic ligands in non-polar regions of the enzyme. So, there is a need for both numerical and structural analysis in the docking interpretation.

Docking can be used in environmental biotechnology to help hypothesize. It cannot prove enzyme activation or inhibition alone, but can identify the ligands which warrant further molecular dynamics simulation, enzyme assays or microbial validation. In the case of nitrogen-removal enzymes, docking can give a preliminary idea of whether or not the molecules chosen interact with the catalytic pockets or with important residues of the structure. This is especially helpful when testing in the field is time consuming or expensive or not available immediately. Docking is thus a preliminary screening method which restricts the number of compounds to be tested in the laboratory stage.

1.5 Natural Bioactive Ligands in the Present Study

Selected naturally occurring polyphenolic and flavonoid compounds, such as quercetin, catechin, kaempferol, apigenin and naringenin, are the focus of the present study. The compounds are extensively investigated due to their hydroxylated aromatic groups that can interact with proteins through π-mediated interactions, hydrophobic contacts, and hydrogen bonding. Flavonoids are a group of structurally heterogeneous plant secondary metabolites with antimicrobial, anti-inflammatory, enzyme interaction and antioxidant properties (Panche

et al., 2016). Their chemical structures make them potential candidates for molecular docking as there are several functional groups to interact with the amino acid residues in the enzyme binding pocket.

A flavanol with multiple hydroxyl groups and a planar aromatic structure. These features enable it to make hydrogen bond and π interactions with protein residues. Catechin is a flavan-3-ol that contains more hydroxyl groups than planar flavanols and has a more flexible 3D structure. Kaempferol is structurally similar to quercetin, but differs in the hydroxyl substitution pattern, which may affect its hydrogen-bonding capacity and binding orientation. Apigenin is a flavone with less hydroxyl groups and its relatively planar structure might facilitate a stable fit into a pocket. Naringenin is a non-planar flavanone which can engage in different interactions than flavanols and flavones. These differences in structure make it possible to compare the docking of these ligands, as they can have different binding affinity and interaction sites.

Natural ligands in this study are not meant to be directly released into the aquaculture wastewater. Instead, they serve as model bioactive molecules for understanding the ligand-compatibility of the bacterial enzymes of nitrogen metabolism. Using molecular docking can determine if these compounds bind to the functional pockets of enzymes and if certain structures are more closely associated with better binding. The kind of analysis could be useful for future studies of enzyme modulation, biofilter performance, microbial regulation and/or natural product environmental biotechnology.

The chosen ligands are also a helpful comparison set since they are not the same, but are closely related in chemical structure. The differences in hydroxylation, molecular planarity, polarity and ring arrangement can have an effect on the docking results. The study can then determine which structural features are linked to greater binding to the bacterial enzyme by comparing the results to the chosen enzyme. By using a comparative approach, more information can be gained than by analysing a single compound, as it enables the results of the docking to be interpreted in terms of chemical structure.

1.6 Rationale of the study

Even though microbial nitrogen transformation plays a prominent role in aquaculture wastewater treatment, the majority of treatment discussions in practice are centered on reactor design, water-quality parameters, microbial community composition or removal efficiencies.

These are important but not exhaustive parameters of a molecular interaction that could affect the behavior of an enzyme. The interaction between bacterial enzymes and small molecules should be systematically studied because it is enzyme-mediated.

In the light of this, the present study was used as a rationale that in silico molecular docking can serve as a preliminary insight to the binding behaviour of natural ligands with the bacterial enzymes involved in nitrogen removal. There are three reasons for such an approach. For the first time, it connects aquaculture wastewater treatment to molecular biotechnology, rather than just system-level performance, and investigates enzyme-level interactions. Second, it offers a cost-efficient screening technique for screening several different ligands prior to experimental validation. Third, it assists in the identification of candidate molecules and interactions which could inspire future research into microbial nitrogen metabolism.

This study is not intended to make a direct deduction of the amount of nitrogen removed from the docks. Docking is not a substitute for enzyme assays, microbial growth studies, biofilter trials or wastewater-scale validation. Docking, however, can offer a rational approach to target compound selection and understanding their probable binding to the target enzymes. The binding-affinity data and residue-level analysis of interactions provides a structured computational basis for future experimental work.

1.7 Aim of the Study

The aim of the present study is to perform an in silico molecular docking analysis of selected natural bioactive ligands against the nitrite reductase enzyme prepared as the receptor for docking, and to evaluate the predicted binding affinity and protein–ligand interaction patterns using a PyRx/Auto Dock Vina-based computational workflow.

1.8 Objectives of the Study

The study is guided by the following three objectives:

1. To prepare the selected bacterial nitrogen-removal enzyme and natural ligands for molecular docking using standard in silico procedures, including receptor preparation, ligand energy minimisation, and PDBQT conversion.

2. To evaluate the binding affinity of selected natural ligands, including quercetin, catechin, kaempferol, apigenin, and naringenin, against the cd1 nitrite reductase NirS using Auto Dock Vina through the PyRx platform.

3. To analyse and compare the protein–ligand interaction profiles of the docked complexes in terms of hydrogen bonding, hydrophobic interactions, binding orientation, and relative ligand ranking.

1.9 Significance of the Study

The value of the present investigation is the combination of aquaculture wastewater treatment, bacterial nitrogen metabolism and computational molecular analysis. The molecular basis of enzyme–ligand interactions is a topic yet to be thoroughly explored in the context of nitrogen removal, which is a key component of sustainable aquaculture. The study provides a more mechanistic understanding of interactions between selected natural compounds and nitrogen-removal enzymes by using a bacterial enzyme that is associated with nitrogen transformation.

The research is relevant to environmental biotechnology because it shows the applicability of computational screening in the preliminary steps of wastewater research involving enzymes. Docking can be used to find the ligands that have better predicted binding and interaction profile, which can be used to guide the researchers to perform experimental screening. This type of data can be used to prioritize compounds for molecular dynamics simulation, enzyme assays, microbial culture testing or biofilter-based validation.

The study is significant for aquaculture sustainability too. Efficient nitrogen removal minimizes the ammonia and nitrite toxicity, increases the stability of the culture-water, promotes the good health of the fish and shrimp, and decreases the release of nutrients to the natural environment. The current research is computational; however, its applications are far-reaching and can aid in further study for better microbial nitrogen-removal systems. This enzyme level knowledge can, over time, help to design improved biofilters, select microbial inoculant and nitrogen-management strategies.

Academically, the study offers a systematic case example of the use of molecular docking in an alternative context to drug discovery. It brings into aquatic culture practice the concept of docking and uses bacterial enzymes as molecular targets, in environmental-biotechnology

studies. This could stimulate greater collaborative efforts among the fields of computational biology, microbiology, wastewater treatment, and aquaculture science.

1.10 Scope and delimitations of the study

The study is limited to computational molecular docking analysis. The study is now concentrated on some of the natural ligands and one bacterial enzyme involved in the nitrogen removal. This study consists of receptor preparation, ligand preparation, docking execution, comparison of binding affinity and interpretation of interaction-profile. The results are designed to detect relative binding trends of the selected ligands and to give a preliminary insight into enzyme–ligand compatibility at the molecular level.

Molecular dynamics simulation, quantum chemical calculation, enzyme kinetics, microbial culture experiments, toxicity testing, and real aquaculture wastewater treatment experiments are not included in the study. Thus, the results should be regarded as preliminary and predictive. A good docking score doesn't mean a good biological activation or inhibition and the binding of a ligand to an enzyme does not necessarily mean that the enzyme will have better nitrogen removal. No conclusions can be drawn on treatment performance without it being experimentally validated.

Other factors that cause restrictions in the study are the quality of selected protein structure, docking parameters, scoring function and method of preparation of the ligand. Docking does not necessarily reflect true molecular interaction in simplified computational environments and may not account for the effects of the solvent, protein flexibility, microbial cell environment or wastewater chemistry. Taking all these drawbacks into consideration, docking proves to be a useful first stage screening tool if interpreted wisely and used along with the subsequent experiments.

1.11 Organization of the Thesis

The thesis is organised into six chapters. Chapter 1 portrays the research problem, justification, objective, significance, and scope of the study with regard to the relevance of the removal of nitrogen from aquaculture effluent and also the role of bacterial enzymes. Chapter 2

summarizes the literature on aquaculture wastewater, nitrogen-cycle processes, bacterial enzymes that participate in the nitrification and denitrification reaction, natural bioactive ligands, and molecular docking in environmental biotechnology. The materials and methods to retrieve, prepare protein, select and prepare ligand, use of docking software, grid parameters, docking protocol and procedure for the analysis of interactions are explained in chapter 3. The docking results are shown in Chapter 4 in terms of binding affinity, ranking of the ligand and 2D/3D interaction profiles. The findings are discussed in Chapter 5 in terms of the biology of nitrogen removal, the structure of the ligands, binding of ligands to the enzymes, and the relevance to environmental-biotechnology. The conclusion along with the significant findings, limitations, and future research directions are presented in Chapter 6.

In conclusion, this chapter has demonstrated that N pollution is a major environmental and production problem in the aquaculture wastewater, the key role of bacterial enzymes in the process of biological N removal, and the potential of molecular docking for studying enzyme–ligand interactions through a rational computational approach. Building on this structure, the study aims to assess selected natural ligands to a bacterial nitrogen-removal enzyme by means of a structured in silico docking workflow.

CHAPTER 2: REVIEW OF LITERATURE

2.1 Introduction

This chapter aims to provide a review of the literature related to the in-silico analysis of the selected natural bioactive ligands for a bacterial enzyme for the removal of nitrogen from aquaculture effluents. The review is divided into four interconnected topics: aquaculture wastewater and nitrogen pollution, biological nitrogen-removal pathways, bacterial enzymes for denitrification and nitrification and preliminary screening of enzyme-ligand interactions by molecular docking. The relevance of the selected natural ligands (apigenin, catechin, naringenin, quercetin and kaempferol) and the reason of the choice of cd1 nitrite reductase NirS, as a target for a computational study of nitrogen transformation, are also presented.

The literature indicates that aquaculture wastewater management is not just a water exchange or physical filter issue. Nitrogenous waste is generated throughout the feed input, animal metabolism, faecal discharge, microbial decomposition and organic matter mineralisation. Failure to efficiently transform this nitrogen load can result in accumulation of ammonia and nitrite and lead to toxicity within the culture system, and discharged nitrate and organic nitrogen can lead to eutrophication in the receiving environment. Hence, the biological and enzymatic mechanism of the conversion of nitrogen is at the heart of sustainable aquaculture wastewater treatment (Preena et al., 2021; Crab et al., 2007; van Rijn, 2013).

