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THEME

Production Of Bioethanol From Microalgae Biomass(*Arthrospira Platensis*)

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

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In the name of Allah

All praise and gratitude are due to Allah SWT, Lord of the Worlds, for bestowing upon us the strength, knowledge, and perseverance to complete this Master 's thesis. Without His divine guidance and blessings, this accomplishment would not have been possible. Alhamdulillah.

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Dedication

We are profoundly indebted to our beloved parents for their endless love, prayers, sacrifices, and constant encouragement throughout our lives and academic pursuits. Their belief in us has always been our greatest motivation. May Allah grant them the best in this life and the Hereafter.

To our siblings, thank you for your support, understanding, and for always being there for us.

Finally, we pray that this work contributes positively to the field and benefits the wider community. Any errors or shortcomings in this thesis are solely our own.

الملخص

في ظل الاهتمام المتزايد بمصادر الطاقة المتجددة لمواجهة تحديات تغير المناخ والاعتماد على الوقود الأحفوري، هدفت هذه الدراسة إلى تقييم إمكانية استخدام الكتلة الحيوية للطحلب الدقيق *Arthrospira platensis* (سبيرولينا) كمادة خام مستدامة لإنتاج الإيثانول الحيوي. تم تبني منهجية متكاملة تجمع بين التحليل الحسابي (In-silico) والتجارب العملية (In-vitro). تضمن التحليل الحسابي إجراء إرساء جزيئي لتقييم التآثر بين إنزيم الجلوكو أميلاز من فطر *Aspergillus niger* وركيزة المالتوز، حيث أظهرت النتائج ألفة ارتباط قوية ومفضلة ديناميكياً (ΔG = -7.0 كيلو كالوري/مول)، مع تحديد الروابط الهيدروجينية والأحماض الأمينية الرئيسية (ARG596, GLU597, THR614, ARG616) المسؤولة عن استقرار المعقد. أما التجارب العملية، فشملت التحلل المائي الإنزيمي للكتلة الحيوية للسبيرولينا باستخدام إنزيمات *A. niger* الخام، متبوعاً بعملية التخمير باستخدام خميرة *Saccharomyces cerevisiae*. أسفرت هذه العملية عن مردود إيثانول حيوي مشجع بلغ 62.5%. وأكد اختبار الاحتراق للإيثانول المنتج كفاءة احتراق جيدة، على الرغم من ملاحظة وجود شوائب معدنية طفيفة تستدعي تحسين خطوات التنقية. تبرهن نتائج هذه الدراسة على الجدوى العلمية والعملية لاستخدام *Spirulina platensis* وإنزيمات *A. niger* في إنتاج الإيثانول الحيوي، وتؤكد على إمكانات الطحالب الدقيقة كمصدر واعد للجيل الثالث من الوقود الحيوي، مما يفتح آفاقاً لتطوير تقنيات طاقة أكثر استدامة.

الكلمات المفتاحية: إيثانول حيوي، *Arthrospira platensis* (سبيرولينا)، *Aspergillus niger*، تحلل مائي إنزيمي، تخمير، *Saccharomyces cerevisiae*، إرساء جزيئي.

Abstract

Amidst growing interest in renewable energy sources to address climate change challenges and fossil fuel dependency, this study aimed to evaluate the potential of using the microalga *Arthrospira platensis* (Spirulina) biomass as a sustainable feedstock for bioethanol production. An integrated methodology combining computational analysis (In-silico) and practical experiments (In-vitro) was adopted. The computational analysis involved molecular docking to assess the interaction between the glucoamylase enzyme from the fungus *Aspergillus niger* and the maltose substrate. Results showed a strong and thermodynamically favorable binding affinity (ΔG = -7.0 kcal/mol), identifying hydrogen bonds and key amino acids (ARG596, GLU597, THR614, ARG616) responsible for complex stability. Practical experiments included enzymatic hydrolysis of Spirulina biomass using crude *A. Niger* enzymes, followed by fermentation using Saccharomyces cerevisiae yeast. This process yielded an encouraging bioethanol yield of 62.5%. Combustion testing of the produced bioethanol confirmed good combustion efficiency, although slight mineral impurities were observed, indicating a need for optimizing purification steps. The findings of this study demonstrate the scientific and practical feasibility of using Spirulina platensis and *A. Niger* enzymes for bioethanol production, confirming the potential of microalgae as a promising source for third-generation biofuels, thereby opening prospects for developing more sustainable energy technologies.

Keywords: Bioethanol, *Arthrospira platensis* (Spirulina), *Aspergillus Niger*, Enzymatic Hydrolysis, Fermentation, *Saccharomyces cerevisiae*, Molecular Docking.