In the present thesis, the interaction between selected natural ligands and cd1 nitrite reductase NirS is examined, which lies in this body of literature. This docking-based approach is not a substitute for wastewater tests, microbial testing or enzyme kinetics. It rather offers a preliminary screening test at the molecular level to help in the identification of the ligands and enzyme compatibility, and to pave the way for further testing and validation. This literature review, therefore, provides the scientific foundation for linking aquaculture wastewater treatment and bacterial denitrification enzymes, natural flavonoids and computational molecular docking.

2.2 Aquaculture Growth and Wastewater Management Challenge

Aquaculture is now a significant part of the world's aquatic food production system. The Food and Agriculture Organization (FAO) reported that in 2022, aquaculture production has hit a new high and surpassed that of catch fisheries for aquatic animals for the first time (FAO,

2024). This is a change in the trends of global food systems, which are becoming more reliant on farmed fish, shrimp, shellfish and other aquatic organisms. But the same expansion has led to a growing concern for wastewater discharge, nutrient load, disease pressure and ecological sustainability.

The composition varies from wastewater from different aquaculture systems, and is biologically active, constantly modified by feed, animal biomass, microorganisms, water exchange rate and system design. The most significant wastes in intensive systems are suspended solids, feed not eaten, faecal particles, dissolved organic carbon, ammonia, nitrite, nitrate, phosphate and microbial biomass. Of special concern is the fraction of nitrogen since it directly impacts water quality and culture performance. Most feed used in aquaculture is high protein feed, and only a fraction of the feed nitrogen is retained in the animal. The rest goes into the water column as dissolved ammonia, organic nitrogen, faecal nitrogen or as decomposed particulate material (Crab et al., 2007; van Rijn, 2013).

Aquaculture waste can have both internal (on-site) and external (off-site) environmental impacts. The build-up of nitrogen inside reduces growth, affects physiological processes, introduces stress, disrupts biofiltration and makes the culture organisms more susceptible to disease. Untreated or inadequately treated discharge may add nitrogen and phosphorus to receiving waters, which can lead to an increase in algal growth and reduce water oxygen levels. To achieve sustainable aquaculture, the treatment system must be able to accommodate production of animals while minimizing nutrient discharge to the environment (Crab et al., 2007; van Rijn, 2013).

To help mitigate these concerns, part of the reason that recirculating aquaculture systems have been developed is because they will have lower water usage and better waste removal. In these systems, water is treated by mechanical filtration, biological filtration, gas exchange, and disinfection and/or denitrification in some cases and reused. Recirculation eliminates the need for all the time water exchange, but it makes biofiltration even more significant because if biofiltration is not occurring, then ammonia and nitrite may accumulate rapidly. Nitrogen-removal biology is hence an important design element in intensive aquaculture systems (Preena et al., 2021; van Rijn et al., 2006).

2.3 Nitrogenous compounds in aquaculture waste water

The major nitrogenous waste products in aquaculture effluents are ammonia, nitrite, nitrate, dissolved organic nitrogen and particulate organic nitrogen. The production of ammonia is primarily from protein metabolism and the microbial breakdown of organic material. Ammonia nitrogen (NH3-N) in water is present in both unionised and ionised forms. These two forms are in a balance, which is affected by pH, temperature and salinity. The toxic effects of unionised ammonia are greater due to its greater ability to cross biological membranes, which can disrupt respiration, ion regulation, neurological function and overall metabolism in aquatic organisms (Crab et al., 2007; van Rijn, 2013).

Nitrite is typically generated as a transition product between the processes of ammonia oxidation (nitrification) to nitrite by ammonia-oxidising microorganisms, and nitrite to nitrate by nitrite-oxidising microorganisms. Nitrite is toxic because it causes the haemoglobin to become methaemoglobin which is the process that is often associated with brown blood disease in fish. Nitrate is usually not as acutely toxic as ammonia or nitrite but its accumulation must be avoided, either because it may be toxic at high concentrations for aquatic organisms, or because it can cause eutrophication when it is discharged outside the production system (Kroupová et al., 2005; Preena et al., 2021; van Rijn et al., 2006).

Organic N also contributes significantly due to the ability to mineralise organic N to ammonia as a result of microbial degradation. The uneaten feed and faecal solids are thus still a continuing source of nitrogen after cessation of feed. This load can be reduced by mechanical removal of solids, but still needs to be processed biologically as a dissolved substance. To control nitrogenous compounds in aquaculture waste water, a multi-disciplinary approach to control of solids, microbial biofiltration, oxygen, pH, carbon balance and hydraulic retention is required (Crab et al., 2007; van Rijn, 2013).

2.4 Biological Nitrogen Removal in Aquaculture Systems

Dissolved inorganic nitrogen is mainly transformed in aquaculture systems via biological nitrogen removal. While physical process can remove suspended solids and chemical process can alter the water chemistry, microbial process can transform ammonia, nitrite, and nitrate into less toxic and/or removable forms. The most popular biological pathway is nitrification followed by denitrification. Nitrification transforms ammonia to nitrite and then to nitrate under

aerobic conditions and denitrification to gaseous nitrogen compounds under oxygen limited and even oxygenic conditions (Kuypers et al., 2018; Preena et al., 2021; van Rijn et al., 2006).

Typically, in a conventional biofilter the nitrogen-control process is nitrification. Ammonia-oxidising bacteria (and archaea) transform ammonia to nitrite, nitrite-oxidising bacteria transform nitrite to nitrate. This is necessary as ammonia and nitrite toxicity is abated. But the nitrogen is not eliminated from the system by nitrification, it only alters the form of the nitrogen. Nitrate can build up if it is not removed by water exchange, plant uptake, algae, denitrification or other treatment routes, as described by Crab et al., 2007 and van Rijn, 2013.

Denitrification is significant when nitrate buildup is a problem. During denitrification, the nitrate is converted in a series of steps to nitrite, nitric oxide, nitrous oxide and finally dinitrogen gas. This pathway allows for the transfer of nitrogen out of the water column as a gas and thus is important for the full removal of nitrogen. The conditions for denitrification, however, are not the same which include electron donors, suitable redox conditions, and functional denitrifying microbial communities. Incomplete denitrification can result in the accumulation of intermediates like nitrite, nitric oxide or nitrous oxide (Zumft, 1997).

Aquaculture systems are also interested in heterotrophic nitrification, aerobic denitrification, biofloc systems, integrated multi-trophic systems and microbial fuel cell-based nitrogen removal. These approaches demonstrate the need for adaptable systems which can cope with the constantly changing parameters of aquaculture, such as oxygen, organic carbon, microbial biomass, and water circulation. However, in either treatment configuration, nitrogen conversion requires the microbial metabolism of microbes that are able to metabolize in an enzyme-mediated fashion. This is one of the reasons why understanding of the level of enzymes is crucial to understand and optimize biological nitrogen removal (BNR) (Crab et al., 2007; Kuypers et al., 2018; Preena et al., 2021).

2.5 Enzymatic Basis of Nitrification and Denitrification

Nitrogen removal is not just a community-level process, it's a process that is catalysed by specific enzymes which catalyse individual chemical transformations. Amoeba undergoes nitrification, in which ammonia is converted to hydroxylamine by the action of the enzyme ammonia monooxygenase. Further oxidation of hydroxylamine leads to the formation of ammonia, in which the hydroxylamine oxidoreductase (HAMX) is the most central enzyme in ammonia oxidizing bacteria. Nitrite oxidoreductase is an enzyme that acts as a catalyst in a

chemical reaction that oxidizes nitrite to nitrate in bacteria that can oxidise nitrite. These reactions help to turn harmful ammonia and nitrite into less toxic nitrate (Kraft et al., 2011; Pajares & Ramos, 2019).

Another series of enzymes are involved in denitrification. Nitrate is converted to nitrite by nitrate reductase. Nitrite is reduced to nitric oxide by the action of nitrite reductase. Nitric oxide reductase acts to convert nitric oxide to nitrous oxide. Nitrous oxide is converted to dinitrogen gas by nitrous oxide reductase. The simultaneous activity of these enzymes will decide if a complete denitrification or if it will stop at a partial level. N2O is a greenhouse gas and incomplete denitrification might represent a second environmental issue while nitrate removal from wastewater occurs, as the process is environmentally relevant (Kuypers et al., 2018; Zumft, 1997).

The enzyme targeted for this thesis is the cd1 nitrite reductase NirS which is part of the denitrification pathway. This catalyses the reduction of nitrite to nitric oxide, which is an important step in the anaerobic respiration of nitrate. The RCSB Protein Data Bank entry 6TSI describes a nitrite reductase NirS structure from Pseudomonas aeruginosa PAO1 (Kluenemann & Blankenfeldt, 2020) with dihydro-heme d1 (heme d1) bound. This enzyme is of structural relevance as cd1 nitrite reductases contain haem cofactors and a well-defined architecture of the active site which enables the reduction of nitrite. Nitrite, being a toxic compound in aquaculture systems, is directly relevant to the biological logic of the research on nitrogen-removal, because one of the enzymes involved in conversion of nitrite to nitrate is relevant to aquaculture.

NirS is thus a scientifically valid docking target for this study focusing on enzymes for nitrogen removal. It relates the environmental issue of nitrite build up to a particular bacterial enzyme involved in denitrification. But, it is necessary to differentiate between the enzyme binding and wastewater performance. The stronger the binding of the ligand in a docking model, the better the nitrogen removal is not always the case. It does not imply that a ligand could bind to a region of the enzyme that is structurally relevant, though, and would need to be tested further by molecular dynamics, enzyme assays, microbial testing and wastewater-scale experiments.

2.6 Structural Databases and Protein Selection for Docking.

The key to computational docking is a reliable three-dimensional structure of a receptor. The PDB is the main repository of experimentally determined structures of biological macromolecules and is well utilized in structural biology, molecular modelling and docking studies (Berman et al., 2000). The structures of proteins deposited in the PDB enable the researchers to explore binding pockets, active site residues, cofactors and the overall architecture of the protein. When conducting enzyme-related docking studies, it is necessary to have a structurally resolved receptor at hand since the docking prediction is highly dependent on the quality of the receptor model.

In this thesis, the structure of the receptor will be based on PDB ID 6TSI. The receptor in this thesis' methods chapter is the enzyme nitrite reductase, because the original protein structure was cleaned prior to docking using AutoDock Vina. The atom types, atomic coordinates, torsion data for ligands, and charge-related data needed by AutoDock-family programs are stored in PDBQT files. The preparation of the receptor is therefore not a small technical detail but has a direct impact on the search space of the docking, the generation of the binding poses and the output of the score (Forli et al., 2016; Morris et al., 2009).

Typical steps in the preparation of protein involve the removal of irrelevant water molecules, deletion of nonessential heteroatoms, addition of polar hydrogens, charging and conversion to the desired docking format. These steps should be repeated consistently as to avoid bias in the receptor preparation in ligand comparisons. All the ligands in the present thesis were docked into the same prepared receptor, making the docking scores presented in this thesis more comparable. The main drawback is that the receptor was assumed to be static, which is a commonly used approximation in the initial stages of virtual screening, but does not fully capture the flexibility of a protein in a biological environment (Forli et al., 2016; Trott & Olson, 2010).

2.7 Natural Bioactive Ligands and Flavonoid Chemistry

The ligands that were chosen for use in this thesis are part of a group of naturally occurring polyphenolic compounds: apigenin, catechin, naringenin, quercetin and kaempferol. Flavonoids are chemicals found in plants that vary in their phenolic structures, but share the same carbon skeleton, which can be substituted by a variety of compounds and/or can engage in a variety of interactions. They can be found in various plants, such as fruits and vegetables,

grains, tea, flowers, etc. With the structural diversity, the different flavonoids might exhibit different antioxidant, antimicrobial, anti-inflammatory and enzyme-interaction properties (Panche et al., 2016).

The chosen ligands are structurally similar but different flavonoids subclasses. Apigenin is a flavone, Catechin is a flavan-3-ol, Naringenin is a flavanone, Quercetin and Kaempferol are flavonols. Such class differences are not just descriptive, but also affect the planarity, hydroxylation pattern, polarity, flexibility and hydrogen bonding or aromatic interactions of the molecules. In molecular docking, these structural attributes may affect the orientation of the ligand in the binding site, the ability to form stable hydrogen bond(s) and the orientation of aromatic rings for hydrophobic or π-mediated contacts (Panche et al., 2016).

Quercetin has a number of hydroxyl groups and is relatively planar, offering several possibilities for hydrogen-bonding. Kaempferol belongs to the flavonol category of polyphenols, and is a member of a structural class that shares at least one hydroxyl substitution with quercetin but also differs in several other substitutions, which may lead to different hydrogen-bonding opportunities. But, apigenin contains fewer hydroxyl groups and has a planar flavone scaffold that could facilitate the accommodation in a compact pocket. The structure of naringenin is less planar than that of flavones and flavonols, and has a flavanone scaffold. The molecular structure of catechin is a flavan-3-ol that has a higher conformational flexibility. The differences warrant comparative docking since the molecule with the highest number of hydroxyl groups may not be the strongest binder; rather, binding will depend on fitting the binding pockets of functional groups into the receptor residues.

Natural ligands do not constitute an instant suggestion to incorporate these compounds to aquaculture wastewater itself as discussed in this thesis. The role they play is best seen as being that of model bioactive compounds for computational screening. By docking them against cd1 nitrite reductase NirS it becomes possible to determine if they are compatible with the enzyme-binding environment as predicted. When a ligand has favourable binding and any meaningful residue level interactions, then it may be prioritised for further molecular dynamics simulation, enzyme inhibitory or activating assays, microbial denitrification assays, and toxicity assessments.

2.8 Molecular Docking as a Screening Tool

Molecular docking is a computational technique that has been developed to be able to predict the preferred orientation of a ligand that is bound to a receptor and to estimate the relative binding strength of the complex. It is applied to drug discovery, but its reasoning is applicable to environmental biotechnology, as in many processes in the environment, interactions between enzyme and substrate or between enzyme and inhibitor or enzyme and pollutant are at play. Docking can also be used to predict if a molecule will bind in a pocket, have stabilising contacts, and interacting with residues which can be critical for enzymatic activity (Forli et al., 2016; Pagadala et al., 2017).

AutoDock Vina is the most popular molecular docking software, due to the fact that it is efficient in conformational searching and is based on an empirical scoring function which can provide an estimate on the binding affinity in kcal/mol. Negative binding scores are a better prediction of binding under the scoring model. Vina's docking speed and accuracy were superior to previous methods and is suitable for use in practical virtual screening workflows (Trott & Olson, 2010). The PyRx platform with its graphical user interface to load receptors, prepare ligands, to conduct virtual screening, and manage docking results were used for AutoDock Vina in the present thesis.AutoDock Vina was used through the PyRx platform, which has a graphical user interface to load receptors, prepare ligands, conduct virtual screening, and manage the results of the docking. (Dallakyan & Olson, 2015).

One of the important aspects of docking validity is the preparation of the ligands. PubChem is a large-scale chemical database that can be used to search for small molecules, including structures and information about the compounds, which can be useful for computational studies to find ligand structures (Kim et al., 2023). Ligands are usually subjected to energy minimisation after retrieval to relieve unrealistic strain prior to docking. The Universal Force Field is often used for global parameterization of small molecules since it has been developed to be applicable to the entire periodic table (Rappe et al., 1992). Open Babel can handle the conversion of chemical files and interconversion of molecular formats, e.g., where the structures need to be converted to docking formats (O' Boyle et al., 2011).

Docking outputs should be taken with a pinch of salt. The binding affinity values can be useful for the comparative ranking of the docked ligands if they are docked against the same receptor in the same conditions. But a docking score by itself does not provide an indication of biological effect. Hydrogen bonding, hydrophobic contacts, π interactions, involvement of

residues, and binding orientation can only be studied by interaction analysis. Despite this, the behavior of the solvent, flexibility of the protein, conditions within the microbial cell, metabolism of the ligand, bioavailability or chemical properties of the wastewater cannot be entirely explained by docking. As such, docking is more of a hypothesis generating technique than a definitive proof of function (Forli et al., 2016; Pagadala et al., 2017; Trott & Olson, 2010).

2.9 Relevance of Docking to Environmental Biotechnology and Aquaculture Wastewater

Molecular tools are now more and more employed in environmental biotechnology to elucidate the roles enzymes play in changing pollutants, nutrients and bioactive molecules. Enzymes play a key role, especially in wastewater studies, where they will dictate whether chemical transformations occur efficiently or not. In aquaculture wastewater, the nitrogen removal is accomplished by enzymes that catalyze the conversion of ammonia, nitrite, nitrate, nitric oxide, nitrous oxide and dinitrogen in specific steps. The ability to analyse the docking of selected ligands with these enzymes before embarking on more costly experimental work, makes it a useful tool.

The importance of docking in the present thesis is that it could be used to link the molecular level interaction to the questions of treatment on the system level. Strong binding of a ligand to a nitrogen removal enzyme could lead to additional questions: Is the ligand in the vicinity of a catalytic region? Does it interact with residues associated with binding to its substrate? Does the ligand bind to or mask the binding pocket? Are the bonds in the complex not broken under molecular dynamics simulation? Does the ligand affect the activity of the enzyme in-vitro? Is there any influence on the transformation of nitrogen in bacterial culture? While docking is a logical starting point to design such follow-up studies, Docking cannot be used to answer these questions.

This method is especially suitable for natural compounds since a lot of flavonoids have properties which facilitate their interaction with proteins. They can engage in π-mediated or hydrophobic contacts and in hydrogen bonding. The same properties, however, are not indicative of beneficial biological effects. There are several possibilities for a ligand to inhibit an enzyme, but not have a meaningful effect in the cell, not get to the enzyme in a microbial system or be toxic at the effective concentrations. Hence, there is a need to validate the results

of docking with microbiological, biochemical and environmental tests before any conclusion is drawn to apply it.

The docking results in this thesis should be regarded as a preliminary ranking of the ligand compatibility to cd1 nitrite reductase NirS. The later results chapter shows that the highest predicted binder of the selected ligands was apigenin followed by catechin, naringenin, quercetin and kaempferol. The results found in this chapter are explained by the literature reviewed in this chapter, which helps to interpret the results by explaining why nitrite reductase is relevant, why the structure of flavonoids determines the results, and why the results cannot be seen as confirmatory but as predictive.

2.10 Research Gap

Although removal of nitrogen, biofiltration, nitrification, denitrification and manipulation of microbial populations have been previously well addressed in relation to aquaculture effluents, most of these papers focus primarily on the design of the reactor, water quality, microbial communities and remove efficiencies, which is all important and essential information, yet fails to cover interactions between ligands in the environment and bacteria's enzymes involved in the process at a molecular level. The literature involving molecular docking, however, is already thoroughly developed for the field of drug discovery, and has barely ever been utilized in the field of aquaculture wastewater enzymes.

This omission of research means there is a crucial gap in between a variety of fields, namely between structural biochemistry and environmental biotechnology. Aquaculture wastewater has yet to develop a comprehensive understanding, driven by a mechanism, of what types of ligands are compatible with the appropriate bacteria's enzymes, and computational docking models still have to be developed for environmental enzymes rather than just clinical ones.

The aim of this project has been to fulfill this research gap by conducting an in silico analysis involving molecular docking between various phytochemicals isolated from plant life and bacterial cd1 nitrite reductase (NirS) – an enzyme essential in the rate-limiting step of reducing poisonous nitrite during denitrification. The analyze of specific interactomes by this project results in an entirely novel, interdisciplinary, computational method linking engineering and structural biochemistry.

This study is based on three main ideas that are all connected to each other:

1. Ecotoxicological Imperative**:** Within intensive aquaculture systems, the accumulation of toxic nitrogenous intermediates—specifically ammonia and nitrite—represents the primary

driver of water quality degradation, inducing severe physiological stress, toxicity, and mortality in aquatic stock.

2. Enzymatic Dependency**:** The structural transformation and eventual detoxification of these hazardous nitrogen species are not passive chemical events, but are actively driven by specific, rate-limiting bacterial enzymes embedded within the microbial consortia of biofilters.

3. Predictive Chemical Bottlenecking**:** Utilizing *in silico* molecular docking allows for the high-throughput mapping of structure–activity relationships (SAR). By identifying discrete binding configurations, spatial orientations, and thermodynamic affinities, this framework generates mechanism-driven hypotheses to screen and prioritize candidate molecules, eliminating arbitrary and expensive laboratory trial-and-error.

2.11 Chapter Summary

The literature reviewed in this chapter was to support the thesis. As aquaculture grows in significance, so does the need for wastewater management including control of nitrogen. Nitrogenous compounds, like ammonia, nitrite, and nitrate, affect the quality of discharges in the environment, microbial stability, and animal health. Relevant enzymes are biological nitrogen removal enzymes, particularly nitrification and denitrification enzymes, such as cd1 nitrite reductase NirS, which is involved in the reduction of nitrite to nitric oxide, which is a part of denitrification and nitrogen transformation.