Resume

Dans le contexte de l'intérêt croissant pour les sources d'énergie renouvelables afin de relever les défis du changement climatique et de la dépendance aux combustibles fossiles, cette étude visait à évaluer la possibilité d'utiliser la biomasse de la microalgue Arthrospira platensis (Spiruline) comme matière première durable pour la production de bioéthanol. Une méthodologie intégrée combinant l'analyse computationnelle (In-silico) et les expériences pratiques (In-vitro) a été adoptée. L'analyse computationnelle comprenait l'exécution d'un amarrage moléculaire pour évaluer l'interaction entre l'enzyme glucoamylase du champignon *Aspergillus niger* et le substrat maltose. Les résultats ont montré une forte affinité de liaison thermodynamiquement favorable (ΔG = -7,0 kcal/mol), identifiant les liaisons hydrogène et les acides aminés clés (ARG596, GLU597, THR614, ARG616) responsables de la stabilité du complexe. Les expériences pratiques comprenaient l'hydrolyse enzymatique de la biomasse de Spiruline à l'aide d'enzymes brutes d'A. niger, suivie d'une fermentation à l'aide de la levure *Saccharomyces cerevisiae*. Ce processus a abouti à un rendement encourageant en bioéthanol de 62,5 %. Le test de combustion du bioéthanol produit a confirmé une bonne efficacité de combustion, bien que la présence de légères impuretés minérales ait été observée, nécessitant une optimisation des étapes de purification. Les résultats de cette étude démontrent la faisabilité scientifique et pratique de l'utilisation de Spirulina platensis et des enzymes d'A. niger pour la production de bioéthanol, et confirment le potentiel des microalgues comme source prometteuse de biocarburants de troisième génération, ouvrant ainsi des perspectives pour le développement de technologies énergétiques plus durables.

Mots-clés : Bioéthanol, *Arthrospira platensis* (Spiruline), *Aspergillus niger*, hydrolyse enzymatique, fermentation, *Saccharomyces cerevisiae*, amarrage moléculaire.

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List of Abbreviations

- ARG: Arginine
- CAZy: Carbohydrate-Active enZymes database (Referenced in discussion)
- CID: Compound Identifier (PubChem)
- ΔG: Gibbs Free Energy change
- EM: Electron Microscopy (Implied in QFDE-EM)
- GC-MS: Gas Chromatography-Mass Spectrometry (Referenced in discussion)
- GLU: Glutamic Acid
- HCl: Hydrochloric Acid
- H_2SO_4: Sulfuric Acid
- ICP-MS: Inductively Coupled Plasma Mass Spectrometry (Referenced in discussion)
- IEA: International Energy Agency
- in silico: Performed on computer or via computer simulation
- in vitro: Performed in a test tube, culture dish, or elsewhere outside a living organism
- kcal/mol: Kilocalories per mole
- mito: Mitochondria (Figure caption)
- OH: Hydroxyl group
- PDB: Protein Data Bank
- PEF: Pulsed Electric Field
- pm: Plasma Membrane (Figure caption)
- QFDE-EM: Quick-Freeze Deep-Etch Electron Microscopy
- Rmsd L.B.: Root Mean Square Deviation Lower Bound
- Rmsd U.B.: Root Mean Square Deviation Upper Bound
- THR: Threonine
- YPD: Yeast Extract Peptone Dextrose

Introduction

Introduction:

The global demand for renewable energy sources has experienced significant growth in recent years, driven by the urgent need to combat climate change and decrease reliance on fossil fuels, As reported by the International Energy Agency (IEA), renewable energy contributed to nearly 30% of worldwide electricity generation in 2022, with biofuels playing a pivotal role in decarbonizing transportation sectors **(International Energy Agency [IEA], 2022)**. Among biofuel alternatives, bioethanol stands out due to its compatibility with existing infrastructure and potential for carbon neutrality **(Singh, Nigam, & Murphy, 2021).** However, conventional bioethanol production from crops such as corn and sugarcane face challenges, including competition with food resources and substantial environmental costs **(Singh, Nigam, & Murphy, 2021).**

Microalgae represent a promising source for the production of numerous bioproducts, ranging from fuels and chemicals to materials, cosmetics, animal feed, and dietary supplements **(Singh, Nigam, & Murphy, 2021).** The biomass derived from microalgae holds great potential for use in various energy-related processes, offering notable benefits compared to traditional feedstocks **(Dash et al., 2022),** Moreover, microalgae can thrive in diverse water sources. Importantly, they can be grown on marginal lands unsuitable for agriculture, thereby avoiding conflicts with food production systems and enhancing their sustainability profile **(Xu et al ., 2011;Cazzaniga et al., 2014).**

Among microalgae, *Arthrospira platensis* (commonly known as Spirulina) is a promising candidate for bioethanol production. This cyanobacterium can accumulate up to 65% of its dry weight as glycogen, a carbohydrate readily convertible into fermentable sugars **(Bautista & Laroche, 2021).** Under nutrient-limited conditions, Furthermore, *A. platensis* can be cultivated in large-scale outdoor systems with robust growth and contaminant-free cultures, facilitating

industrial bioethanol production **(Aikawa et al., 2018; Sathya et al., 2023; Singh & Sreevidya, 2024).**

Saccharomyces cerevisiae is the primary microorganism utilized in industrial bioethanol production due to its remarkable capacity to convert sugars into ethanol with high efficiency and its ability to endure the challenging conditions of large-scale fermentation. This yeast flourishes in high-sugar environments, tolerates elevated ethanol concentrations, and ferments effectively under anaerobic conditions, rendering it the preferred choice over alternative microbes for ethanol production.