The chapter also examined the selected natural ligands, and discussed the various contributions of the class, hydroxylation patterns, aromaticity and geometry of the molecules to the docking behaviour. The molecular docking was mentioned as a preliminary screening method, which provided an estimation of the pose, the binding affinity and the interaction profile. The literature provides a validation of the use of PyRx, AutoDock Vina, PubChem, Open Babel and UFF-based ligand preparation as proper tools for a comparative in silico workflow. Overall, the review sets the ground for the current study: docking selected natural ligands with cd1 nitrite reductase NirS (structure 3D models) can offer a useful preliminary molecular insight into the interactions between the enzyme and the ligand related to nitrogen-removal processes in aquaculture wastewater.

CHAPTER 3: MATERIALS AND METHODS

3.1 Research Design

In the present study, an in silico molecular docking-based study was carried out to assess the interaction of selected natural bioactive ligands with bacterial enzyme involved in the removal of nitrogen from aquaculture effluents. The approach was virtual screening based on the molecular structure, using a three-dimensional structure of the protein as receptor and the small molecules selected for virtual screening were docked into the defined binding region of the protein. It is suitable for this kind of study because molecular docking can give a preliminary estimate of the binding potential of a ligand to a protein, its preferred binding mode, and modes of non-covalent interactions between the proteins and the ligand before experimental attempts are made (Trott & Olson, 2010).

The target enzyme for this study was the nitrite reductase enzyme cd1 nitrite reductase NirS, which is directly related to nitrogen transformation, where NirS catalyses the reduction of nitrite to nitric oxide which is a key step in bacterial denitrification and anaerobic nitrate respiration. The cd1 nitrite reductase NirS with bound dihydro-heme d1 structure (PDB ID: 6TSI) was identified by the RCSB Protein Data Bank. Protein Data Bank is a standard global repository of experimentally determined biological macromolecular structures with wide usage in the retrieval of receptor for docking studies (Berman et al., 2000).

Five naturally occurring ligands – apigenin, catechin, naringenin, quercetin, and kaempferol – were screened in the study. These ligands were chosen as they have a structural similarity to flavonoids and polyphenolic compounds, and contain hydroxylated aromatic frameworks that can hydrogen bond, hydrophobic contact or π-mediated interact with protein residues. The docking workflow included receptor selection, preparation of the protein, preparation of the ligand, energy minimisation, definition of the docking grid, molecular docking by AutoDock Vina software in PyRx and post-docking interaction analysis.

3.2 Selection of Target Protein

For the docking target receptor, the cd1 nitrite reductase NirS was selected from the PDB ID: 6TSI. The choice of NirS was based on its biological role in denitrification, particularly its involvement in the reduction of nitrite to nitric oxide. It is important in the removal of nitrogen because the accumulation of nitrite is a toxic intermediate which may occur in wastewater and aquatic systems if the microorganisms are not able to remove nitrogen. The efficient conversion

of nitrite is critical in aquaculture wastewater treatment as nitrite can affect the oxygen transport in aquatic organisms and can negatively affect the performance of biofilters.

The receptor was chosen since it is a bacterial enzyme directly involved in the process of nitrogen removal and not a general metabolic enzyme. Structurally resolved receptors enable docking study to be targeted to the 3D binding environment of the enzyme. The receptor in the docking protocol was prepared and referred to as the nitrite reductase enzyme, which implies that the protein structure was already cleaned and was in the format necessary for docking with AutoDock Vina: PDBQT. The PDBQT format stores atomic coordinates, atom types, and partial charge information needed by AutoDock-family docking programs (Morris et al., 2009).

The receptor was considered as a rigid structure, and the ligands were considered as flexible molecules. This is often employed in initial virtual screening as it reduces the computational complexity and still enables conformational sampling of the ligand torsions in the search space for the docking. The problem with receptor rigidity is not a problem if the primary goal of this first stage comparative docking study is to rank the ligands based on the same docking conditions.

3.3 Retrieval and Preparation of Protein Structure

The 3D protein structure for PDB ID: 6TSI was downloaded from the RCSB Protein Data Bank. The PDB file has been checked prior to docking for suitability for receptor preparation. The preparation of the receptor described was a receptor preparation procedure that included the removal of non-essential molecules, correction of receptor format, introduction of polar hydrogens and the assignment of charges. The raw PDB structures often have crystallographic water molecules, co-crystallised ligands, ions or other heteroatoms that can affect the docking if they are not in the protocol of the intended docking.

In the present study the receptor used was as the enzyme nitrite reductase. The cleaned receptor was converted to PDBQT format for compatibility with AutoDock Vina during the preparation of the protein. The conversion to PDBQT is crucial because these atom-type and charge related data are required by AutoDock Vina in the receptor and ligand files. The generated receptor was then added to PyRx as macromolecule for docking.

The cleaning stage was carried out to assure the docking of the ligands to the protein receptor, and not to any crystallographic artefacts. Water had no explicit representations in the docking procedure and water molecules were not included. Unless they were biologically relevant molecules within the catalytic domain of the receptor, non-structural molecules were

removed. The receptor was then cleaned for all subsequent docking runs of the ligands to avoid any differences in receptor preparation which would affect the binding-affinity comparisons..

3.4 Selection and Preparation of Ligands

The following five ligands, apigenin, catechin, naringenin, quercetin and kaempferol were chosen for the study of docking. These compounds are natural polyphenolic molecules with established biological significance and multiple functional groups that are able to interact with proteins. Because of their structural similarity they were appropriate for comparative docking, as difference in hydroxylation, ring planarity and geometry of the molecule could be correlated with difference in binding affinity, interaction profile.

The three-dimensional structure of the ligands was obtained from a public chemical information database, PubChem, that is commonly used for obtaining the small-molecule structure and identifier for computational studies (Kim et al., 2023). Chemical structure, molecular formula and chemical identifier, among other types of related compound data, are available for ligands for use in the docking process from PubChem. The structures of the ligands were retrieved in SD format and imported to PyRx to prepare.

The preparation of the ligands involved energy minimisation and conversion to PDBQT format. The Universal Force Field (UFF) was used to perform the energy minimisation for small molecules, which are known to have a good parameter coverage across the periodic table of elements (Rappé et al., 1992). The unfavourable bond angle, torsional strain and steric clashes are minimized in the structure of the ligand before docking. The ligands were minimized, and then converted to PDBQT format using the tools that are built into PyRx and Open Babel. Open Babel is an open source cheminformatics toolbox for converting, atom typing and interconverting between molecular file formats in cheminformatics pipelines (O' Boyle et al., 2011).

To ensure method uniformity all five ligands were prepared following the same procedure. In the preparation, no manual favouring was done on any of the ligands, and no docking parameter was changed for single compounds. This was a requirement as the aim of the study was to compare the performance of the ligands under the same docking conditions.

Table 3.1

Ligands Selected for Molecular Docking

Ligand	Compound Class	Purpose in Study
Apigenin	Flavone	Natural ligand screened against NirS
Catechin	Flavan-3-ol	Natural ligand screened against NirS
Naringenin	Flavanone	Natural ligand screened against NirS
Quercetin	Flavonol	Natural ligand screened against NirS
Kaempferol	Flavonol	Natural ligand screened against NirS

3.5 Docking Software and Computational Tools

Virtual screening was carried out by molecular docking using PyRx software that includes AutoDock Vina for docking calculation, and this software has a graphical virtual screening interface. PyRx is widely used to perform virtual screening of small molecules because it facilitates loading the receptor, preparing the ligands, minimising the energy of the ligands, converting files between formats, setting up the docking, and docking without the need to change the computational environment (Dallakyan & Olson, 2015).

Docking calculations were performed with the docking program AutoDock Vina, which is used to predict the docking pose of a ligand and to estimate binding affinity using an empirical scoring function. The improved speed, multithreading capacity and the efficient optimisation algorithm of AutoDock Vina make it widely used in comparison with previous docking tools (Trott & Olson, 2010). The binding affinity values obtained with Vina are expressed in units of kcal/mol with more negative numbers representing more favorable predicted binding of the ligand to the receptor.

Ligand conversion workflow: Open Babel was used. It was used as a key step in the processing of ligand files to be compatible with the docking platform and for preparing the molecules for docking processes (O' Boyle et al., 2011). Visual inspection and interpretation of interaction after docking were performed by the use of molecular visualisation software like BIOVIA Discovery Studio visualiser. Discovery Studio Visualizer can be used to perform visual analyses of protein and ligand structures, and is often used to examine the hydrogen bonds, hydrophobic contacts, π interactions, and other aspects of ligand/protein interactions.

3.6 Docking Grid Parameters

A docking grid box was defined to cover the relevant binding region of the prepared cd1 nitrite reductase receptor. The grid box determines the three-dimensional search space within which AutoDock Vina explores possible ligand orientations and conformations. If the grid box

is too small, relevant binding regions may be missed. If it is too large, the search becomes less focused and may increase the possibility of non-specific binding poses. Therefore, the grid parameters were selected to include the receptor-binding region adequately while maintaining a consistent docking space for all ligands.

The docking protocol used the following grid settings:

Table 3.2: **Docking Grid Parameters Used in PyRx/AutoDock Vina**

Parameter	Value
Receptor file	nitrite reductase enzyme
Exhaustiveness	8
center_x	27.0937343505
center_y	11.0384249683
center_z	28.1098273286
size_x	93.0235512284
size_y	105.609879737
size_z	118.298873886

The default search-intensity (exhaustiveness) value in docking workflows using AutoDock Vina was selected, as is often done in these docking applications (8). Exhaustiveness is used to limit conformational search in docking. The greater the number the deeper the search will go, but the longer the computer will take to do it. For consistency, exhaustiveness 8 was kept for all the ligands in this study.

All of the ligands were placed in the same grid box. This was necessary as the grid size and centre coordinates of the individual ligands would be changed, which would not allow the docking scores to be compared. The study used a single receptor, a single docking grid and a single exhaustiveness value on all ligands to allow the differences in predicted binding affinity to reflect the behaviour of the ligand in identical computational conditions.

3.7 Molecular Docking Procedure

Docking was done in a step-by-step process. The enzyme receptor (prepared nitrite reductase enzyme) was loaded as a macromolecule into PyRx. The 5 prepared ligands were then loaded

into the ligand workspace. All ligands were validated after import to make sure that they are in the proper format and that they have been energy minimised prior to docking.

Secondly, the size and centre of the docking grid were set. All ligand docking runs were carried out with the same grid settings. The docking analysis was then carried out by using AutoDock Vina via PyRx. While docked, Vina generated possible binding conformations for each ligand in the defined grid box and ranked the poses by their predicted binding energy.