And we use **Molecular docking** is a powerful bioinformatics approach used to predict the conformation and interaction affinity between two molecules.

This research is significant because it has the potential to provide a sustainable and cost-effective alternative to conventional bioethanol production methods. The study's objective is to leverage the inherent capabilities of *Aspergillus niger* enzymes to develop a more efficient and environmentally sustainable bioethanol production process. Beyond that, the findings could have wider benefits for industries that rely on biofuels, helping to push forward global efforts to adopt renewable energy and build a more sustainable future. This thesis is structured as follows:

Chapter I provides a bibliographic synthesis focused on bioethanol production, microalgae as a biofuel feedstock.
Chapter II describes the materials and methods, detailing the experimental design and analytical techniques employed to conduct the research effectively.
Chapter III presents the results and discussion, highlighting key findings and comparing them with data from existing literature.
Chapter IV concludes with a general summary and outlines future perspectives aimed at improving and extending the present work

Chapter 01:
Microalgae and Biotechnology

1.Overview of Microalgae as a Feedstock for Biofuels:

1.1Definition of Microalgae:

Microalgae are a diverse group of photosynthetic microorganisms that can be found in various aquatic environments, ranging from freshwater to marine ecosystems, They are considered among the most primitive forms of plants, lacking conventional plant structures such as stems, roots, and leaves, and are classified as thallophytes **(Sharma et al., 2025).** These microscopic organisms are typically unicellular, although some species form colonies or filaments. Their size ranges from a few micrometers to a few hundred micrometers, making them invisible to the naked eye but capable of forming visible colonies or blooms when present in high concentrations **(Bora et al., 2024).**

Taxonomically, microalgae encompass a wide range of organisms, including eukaryotic species (e.g., green algae, diatoms, red algae) and prokaryotic cyanobacteria (often referred to as blue-green algae). Despite their taxonomic diversity, microalgae share the fundamental characteristic of performing oxygenic photosynthesis, converting solar energy, carbon dioxide, and nutrients into biomass and oxygen (Wang et al., 2024). This photosynthetic capability positions them as primary producers in aquatic ecosystems and as potential "photosynthesis-driven factories" for biotechnological applications **(Sharma et al., 2025).**

Among the commonly used species in energy-related applications are *Spirulina* and *Chlorella* . These species have high concentrations of fermentable materials such as sugars and show good enzymatic processability, facilitating subsequent biodegradation and fermentation processes **(Ho et al., 2013)**. Additionally, their cultivation does not require fertile land or large quantities of freshwater, and marginal lands or polluted water can be utilized, providing a sustainable environmental alternative compared to traditional agricultural crops **(Guldhe et al., 2019).**

Furthermore, microalgae serve as an effective environmental tool for absorbing carbon dioxide, offering a dual benefit in reducing greenhouse gas emissions and contributing to sustainable development goals related to energy and the environment.

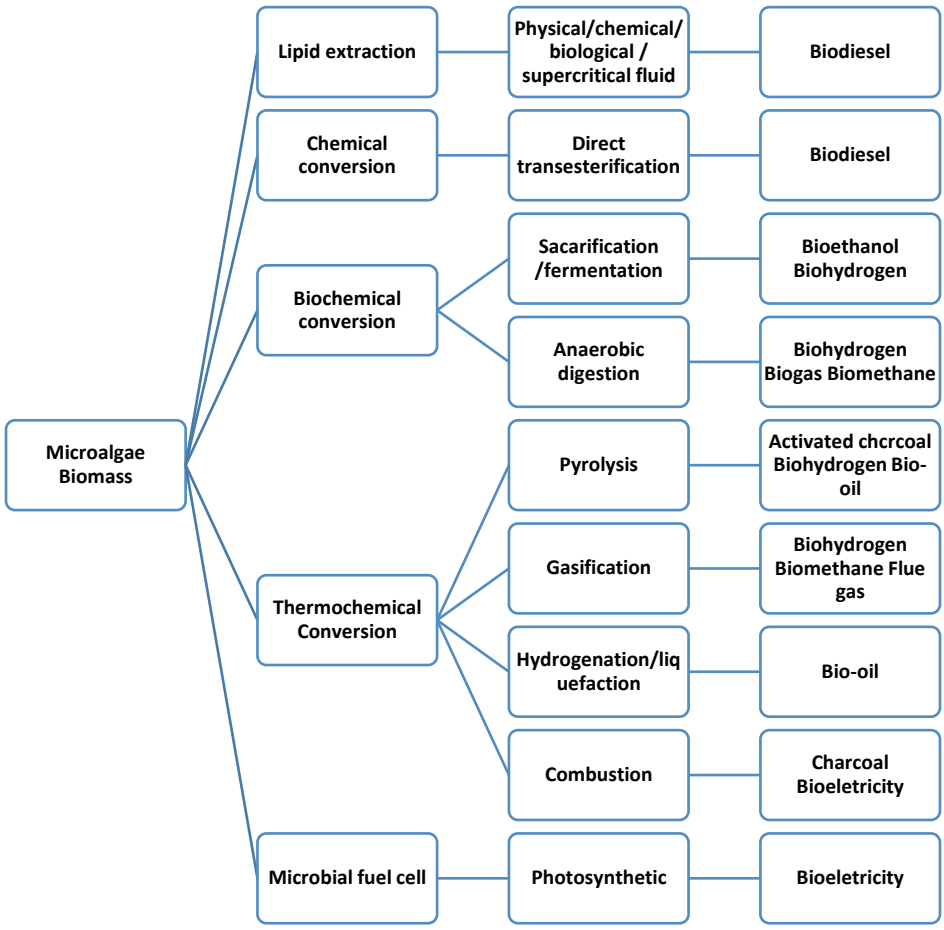

*Figure 1 : Scheme of Bio-fuel production from microalgae biomass (**Nunes et al.,2021**).*

1.2. Historical Milestones in Microalgal Biofuel Research:

The exploration of microalgae as a potential source of biofuels has evolved significantly over the past several decades, reflecting broader trends in renewable energy research and development. The historical trajectory of microalgae-based biofuel research can be divided into several distinct phases, each characterized by specific technological advancements and shifting research priorities.

The initial scientific interest in microalgae as a potential energy source emerged in the mid-20th century. During the 1950s, researchers began investigating the photosynthetic efficiency of microalgae and their potential for mass cultivation **(Borowitzka, 2013).** However, it was not until the energy crisis of the 1970s that significant attention turned toward microalgae as a potential renewable fuel source. The U.S. Department of Energy established the Aquatic Species Program (ASP) in 1978, which focused specifically on developing renewable transportation fuels from algae. This program, which continued until

1996, laid the groundwork for much of the subsequent research in algal biofuels, particularly in the areas of species selection, cultivation techniques, and lipid production **(Sheehan et al., 1998).**

The past decade (2015-2025) has witnessed a significant shift in research focus toward addressing the economic and technical challenges that have hindered the commercial viability of microalgae-based biofuels. Recent advancements have focused on several key areas:

1- genetic engineering to enhance lipid productivity and stress tolerance **(Saravanan et al., 2022).**

2- development of more efficient and cost-effective cultivation systems, including photobioreactors and hybrid systems **(Wang et al., 2024).**

3- integration of microalgae cultivation with wastewater treatment and carbon dioxide mitigation from industrial sources **(Bora et al., 2024).**

4- biorefinery approaches that maximize the value of microalgal biomass through the co-production of biofuels and high-value compounds **(Chew et al., 2023).**

Contemporary research increasingly emphasizes the sustainability aspects of microalgae-based biofuels, including life cycle assessment, water and nutrient recycling, and energy balance considerations. The integration of microalgae cultivation with environmental remediation processes, such as wastewater treatment and carbon dioxide capture from industrial emissions, represents a promising approach to enhance the economic feasibility and environmental benefits of microalgae-based biofuels **(Bora et al., 2024; Wang et al., 2024).**

1.3.Comparative Advantages of Microalgae Feedstocks:

Microalgae offer numerous advantages over conventional first and second-generation biofuel feedstocks, positioning them as a promising sustainable alternative for future energy production. These advantages span various dimensions, including productivity, resource utilization, environmental impact, and versatility.

2.Taxonomic Classification and Diversity of Microalgae:

2.1. Major Taxonomic Groups and Their Relevance:

Microalgae represent an extraordinarily diverse group of organisms, spanning multiple kingdoms and phyla, each with unique physiological and biochemical characteristics. This diversity offers a vast resource for identifying species with optimal traits for biofuel production. Understanding the major taxonomic groups is crucial for selecting appropriate strains and developing tailored cultivation and processing strategies **(Heimann & Huerlimann, 2015).**