Third, the highest scoring pose for each ligand in the docking was chosen to be interpreted. The pose with the lowest binding affinity was decided as the best pose. This convention is followed broadly in docking interpretation due to the fact that the more negative the binding energy, the more likely it is to form a ligand-receptor complex using AutoDock Vina's scoring function (Trott and Olson, 2010). But the meaning of binding energy was not taken alone. For each of all the selected poses, the type of interaction, residue participation, the orientation of the binding, and visual accommodation inside the receptor pocket were analyzed.

The output files obtained from docking were saved for post docking analysis. Using visualisation tools, the docked complexes were analysed to find interacting residues and binding patterns. For the interaction analysis, hydrogen bonding, hydrophobic contacts, van der Waals interactions, π–π interactions, π–alkyl interactions, and other interactions known to be stabilizing were analyzed in the docked complexes.

3.8 Binding Affinity Evaluation

Each docked ligand was given a binding affinity in kcal/mol. The more negative or lower the binding energy is in the AutoDock Vina output, the greater the predicted interaction between the ligand and receptor. For instance, a binding energy of −9.2 kcal/mol is a stronger prediction of binding than a binding energy of −7.1 kcal/mol under the same docking conditions. The docking scores of the five ligands could be comparatively ranked as all of them were docked onto the same receptor using the same grid and exhaustiveness value.

The binding affinity ranking was employed to select the top predicted binder. The ranking was however not considered as proof of biological activity but rather a computational prediction. Molecular docking provides information on the possible interaction potential under the simplified conditions and does not account for the full flexibility of the protein, an environment inside the bacteria, the specific environmental conditions in wastewater, or the effects of

solvents. Thus, docking results were only used for initial structure–activity considerations of the ligand–enzyme compatibility.

The docking scores were interpreted along with interaction profiles. A ligand that scores well but doesn't dock well or has weak interactions with residues at a particular position would need to be interpreted with caution. On the other hand, a ligand that could have more hydrogen bonding and hydrophobic stabilisation as well as was appropriate for pocket would be deemed more probable to be a viable candidate for either computational or experimental testing.

3.9 Protein–Ligand Interaction Analysis

Once docked, the best pose of each ligand was then visualised to determine the molecular interactions in the receptor-binding region which were responsible for stabilization. Both 2D and 3D interaction views were analysed. Two dimensional interaction diagrams were used to determine specific amino acid residues involved in the binding of a ligand, and three dimensional, to understand the spatial position of the ligand within the protein structure.

Hydrogen bonds were deemed to have directional interaction between the receptor and ligand atoms, and were therefore thought to be important. Hydrophobic contacts were also observed, as they also help to stabilize the ligand in the non-polar part of the binding pocket. If present, aromatics interactions like π–π stacking or π–alkyl interactions were noted, particularly with the ligands chosen that include aromatic ring systems capable of π–π interactions or π–alkyl interactions.

Interaction analysis was separately carried out for each ligand. The last interpretation compared the three ligands based on three criteria: the predicted binding affinity, the number and type of interactions, and the orientation of the binding in relation to the receptor region. This way, the results chapter would not be based solely on the docking scores, but would also explain the molecular basis of the ranking of ligands.

3.10 Data Organisation and Presentation

The information on the docking was presented in tables and figures. The first level of data organisation is the binding-affinity table that shows the docking results from the best score. The second level was an interaction-summary table that listed the amino acid residues involved in hydrogen bonding, hydrophobic contacts, etc. that form the stabilising interactions. The third level contained images of the docked conformations, both two and three dimensional.

All of the ligands were ranked by predicted binding affinity in order of strength. The ranking was done according to the worst binding score that was found for each ligand. The interaction diagrams were employed as aids for the numerical docking scores. This was a joint presentation that enabled the results to be presented without any such vague comments as “good binding” or “better interaction” being made without substantiation.

3.11 Methodological Validity and Control of Bias

Several changes are made to enhance the reliability of the workflow for docking. The first is that the same receptor file was used for all the ligands. Secondly, all the ligands were prepared by the same minimisation and conversion process. Third, the same grid box was used for all the docking runs and the same exhaustiveness value was used. Fourth, binding energy and interaction analysis were used to interpret the docking results as opposed to binding score.

Docking parameters were not changed for individual ligands as this would have added bias to the comparative ranking. The ranking of the ligands in the database thus was done on the basis of the same docking protocol. A consistency of methodology was also provided by using established computational molecular docking software, such as PyRx, AutoDock Vina, Open Babel and PubChem-supported ligand retrieval, which are widely used in computational molecular docking workflows (Dallakyan & Olson, 2015; Kim et al., 2023; O’Boyle et al., 2011; Trott & Olson, 2010).

3.12 Ethical Considerations

Human subjects, animal research, clinical samples, or collection of biological specimens from aquaculture facilities were not involved in the present study. Thus, there was no formal human or animal ethical approval necessary. All research was conducted using structural and chemical databases that were publicly available and computational analysis. Structures of proteins were taken from the Protein Data Bank database and the structures of ligands were obtained from the PubChem database. All of the material used was derived from the database for academic and/or computational purposes.

3.13 limitations

There are some limitations in the methodology. Predictive approach and does not prove biological activity. The receptor was rigidly treated, meaning that all protein flexibility was neglected. There was also no mention of explicit solvent modeling, molecular dynamics simulation, enzyme kinetics, microbial culture testing or real aquaculture wastewater testing in

the docking protocol. Hence, the conclusion with respect to docking may be considered as preliminary computational proof.

In spite of these drawbacks, the method is suitable for the purpose of the study since it is a highly structured and reproducible approach to comparing selected natural ligands with a bacterial nitrogen-removal enzyme. The outcome can be used to inform future investigations using molecular dynamics simulation, binding free-energy estimation, in vitro enzyme assays, and microbial N-removal experiments.

CHAPTER 4: RESULTS

4.1 Overview of Molecular Docking Results

This chapter presents the molecular docking results obtained for five selected natural ligands, namely apigenin, catechin, naringenin, quercetin, and kaempferol, evaluated against the prepared nitrite reductase enzyme receptor. The target receptor corresponds to the structural model of the bacterial *cd1* nitrite reductase (NirS) enzyme, a critical catalyst involved in biological nitrogen removal pathways within aquaculture wastewater systems. The resulting docking outputs were analyzed using three distinct diagnostic parameters: the predicted binding affinity values, the spatial configuration of the ranked docking poses, and the characterization of two-dimensional and three-dimensional protein–ligand interaction patterns. Because all five phytochemicals were screened against an identical receptor pocket under uniform grid box dimensions and simulation constraints, their binding energies were interpreted comparatively. Under the AutoDock Vina scoring matrix, a higher negative binding affinity reflects a thermodynamically more stable ligand–receptor complex within the designated search coordinates.

The computational screening demonstrated that all five natural compounds successfully occupied the catalytic site of the NirS receptor, yielding distinct binding energies. The highest binding efficiency was achieved by apigenin, followed in descending order by catechin, naringenin, quercetin, and kaempferol. These thermodynamic profiles indicate that apigenin possesses the highest structural compatibility with the bacterial enzyme under the simulated conditions. While catechin, naringenin, and quercetin also demonstrated strong docking scores, kaempferol displayed the weakest interactive potential among the group. This hierarchical variation strongly suggests that the specific structural topology of the ligands—including hydroxyl substitution patterns, aromatic ring orientation, and spatial fit within the pocket—governs their binding efficacy.

Ultimately, these results serve exclusively as computational predictions within an *in silico* framework. A favorable docking score cannot independently confirm the biological activation, inhibition, or practical enhancement of nitrogen removal pathways in a functional aquaculture wastewater treatment system. Instead, this evaluation provides a molecular-level screening bottleneck designed to prioritize promising phytochemical candidates for subsequent experimental validation. Future research must expand upon these static findings

through molecular dynamics simulations, enzyme kinetics assays, microbial culture studies, and bioreactor experiments. This transition is essential because molecular docking operates under rigid, simplified conditions that do not replicate solvent dynamics, protein flexibility, microbial cellular physiology, or the complex chemical matrices of real-world wastewater.

4.2 Binding Affinity Ranking of the Selected Ligands

The primary docking result was the binding affinity value generated by AutoDock Vina through the PyRx virtual screening interface. The top-ranked pose for each ligand was identified as the pose with the most negative binding energy. Table 4.1 presents the comparative binding affinity of the five ligands. The values were taken from the PyRx docking result panels generated for each docked complex.

Table 4.1

Binding affinity ranking of selected ligands against cd1 nitrite reductase NirS

Rank	Ligand	Best binding affinity (kcal/mol)	Docking mode selected	Result interpretation
1	Apigenin	-9.2	Mode 0	Strongest predicted binder
2	Catechin	-8.6	Mode 0	Strong predicted binding
3	Naringenin	-8.4	Mode 0	Strong predicted binding
4	Quercetin	-8.3	Mode 0	Strong predicted binding
5	Kaempferol	-7.1	Mode 0	Lowest score among screened ligands

The binding affinity ranking shows a clear separation between the best-performing ligand and the weakest ligand. Apigenin produced a binding affinity of -9.2 kcal/mol and ranked first. Catechin, naringenin, and quercetin produced closely grouped scores of -8.6, -8.4, and -8.3 kcal/mol, respectively, indicating comparable predicted binding strength. Kaempferol showed a binding affinity of -7.1 kcal/mol, which was less favourable than the other four ligands. Although the difference between catechin, naringenin, and quercetin was small, the score gap between apigenin and kaempferol was larger, suggesting a stronger distinction in predicted receptor compatibility.

In addition to the primary binding-affinity ranking, the docking modes were statistically summarised to provide a clearer comparison of pose-level variation. Table 4.2 presents the best binding affinity and the mean ± standard deviation calculated from the top five AutoDock Vina poses reported for each ligand.

Table 4.2

Calculated docking-score summary for selected ligands against cd1 nitrite reductase NirS

Rank	Ligand	Top five docking modes (kcal/mol)	Best binding affinity (kcal/mol)	Mean ± SD (kcal/mol)	Result interpretation
1	Apigenin	-9.2, -9.0, -8.5, -8.4, -8.3	-9.2	-8.68 ± 0.40	Strongest predicted binder
2	Catechin	-8.6, -8.0, -7.9, -7.6, -7.6	-8.6	-7.94 ± 0.41	Strong secondary binder
3	Naringenin	-8.4, -8.4, -8.1, -8.1, -8.0	-8.4	-8.20 ± 0.19	Strong intermediate binder
4	Quercetin	-8.3, -7.9, -7.9, -7.8, -7.5	-8.3	-7.88 ± 0.29	Strong but lower than naringenin
5	Kaempferol	-7.1, -7.0, -6.8, -6.8, -6.7	-7.1	-6.88 ± 0.16	Weakest ligand in present set

Note. Mean ± SD was calculated from the five docking modes reported in the PyRx/AutoDock Vina output for each ligand. More negative binding-affinity values indicate stronger predicted binding under the applied docking conditions.