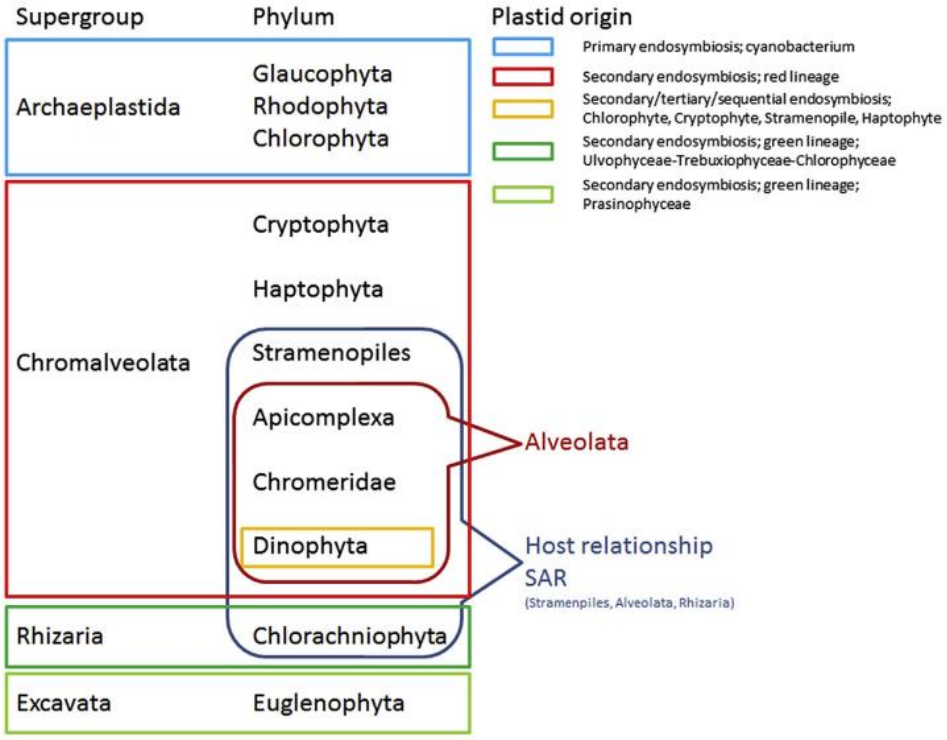

*Figure 2: Taxonomic classification, plastid origins, and host phylogenetic relationships of protistan/algal phyla containing plastid-bearing members (**Heimann & Huerlimann, 2015**).*

2.2.Major phyla/class characteristics of commercial microalgal genera :

a) Chlorophyta:

Chlorophyta, or green algae, constitute one of the largest and most diverse groups of microalgae, encompassing thousands of species found in freshwater, marine, and terrestrial environments, They are characterized by the presence of chlorophylls a and b, similar to higher plants, and typically store energy as starch within their chloroplasts **(Leliaert et al., 2012).**

Green algae are widely considered promising candidates for bioethanol production due to their ability to accumulate high levels of starch, particularly

under stress conditions such as nitrogen limitation **(Wang et al., 2024).** Species like Chlorella vulgaris, Scenedesmus obliquus, and Chlamydomonas reinhardtii have been extensively studied and can accumulate carbohydrates up to 45-55% of their dry weight **(Chew et al., 2023).** Their cell walls are primarily composed of cellulose, hemicellulose, and pectin, which, while requiring pretreatment, are generally amenable to enzymatic hydrolysis using cellulases and related enzymes.

b) Rhodophyta:

Rhodophyta, or red algae, are predominantly marine eukaryotic microalgae characterized by the presence of phycoerythrin, a red pigment that gives them their distinctive coloration, They also contain chlorophyll a and, in some cases, chlorophyll d, along with phycocyanin and allophycocyanin **(Guiry, 2012).**

Red algae primarily store energy as floridean starch, a carbohydrate similar to amylopectin but unique to this group. The carbohydrate content of red algae can range from 30% to 60% of dry weight, with significant variations among species and under different cultivation conditions **(Chew et al., 2023).** While red algae have been less extensively studied for bioethanol production compared to green algae and cyanobacteria, their high carbohydrate content presents potential opportunities.

Genera such as Porphyridium, Rhodella, and Galdieria have been investigated for various biotechnological applications, including biofuel production. Galdieria sulphuraria, in particular, has attracted attention due to its extremophilic nature and ability to grow heterotrophically on various carbon sources, offering flexibility in cultivation strategies **(Chew et al., 2023).**

c) Cyanobacteria (Blue-Green Algae):

Cyanobacteria are prokaryotic microorganisms that perform oxygenic photosynthesis. Although often referred to as blue-green algae, they are taxonomically distinct from eukaryotic algae. They possess chlorophyll a and phycobiliproteins (phycocyanin and phycoerythrin) as photosynthetic pigments and typically store energy as glycogen **(Whitton & Potts, 2012).**

Several cyanobacterial species, including *Spirulina platensis* (*Arthrospira platensis*) and *Synechocystis* spp., have garnered interest for bioethanol production. Under nitrogen limitation, *Spirulina* can accumulate glycogen up to 60-70% of its dry weight **(Bora et al., 2024).** A distinctive advantage of cyanobacteria for bioethanol production is their relatively simple cell wall structure, which lacks the recalcitrant components found in many eukaryotic microalgae, facilitating more efficient hydrolysis and fermentation processes **(Wang et al., 2024).**

d) Bacillariophyta (Diatoms)