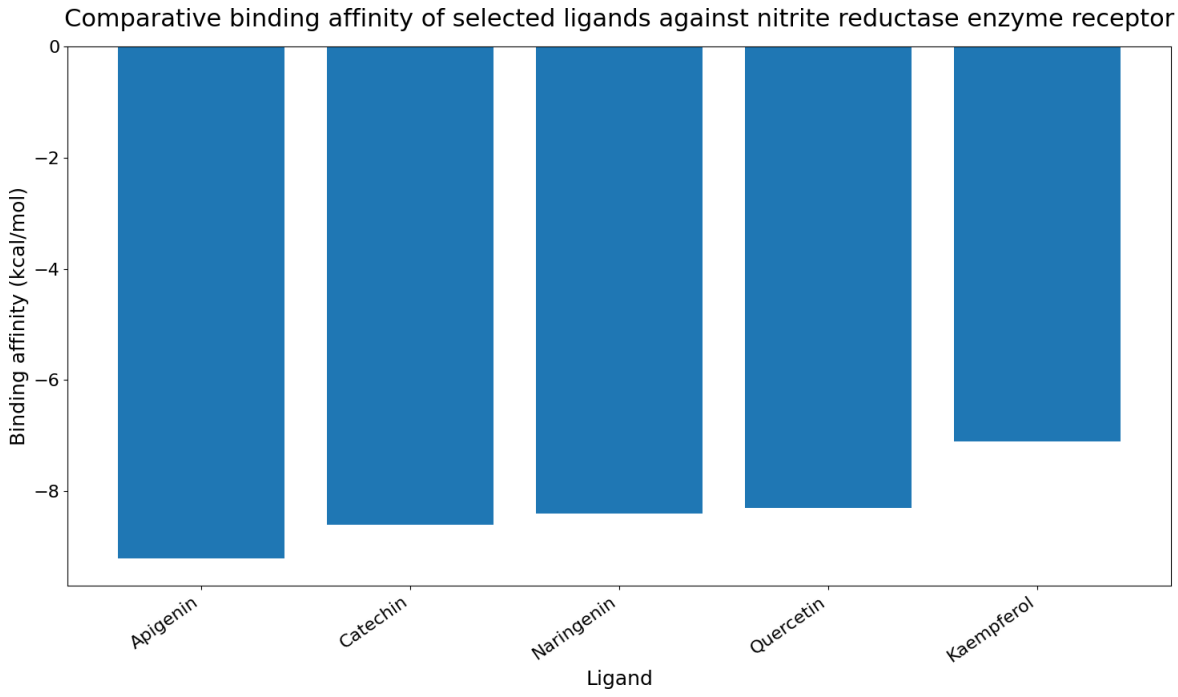


Figure 4.1. Comparative binding affinity profile of apigenin, catechin, naringenin, quercetin, and kaempferol against the prepared nitrite reductase enzyme receptor.

Figure 4.1 graphically confirms the ligand ranking shown in Table 4.1. Since the scores are negative values, bars extending further downward represent stronger predicted binding.

Apigenin is clearly the strongest predicted binder in the present dataset. Catechin, naringenin, and quercetin occupy an intermediate cluster with relatively close docking scores. Kaempferol appears as the weakest ligand in the comparative profile. This ranking provides the basis for the ligand-specific interpretation presented in the following sections.

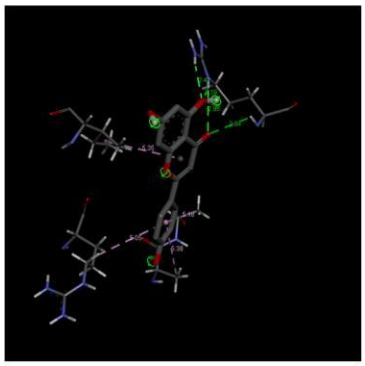
4.1 Docking Result of Apigenin

Apigenin produced the most favourable docking score among the five screened ligands, with a binding affinity of -9.2 kcal/mol. This score identifies apigenin as the top-ranked ligand in the present docking experiment. The PyRx result panel showed the best pose as mode 0, with the selected pose used for subsequent interaction analysis. A score of this magnitude suggests that apigenin had strong predicted compatibility with the receptor-binding region of cd1 nitrite reductase NirS under the applied docking protocol.

The two-dimensional interaction map of apigenin showed that the ligand was positioned within a residue-rich region of the protein. The visible interacting residues included Arg B:372, Gly B:371, Phe B:425, Phe B:533, Gln B:483, Arg B:185, Ile B:183, Ala B:284, His B:182, Arg B:225, His B:327, Tyr B:245, Asp B:328, and Gly B:531. The map indicated the presence of hydrogen-bonding contacts and additional close non-covalent interactions around the flavone scaffold. The 3D interaction view further supported that apigenin was accommodated inside the receptor environment rather than remaining at an isolated external surface.

The stronger binding score of apigenin may be related to the balance between its planar aromatic system and its limited but strategically positioned hydroxyl groups. Unlike larger or more flexible polyphenols, apigenin can fit into compact binding regions while still forming stabilising contacts. The interaction pattern therefore supports the docking score and indicates that apigenin should be treated as the principal computational hit from the present ligand set.

A. Three-dimensional docked pose



B. Two-dimensional interaction map

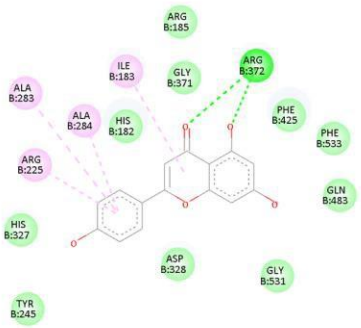


Figure 4.2. (A) Three-dimensional docked pose and (B) two-dimensional interaction map of apigenin docked with cd1 nitrite reductase NirS.

4.2 Docking Result of Catechin

Catechin ranked second among the five evaluated phytochemicals, demonstrating a robust binding affinity of -8.6 kcal/mol. The optimal docking pose chosen for downstream characterization corresponded to mode 0 within the PyRx configuration matrix. Although its binding energy did not exceed that of apigenin, the thermodynamic profile still indicates highly favorable predicted binding stability within the receptor site. The fractional variance of 0.6 kcal/mol between apigenin and catechin suggests that the latter remains an exceptionally competitive ligand within this virtual screening library for bacterial enzyme targeting.

Chromatographic analysis of the two-dimensional interaction topology revealed multiple close-range spatial contacts surrounding the core ligand architecture. The adjacent microenvironment was composed of key coordinating residues, including Val B:317, Ser B:319, Lys B:303, Thr B:318, Ser A:319, Leu B:315, Leu A:315, Leu A:305, Val A:317, Val A:264, Gly B:268, Met B:269, and Gln A:262. This interactome mapping confirms that catechin establishes a highly stabilizing network driven by conventional hydrogen-bonding contacts and non-polar hydrophobic associations. The highly polyhydroxylated scaffold of this flavan-3-ol likely maximizes electrostatic interactions, while its aromatic rings drive non-polar stabilization within the internal catalytic pocket of the enzyme.

These findings are highly significant as they demonstrate that a flexible flavan-3-ol framework can maintain strong thermodynamic compatibility with the target enzyme. Unlike structurally rigid planar flavones, catechin possesses greater conformational freedom and rotatable bonds, which directly influence its spatial orientation within the binding domain. In the present docking output, the ligand achieved optimal geometric accommodation despite these structural degrees of freedom. Consequently, catechin is classified as a primary secondary lead compound behind apigenin for subsequent computational validation aimed at

optimizing nitrogen bioremediation pathways.

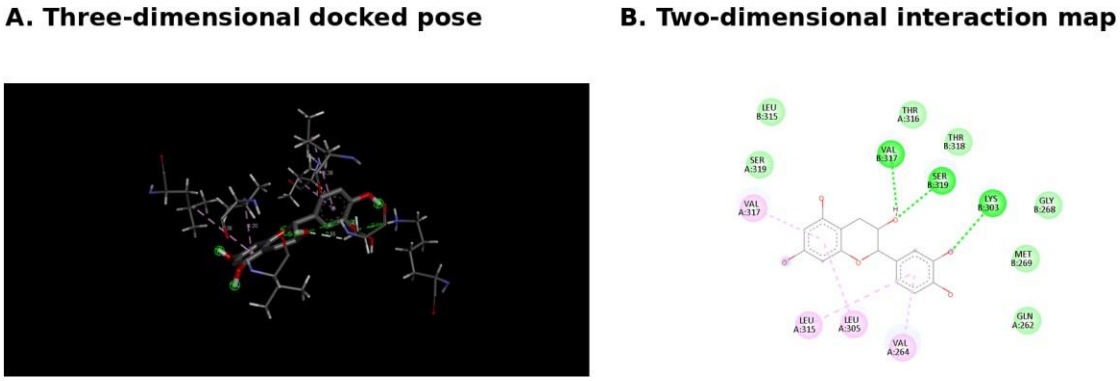


Figure 4.3. (A) Three-dimensional docked pose and (B) two-dimensional interaction map of catechin docked with cd1 nitrite reductase NirS.

4.3 Docking Result of Naringenin

Naringenin obtained 3 rd position out of the 5 tested phytochemicals, with binding affinity -8.4 kcal/mol. These similarities among top 3 hit compounds imply a proper hierarchy ranking of tested phytochemical library and further imply naringenin to be a reliable computational hit for validation purpose despite of its non-planar flavanone skeleton can't make geometrically optimal to maximal binding energy when compare with apigenin in aquaculture wastewater at simulated environment here. The results showed naringenin was able to have multi-centric interaction in a balanced polar/hydrophobic region of the binding site and indicated stabilization by the van der Waals and the directional electrostatic force especially in Met A:269 and its neighboring polar region. This data indicates that naringenin can indeed interact strongly in the binding site of the NirS receptor of bacteria, even though its chiral flavanone skeleton is not fully planar like flavones and flavonols.

As shown in the 2-D interactome, the spatial arrangement between the ligand and its surroundings indicated a tight atom-pair network covering wide micro-environment. Residues in close proximities that interacted with ligand included Met A:269, Gly A:268, Thr A:301, Lys A:303, Leu A:305, Val A:317, Val A:264, Val B:317, Leu A:315, Ser A:319, Ser B:319, Ser B:265, Val B:264, Thr A:266, Val A:298 and Pro A:280. Data showed that naringenin achieved multi-centric interactions within a balanced polar/hydrophobic binding area,

suggesting stabilization via van der Waals and directional electrostatic force particularly involving Met A:269 and its adjacent polar area.

The docking energy -8.4 kcal/mol confirmed that naringenin shows slightly higher predicted binding energy than catechin, while a better binding prediction than quercetin was calculated. These similarities among the top three hit compounds suggested a good hierarchical ranking of the tested phytochemical library and thus indicated that naringenin can serve as a good computationally derived hit for further validation, although its non-planar flavanone skeleton is less geometrically optimized to achieve maximal binding energy when comparing with apigenin within aquaculture wastewater context in this simulated scenario.

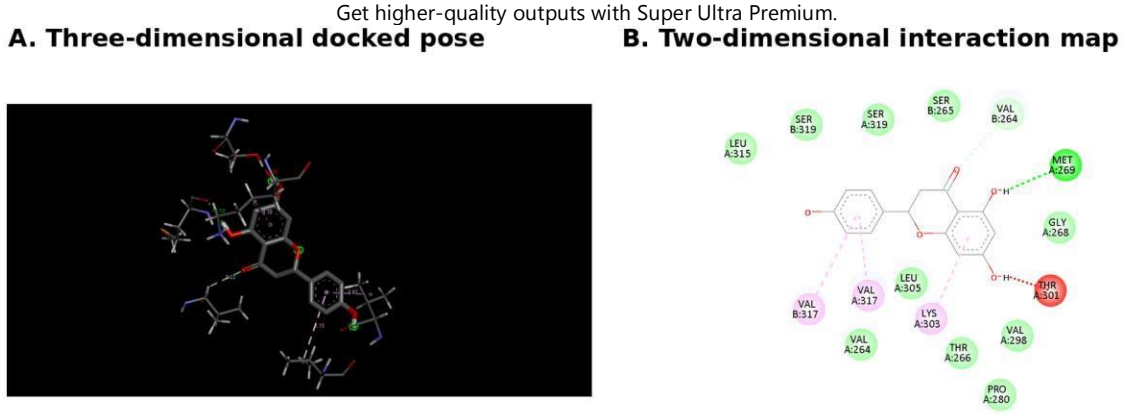


Figure 4.4. (A) Three-dimensional docked pose and (B) two-dimensional interaction map of naringenin docked with cd1 nitrite reductase NirS.

4.5 Docking Result of Quercetin

Therefore merely raising the number of hydroxyl substitutions are not adequate for the highest binding affinity. Though lower than naringenin, its predicted binding affinity is nearly equal to that (differ by only 0.1 Kcal/mol). It shows potent basal binding and though the binding topology, like Q43's has some spatial and conformational penalties which affect the final score, is a good candidate though not a hit in terms of bioremediation in the aquaculture wastewater. As a heavily polyhydroxylated flavonol, the many hydroxyl groups present in different positions within all of the rings, provide theoretical high density of sites for possible interactions via electrostatics and hydrogen bonding networks between the enzyme and amino acid residues.

The 2D interaction map provided revealed a large spread across several different residues as seen in Arg B:267, Gly A:268, Met A:269, Ser A:265, Lys A:303, Val A:317, Val B:317, Leu

A:315, Leu A:305, Thr A:301, Thr B:318, Ser B:319, Gly B:268 and Val A:264. In analyzing the map we see many stabilizing interactions and also specific red outlined ones, which refer to steric interference and energetically unfavored strain of conformations within the software package. This geometric resistance is why it did not rank higher than apigenin and catechin despite its high number of hydroxyl groups.

Thus, not only increasing the number of hydroxyl substitutions are sufficient for highest binding affinity. Interaction complement between receptor and ligand must rely on the ability of the overall molecule fit within the active pocket and the ability for the core molecule to orient itself without spatial or conformal restriction. Quercetin shows strong basal binding but its binding topology does have some positional and conformational penalties that limits its final score, identifying it as a strong candidate but not a hit for aquaculture wastewater bioremediation.

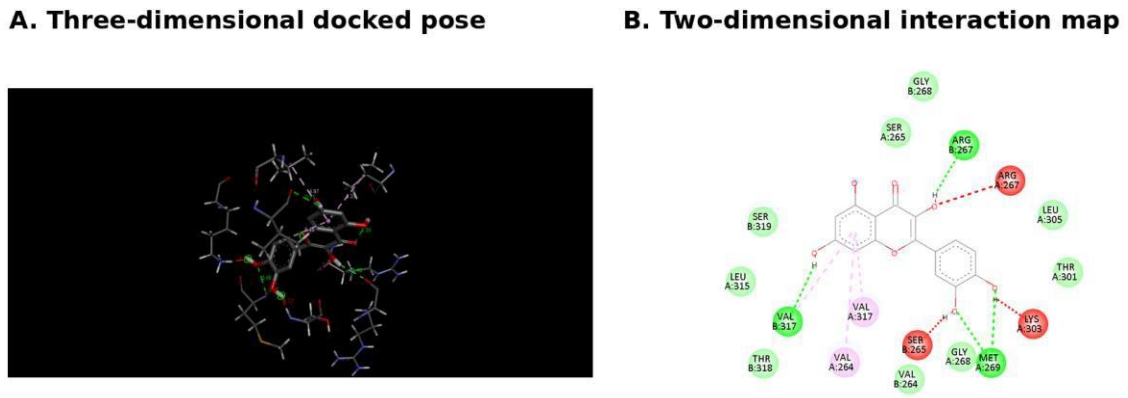


Figure 4.5. (A) Three-dimensional docked pose and (B) two-dimensional interaction map of quercetin docked with cd1 nitrite reductase NirS.

4.7 Docking Result of Kaempferol

Kaempferol produced the lowest binding affinity among the five ligands, with a docking score of -7.1 kcal/mol. The selected best pose was mode 0 in the PyRx output. Although the score still indicated measurable receptor interaction, it was less favourable than the scores obtained for apigenin, catechin, naringenin, and quercetin. The difference between apigenin and kaempferol was 2.1 kcal/mol, making kaempferol clearly weaker than the top-ranked ligand in this study.

The two-dimensional interaction profile of kaempferol showed contacts with residues including Tyr B:276, Arg A:267, Gln A:115, Glu A:118, Pro A:278, Tyr A:276, Arg B:267, Gln B:115, Glu B:118, Trp B:246, Leu B:54, Lys B:56, Thr B:64, Pro B:65, Pro B:113, and Pro B:278. The interaction map displayed hydrogen-bonding contacts as well as hydrophobic or close-contact residues. However, the weaker binding affinity suggests that these interactions were either fewer, less optimally oriented, or less energetically favourable than those observed for the higher-ranking ligands.

Kaempferol is structurally similar to quercetin but differs in its hydroxylation pattern. The weaker score suggests that small differences in substitution pattern can affect ligand orientation and interaction strength. This result is useful because it demonstrates that structurally related flavonoids do not necessarily behave identically during docking. In the present screening set, kaempferol should be considered the least promising ligand for further validation unless additional computational or experimental evidence indicates otherwise.

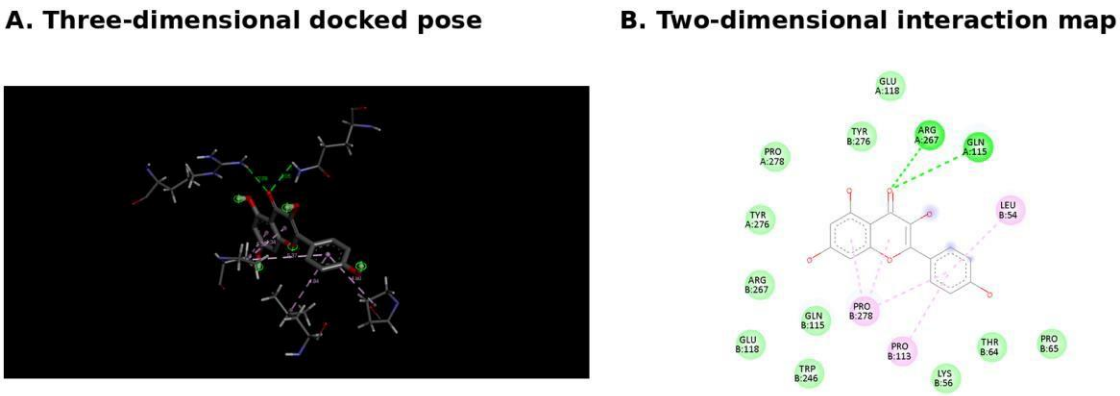


Figure 4.6. (A) Three-dimensional docked pose and (B) two-dimensional interaction map of kaempferol docked with cd1 nitrite reductase NirS.

4.8 Comparative Interaction Summary

The residue-level analysis showed that the ligands interacted with multiple amino acid residues distributed around the receptor-binding region. Several ligands shared contact zones involving residues such as Val, Leu, Ser, Gly, Arg, Met, Lys, and Thr. These residues suggest that the binding environment contained both polar and hydrophobic regions. The presence of hydrogen-bonding residues supports ligand anchoring, whereas hydrophobic and aromatic contacts contribute to ligand stabilisation within the protein environment. Table 4.4 summarises the major residues visible in the two-dimensional interaction maps and the overall interpretation of each ligand complex.

Table 4.3

Summary of major visible protein-ligand interactions from 2D interaction maps

Ligand	Binding affinity (kcal/mol)	Major visible interacting residues	Result interpretation
Apigenin	-9.2	Arg B:372, Gly B:371, Phe B:425, Phe B:533, Gln B:483, His B:182, Arg B:225, Tyr B:245, Asp B:328	Best overall docking profile
Catechin	-8.6	Val B:317, Ser B:319, Lys B:303, Thr B:318, Leu A:305, Val A:264, Gly B:268, Met B:269	Strong secondary candidate
Naringenin	-8.4	Met A:269, Gly A:268, Thr A:301, Lys A:303, Leu A:305, Val A:317, Ser A:319, Pro A:280	Strong intermediate binder
Quercetin	-8.3	Arg B:267, Gly A:268, Met A:269, Ser A:265, Lys A:303, Val A:317, Leu A:315, Thr A:301	Strong but lower than naringenin
Kaempferol	-7.1	Tyr B:276, Arg A:267, Gln A:115, Glu A:118, Pro A:278, Trp B:246, Lys B:56, Thr B:64	Weakest ligand in present set

The interaction summary gives an idea that the ligand ranking wasn't solely based on the numerical docking score. The highest binding affinity and the most number of residues interacting with the receptor was observed for apigenin. A few other contacts were also observed with catechin, naringenin and quercetin, though the predicted binding energies were a bit less favourable. Although there were some receptor contacts, the binding affinity of kaempferol was the lowest among the molecules, indicating that the receptor contacts are not sufficient to give the highest docking score. These contacts should be good or bad, have good or bad shape and contribute with energy.