Diatoms are a major group of eukaryotic microalgae found in oceans, freshwater, and soils worldwide,They are distinguished by their unique cell walls, known as frustules, which are composed primarily of hydrated silicon dioxide (silica) intricately patterned with pores and ribs **(Hildebrand et al., 2012).**

Diatoms typically store energy as lipids and chrysolaminarin, a β-1,3-glucan. While their carbohydrate content is generally lower than that of green algae (typically 8-30% of dry weight), their high lipid content has made them attractive candidates for biodiesel production **(Chew et al., 2023).** The rigid silica frustule presents a significant challenge for cell disruption and carbohydrate extraction, often requiring harsh chemical treatments (e.g., hydrofluoric acid) or energy-intensive physical methods **(Wang et al., 2024).**

e) Other Relevant Groups (Eustigmatophyceae, Cryptophyta, Haptophyta, Euglenophyta) :

Several other taxonomic groups of microalgae have been explored for biofuel applications, albeit to a lesser extent than the major groups discussed above. These include:

- ***Eustigmatophyceae***: This class includes the genus *Nannochloropsis*, extensively studied for biodiesel due to high lipid content, Carbohydrate content (10-20%) suggests potential for bioethanol in a biorefinery context **(Raghupathi & Ravi, 2023).**
- ***Cryptophyta***: These store starch and can grow in low light/cold conditions, offering niche cultivation potential **(Leliaert et al., 2012).**
- ***Haptophyta***: Includes coccolithophores, storing chrysolaminarin and lipids, Carbohydrate content (20-30%) suggests bioethanol potential **(Chew et al., 2023).**
- ***Euglenophyta***: Store paramylon (β-1,3-glucan), *Euglena gracilis* carbohydrate content (14-18%) can increase significantly under specific conditions **(Wang et al., 2024).**

3. Carbohydrates in Microalgae:

3.1. Role of Carbohydrates in Bioethanol Fermentation:

Carbohydrates represent a fundamental component of microalgal biomass and serve as the primary substrate for bioethanol production through fermentation processes. The conversion of microalgal carbohydrates to bioethanol typically involves three main steps: pretreatment to disrupt cell walls, hydrolysis of complex carbohydrates into fermentable sugars, and fermentation of these sugars to produce ethanol **(Chew et al., 2023).** Understanding the carbohydrate content, composition, and structure in microalgae is therefore essential for optimizing bioethanol production processes and maximizing yields.

Microalgal carbohydrates exist in various forms, including structural components of cell walls (e.g., cellulose, hemicellulose, pectin) and storage compounds (e.g., starch, glycogen, chrysolaminarin), The distribution and composition of these carbohydrates vary significantly among different taxonomic groups and are influenced by cultivation conditions **(Wang et al., 2024)**. Storage carbohydrates, particularly starch and glycogen, are generally more readily hydrolyzed and fermented compared to structural carbohydrates, which often require more intensive pretreatment methods to achieve efficient conversion **(Chew et al., 2023).**

3.2.Variability and Composition of Carbohydrates Across Species:

The carbohydrate content of microalgae exhibits remarkable variability across different taxonomic groups, species, and even strains within the same species. This variability is influenced by genetic factors, physiological characteristics, and environmental conditions, presenting both challenges and opportunities for bioethanol production **(Wang et al., 2024).**

Table 1: Typical Carbohydrate Content in Selected Microalgae Species Relevant to Bioethanol Production

Taxonomic Group	Species	Typical Carbohydrate Content (% Dry Weight)	Carbohydrate Content (% Dry Weight, Stress Conditions)	Primary Storage Carbohydrate	Reference(s)
Chlorophyta	*Chlorella vulgaris*	15-25%	Up to 55%	Starch	**Wang et al. (2024),Chew et al. (2023)**
Chlorophyta	*Scenedesmus obliquus*	10-20%	Up to 50%	Starch	**Chew et al. (2023),Bakare et al. (2022)**
Chlorophyta	*Chlamydomonas reinhardtii*	15-25%	Up to 45%	Starch	**Wang et al. (2024), Zhang et al. (2022)**
Chlorophyta	*Dunaliella salina*	20-30%	~30-35% (Less responsive to N-limitation for carbs)	Glycerol, Starch	**Li et al. (2023), Chew et al. (2023)**
Chlorophyta	*Tetraselmis suecica*	25-35%	Up to 45%	Starch	**Chew et al. (2023), Demuez et al. (2022)**
Cyanobacteria	*Spirulina platensis*	15-25%	Up to 60-70%	Glycogen	**Bora et al. (2024), Markou et al. (2023)**
Bacillariophyta	*Phaeodactylum tricornutum*	10-20%	Up to 30%	Chrysolaminarin	**Hildebrand et al. (2012), Chew et al. (2023)**
Eustigmatophyceae	*Nannochloropsis oceanica*	10-15%	~15-20% (Primarily lipid accumulator)	Chrysolaminarin	**Raghupathi & Ravi (2023), Shivakumar et al. (2024)**
Rhodophyta	*Porphyridium cruentum*	30-40%	Up to 55%	Floridean Starch	**Chew et al. (2023)**

3.3.Key Factors Influencing Carbohydrate Accumulation :

The carbohydrate content and composition of microalgae are highly dynamic and can be significantly influenced by various environmental and cultivation factors **(Wang et al., 2024; Chew et al., 2023)**. Understanding these factors and their interactions is essential for developing strategies to enhance carbohydrate accumulation and optimize bioethanol production **(Bora et al., 2024; Wang et al., 2024).**

3.3.1.Nutrient Availability :

Nutrient limitation, particularly nitrogen deprivation, is one of the most effective strategies for enhancing carbohydrate accumulation in many microalgal species **(Chew et al., 2023; Bora et al., 2024).** Under nitrogen limitation, many microalgae redirect their metabolism from protein synthesis to carbohydrate or lipid accumulation **(Chew et al., 2023).** For instance, **Markou et al. (2023)** reported that nitrogen limitation increased the carbohydrate content of *Spirulina platensis* from 15% to 65% of dry weight within 7 days. Similarly, phosphorus limitation has been shown to enhance carbohydrate accumulation in some species, although the effect is generally less pronounced compared to nitrogen limitation **(Wang et al., 2024).**

3.3.2.Light Intensity and Photoperiod :

Light is a critical factor influencing photosynthesis and, consequently, carbohydrate accumulation in microalgae **(Wang et al., 2024)**. High light intensities typically promote carbohydrate synthesis, particularly when combined with nutrient limitation **(Chew et al., 2023).**

Chew et al. (2023) demonstrated that increasing light intensity from 100 to 300 μmol photons m^{-2} s^{-1} enhanced carbohydrate accumulation in *Chlorella vulgaris* by 25%. The photoperiod also influences carbohydrate metabolism, with longer light periods generally favoring carbohydrate accumulation **(Wang et al., 2024).** However, excessive light intensities can lead to photoinhibition and reduced carbohydrate synthesis (Wang et al., 2024).

3.3.3.Carbon Dioxide Concentration:

As the primary substrate for photosynthesis, carbon dioxide availability significantly influences carbohydrate accumulation in microalgae (Bora et al., 2024). Elevated CO_2 concentrations (typically 2-5%) have been shown to enhance carbohydrate synthesis in many species **(Wang et al., 2024).**

Bora et al. (2024) reported that increasing CO_2 concentration from atmospheric levels (0.04%) to 2% enhanced carbohydrate accumulation in *Spirulina platensis* by 30%. However, the optimal CO_2 concentration varies

among species and is influenced by other cultivation conditions **(Chew et al., 2023).**

3.3.4.Temperature :

Temperature affects the metabolic activities of microalgae, including photosynthesis and carbohydrate synthesis **(Chew et al., 2023).** Most microalgal species exhibit optimal carbohydrate accumulation at temperatures between 20°C and 30°C, although the specific optimum varies among species **(Chew et al., 2023).** Temperatures outside the optimal range can reduce carbohydrate synthesis and alter carbohydrate composition **(Wang et al., 2024).** For instance, **Wang et al. (2024)** observed that low temperatures (15°C) reduced starch accumulation in *Chlorella vulgaris* but increased the proportion of soluble sugars.

3.3.5.pH and Salinit :

The pH of the cultivation medium influences nutrient availability, enzyme activities, and cellular metabolism in microalgae **(Bora et al., 2024).** Most species exhibit optimal carbohydrate accumulation at pH values between 7 and 9, although some species, particularly cyanobacteria like *Spirulina platensis*, prefer more alkaline conditions (pH 9-10) **(Bora et al., 2024)**. Salinity stress has been shown to enhance carbohydrate accumulation in some species, particularly marine microalgae **(Chew et al., 2023**). For instance, **Chew et al. (2023)** reported that increasing salinity from 35 to 50 g/L enhanced carbohydrate accumulation in *Tetraselmis suecica*by 20%.