4.9 Overall Result Interpretation

MD simulations should also be performed on the nanosecond timescale, as well as longer, to identify the stability of the complexes and compute correct binding free energies. Stability and activity of these complexes should then be assessed using in vitro enzyme activity assays and small scale aquaculture experiments. Of all the compounds that we screened, it looked like Apigenin was most likely to bind, so we chose this for further investigation. Second on the list for binding was catechin, then naringenin, then quercetin.

Due to the tight cluster of this intermediate set of values, strict numerical ranking should not be used in its totality to eliminate these ligands, as they can serve as crucial backup targets in downstream assays, if the other assays reveal a superior transport, biological compatibility or stability.

Kaempferol achieved the lowest affinity thermodynamically, with a docking score value of -7.1 kcal/mol. The value is not a sign that the docking algorithm failed with kaempferol, but that it predicts it to be less prone to bind, compared to structurally similar molecules under identical simulation constraints. The value has merit as a negative control in that it suggests that structurally similar flavonoids do not interact identically with the bacterial reductase: kaempferol's poor binding might result from intramolecular steric hindrance with its specific hydroxyl orientation and an unfavorable fit within the pocket.

These docking results provide a molecular data foundation for the interface between plant biostimulants and the molecular machinery of denitrifying bacterial consortia in optimising biological nitrogen removal in aquaculture wastewater systems. These screening results serve

only as a preliminary screening bottleneck and not as evidence that treatment will be effective. Apigenin emerged as the most promising candidate for subsequent analysis, followed in order of binding strength by catechin, naringenin, and quercetin.

In the future, multi-nanosecond MD simulations should be run, which will investigate the structural stability of these complexes, and also serve to estimate accurate binding free energies. The next step would be to determine stability and function of these complexes through experimental enzyme activity tests, as well as through small-scale aquaculture treatment experiments.

CHAPTER 5: DISCUSSION, CONCLUSION, LIMITATIONS AND FUTURE SCOPE

5.1 Discussion

To clarify the molecular level interface of certain selected plant-derived bioactive polyphenols to the architecture of bacterial cd1 nitrite reductase (NirS) - a key functional regulator in the biological framework of denitrification pathways in intensive wastewater management - this study was conducted instead of relying upon any ad hoc and/or macroscale adjustment to effluent Chemistry (such as the raw biomass concentration or hydraulic retention times), etc.

The methodology used herewith is to establish an in silico virtual screening bottleneck via PyRx and AutoDock Vina to clarify the ligand-receptor structural complementarity. Elucidation of the binding configurations are of particular importance for the formulation of mechanism-based perspectives as to how to potentially align natural biostimulants with rate-limited enzymes from denitrifying bacteria consortia and this serve as a reference level at molecular level of biological nitrogen removal. The present virtual screening study successfully produced divergent thermodynamical signatures for the whole phytochemical libraries and demonstrates that five selected natural compounds can favorably occupy pockets inside the receptor without causing significant steric strain. The ranking on binding energies were obtained in a tiered format, with the resulting list apigenin (9.2 kcal/mol) > catechin (8.6 kcal/mol) > naringenin (8.4 kcal/mol) > quercetin (8.3 kcal/mol) > kaempferol (7.1 kcal/mol) at favorable, thermodynamically spontaneous pocket accommodation conditions.

Apigenin produced the best (most negative) binding energy in the given set of simulation and it stands out as the top computational hit under uniform simulation parameters due to its most optimal stereochemistry complementarity to the pocket. Other natural phytochemicals fall within the moderate to lower ranking affinities demonstrating the effect of subtle changes in related flavonoid backbones on pocket fitting. Steric fitting analysis indicates the superior docked conformation for apigenin attributed to the spatial features and highplanarity and geometric complementarity of its rigid favone skeleton. The planar flavone moiety smoothly fits into the constrained, residue rich interior pocket of NirS without steric hindrance.

Detailed interactome mapping for apigenin showed extensive interactions with critical coordinating residues such as Arg B:372, Phe B:425, Phe B:533, Gln B:483, His B:182, Arg B:225, Tyr B:245, and Asp B:328. This interaction map consists of both polar and nonpolar forces which include the aromatic interactions of phe and tyr groups for favorable hydrophobic pocket binding and polar interactions via hydrogen bonding and/or electrostatic interactions with the charged and polarized groups of Arg, Gln, His and Asp which accounts for the higher ranking and optimal binding.

The middle binding profiles for catechin and naringenin illustrate the different energetic trade-offs that arise due to the structural flexibility and non-planarity. Catechin docked with strong binding affinity of 8.6 kcal/mol and formed a broad contact profile with Val B:317, Ser B:319, Lys B:303, Thr B:318, Leu A:305, Val A:264, Gly B:268, and Met B:269. However the flexible flavan-3-ol backbone suffers from a greater entropic penalty compared to the rigid flavone scaffold. The many rotatable bonds on catechin provide greater orientation but allow for a greater range of possible poses while multiple hydroxyl positions facilitate a wide range of H-bonds.

Naringenin was able to fit into the active site cleft at 8.4 kcal/mol, illustrating that even with its chiral, non-planar flavanone structure the molecule is physically constrained into the receptor pocket but unlike apigenin, the geometry of naringenin lacks the absolutely plane conformation to obtain optimal binding stability. The poor rank of quercetin (8.3 kcal/mol) compared to naringenin signifies a key structure-activity rule; increasing the number of outer-most peripheral hydroxyl groups does not necessarily correlate to an increase in binding affinity. Rather the key to successfully "sticking" the ligand to a protein active site involves free molecular geometry, unobstructed functional group positioning, and lack of any localized steric strain in the protein active site.

Kaempferol displayed the worst binding affinity of any of the ligands examined in the screen with a score of 7.1 kcal/mol. It bound to an active site domain that consisted of Tyr B:276, Arg A:267, Gln A:115, Glu A:118, Pro A:278, Trp B:246, Lys B:56, and Thr B:64. However, its very poor binding energy showed that the configuration of Kaempferol at the binding site was energetically unfavorable. Kaempferol thus serves as a very useful model for negative structure-activity relationships where a minimal change in outer most hydroxyl topology between very close homologues such as Kaempferol and quercetin has a profound effect on molecularfitand binding strength. This shows that overall shape is a much greater determining factor in ligand-protein interactions than just a plentiful supply of possible H-bonding donors.

Collectively, these in silico data have provided a highly predictive template to pursue environmentally relevant applications utilizing natural products in treating dangerous toxic intermediate compounds such as nitrite in the wastewater systems of aquaculture. This approach has moved beyond adjusting system-level conditions in a traditional sense and by identifying the relationship between plant secondary metabolites and the protein catalysts that drive bacterial metabolism in the nitrogen cycle, an organized molecular design principle has emerged. It is important to consider these computational results as an initial screening hurdle and not as an indication that the molecules can biologically alter the metabolic pathways.

While strong binding affinity predicts that these molecules can enter the binding pocket and interact with the protein of interest they do not necessarily dictate enzyme activity or, most importantly in environmental applications, an improvement in overall biological nitrogen removal in an active wastewater stream. Nevertheless this approach has allowed us to screen out non-viable compounds and reduce the search space greatly to begin more targeted molecular dynamics (MD) simulations, in vitro enzyme studies, and subsequent pilot scale testing.

5.2 Conclusion

With this work we have set a definitive computational benchmark, as the structural match and thermodynamic interactome of the plant derived polyphenolic scaffolds against the bacterial cd1 nitrite reductase (NirS) receptor has been characterized. Assuming that very closely related flavonoids don't necessarily possess identical features, different structure-activity rules

were determined through screening since a slight variation inside the ring core is quite significant in occupying the active site pocket. The enhanced efficacy of apigenin along with strongly self-organized structural networks obtained with the affinity cluster of high affinitycatechin,naringeninand quercetin helped to validate our hypothesis that (tailored hydroxyl-topology and planarity) is responsible for the appropriate interaction between ligand and bacterial denitrification receptor site.

The specific structural differences between compounds within the library provided specific parameters applicable to biochemical principles of biological nitrogen removal. The comparisons displayed reveal how changes in planarity, ring conformation and directional hydrogen bond networks dictate which compounds fit into the active site with the greatest affinity, acting as structural gates. With the realization of these minor stereochemical preferences, the framework of wastewater engineering for aquaculture was moved beyond empirical macroscopic system changes (like controlling biomass, hydraulic retention time, or water changes) to a more specific molecular mechanism focused on enzyme compatibility.

In this way, these predictive models provide a logical mechanism-driven approach for wastewater engineering in aquaculture. As such, the models themselves do not constitute direct proof of accelerated nitrogen removal in the active wastewater, but rather provide the template needed to start testing. This has negated the plethora of unwanted and expensive lab tests that would have been run by providing the defined group of candidates to screen extensively, thereby providing a rational route to utilizing computational biotech towards highly efficient waste water treatment using MD, enzyme kinetics and micro scale assay.

5.3 Limitations

The main disadvantage of this type of virtual screening (though, because of the high resolution structure-activity relationships this is not the case for static, rigid-receptor docking calculations). Although the AutoDock Vina scoring function accounts well for immediate thermodynamical complementarity in the cd1 NirS catalytic site, it does so on vastly simplified structural constraints without the inclusion of time-dependent macromolecular conformational changes. In a physiological setting, proteins are flexible and undergo ligand-induced conformational changes that cannot be emulated by a rigid receptor grid box. In addition, this model doesn't account for the thermodynamical importance of bulk solvent

interactions, water-mediated H-bonding networks, and the competition of unwanted matrix proteins.

5.4 Future Scope

These static in silico data must be translated to empirical and evidence-based environmental biotechnology through a multi-stage validation workflow. The stability of the selected apigenin-NirS complex in the course of time will first be analyzed using extensive MD simulations. End state free energy calculation tools such as Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA) or Generalized Born Surface Area (MM-GBSA) should then be utilized to produce a very accurate calculation of binding free energies taking into consideration all entropic costs and structural changes which a rigid docking model misses. The workflow should then proceed to in vitro biochemical validation where enzymatic kinetics assays need to be performed to quantify the precise regulatory impact of the selected apigenin and the other two lead compounds on the activity of the purified nitrite reductase enzyme.

Beyond simple enzymatic inhibition studies, subsequent downstream work needs to study the effect on actively denaturing microbial communities under these conditions and microbial cultures will be used to examine changes in growth rates, metabolic flux pathways and direct nitrite-reduction rates in response to the tested phytochemical biostimulants.

Further, will also analyze potential rate-limiting other such as nitrate and nitrous oxide reductase within a larger panel of plant secondary metabolites under a broad screening matrix. Robust baseline ecotoxicity testing then allows for this screening process to span from virtual models to application.

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List of publications

Conference Presentation:

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