3.3.6.Combined Stress Conditions :

The application of multiple stress conditions often results in synergistic effects on carbohydrate accumulation **(Wang et al., 2024).** For instance, the combination of nitrogen limitation and high light intensity has been shown to enhance carbohydrate accumulation more effectively than either stress condition alone **(Wang et al., 2024).** Similarly, the combination of nitrogen limitation and elevated CO_2 concentration has been reported to significantly enhance carbohydrate accumulation in various microalgal species **(Bora et al., 2024).**

3.3.7.Growth Phase:

The carbohydrate content of microalgae varies throughout their growth cycle **(Chew et al., 2023).** In many species, carbohydrate content is relatively low during the exponential growth phase and increases during the stationary phase, particularly under nutrient limitation **(Chew et al., 2023).** This temporal variation is an important consideration for harvesting strategies aimed at maximizing carbohydrate content **(Wang et al., 2024).**

Recent research has focused on developing cultivation strategies that balance biomass productivity and carbohydrate accumulation to maximize overall carbohydrate productivity (g carbohydrates per liter per day) **(Markou et al., 2023; Bora et al., 2024).** For instance, **Markou et al. (2023)** proposed a two-stage cultivation strategy for *Spirulina platensis*, involving an initial phase of nutrient-replete conditions to maximize biomass production, followed by a nitrogen-limited phase to enhance carbohydrate accumulation. This approach resulted in a 40% increase in carbohydrate productivity compared to conventional single-stage cultivation.

The manipulation of cultivation conditions to enhance carbohydrate accumulation represents a promising approach for improving the economic viability of microalgal bioethanol production **(Wang et al., 2024).** However, it is important to note that strategies that enhance carbohydrate accumulation often reduce biomass productivity, necessitating a careful balance to maximize overall carbohydrate yield **(Chew et al., 2023; Bora et al., 2024).**

3.4.Evaluating Spirulina platensis as a High-Carbohydrate Feedstock for Bioethanol:

Spirulina platensis (also known as *Arthrospira platensis*) has emerged as one of the most promising candidates for bioethanol production due to its high carbohydrate accumulation potential, relatively simple cell wall structure, and robust growth characteristics, Recent studies have extensively investigated the carbohydrate metabolism of *S. platensis* and strategies to enhance its carbohydrate content **(Markou et al., 2023; Bora et al., 2024).**

Markou et al. (2023) conducted a comprehensive study on the carbohydrate accumulation in S. platensis under various cultivation conditions. They reported that under optimal conditions (nitrogen limitation, high light intensity, and elevated CO_2), S. platensis could accumulate carbohydrates up to 65% of its dry weight within 7 days. The carbohydrates were primarily composed of glucose polymers (glycogen, 60-80%), with smaller amounts of rhamnose, xylose, and galactose. The authors also observed that the carbohydrate composition shifted under stress conditions, with an increase in the proportion of glucose and a decrease in other monomers.

A comparative study by **Chew et al. (2023)** evaluated the carbohydrate content and composition of 15 microalgal species, including S. platensis, under standardized cultivation conditions. They found that S. platensis exhibited moderate carbohydrate content (15-25%) under normal growth conditions but showed remarkable carbohydrate accumulation (up to 60%) under nitrogen limitation. The authors also noted that the carbohydrates in S. platensis were more

readily hydrolyzed compared to those in many eukaryotic microalgae, due to the absence of recalcitrant cell wall components.

Bora et al. (2024) investigated the integration of S. platensis cultivation with wastewater treatment and found that the carbohydrate content of S. platensis grown in wastewater supplemented with additional nitrogen and phosphorus was comparable to that of S. platensis grown in standard medium. However, as the wastewater nutrients were depleted, the carbohydrate content increased significantly, reaching up to 55% of dry weight. This finding suggests that wastewater-based cultivation of S. platensis could potentially combine bioremediation with enhanced carbohydrate accumulation for bioethanol production.

Recent research has also focused on optimizing cultivation strategies to maximize carbohydrate productivity in S. platensis. **Markou et al. (2023)** developed a two-stage cultivation strategy that resulted in a carbohydrate productivity of 0.35 g/L/day, representing a 40% increase compared to conventional single-stage cultivation. Similarly, **Bora et al. (2024)** reported that a semi-continuous cultivation system with periodic nutrient limitation enhanced carbohydrate productivity by 30% compared to batch cultivation.

The carbohydrate composition of S. platensis is particularly favorable for bioethanol production. **Markou et al. (2023)** reported that glucose accounted for 70-80% of the total monomers in S. platensis hydrolysates, with the remainder consisting of rhamnose, xylose, and galactose. The high glucose content facilitates efficient fermentation by conventional yeast strains, while the presence of other monomers suggests potential for enhanced bioethanol yields through the use of engineered microbial strains capable of fermenting multiple sugar types.

4. Cell Wall Composition and Degradation :

Microalgal cell walls represent remarkably diverse and complex structures that have evolved to provide protection, structural support, and selective permeability in various environmental conditions, The composition, architecture, and thickness of these cell walls vary significantly across taxonomic groups, species, and even growth phases, presenting both challenges and opportunities for bioethanol production processes (Shivakumar et al., 2024).The structural complexity of microalgal cell walls is primarily attributed to their multilayered organization and the diverse array of biochemical components they contain. These components typically include polysaccharides (cellulose, hemicellulose, pectin),

proteins, glycoproteins, and in some cases, recalcitrant compounds such as algaenan, sporopollenin, and silica **(Shivakumar et al., 2024).**

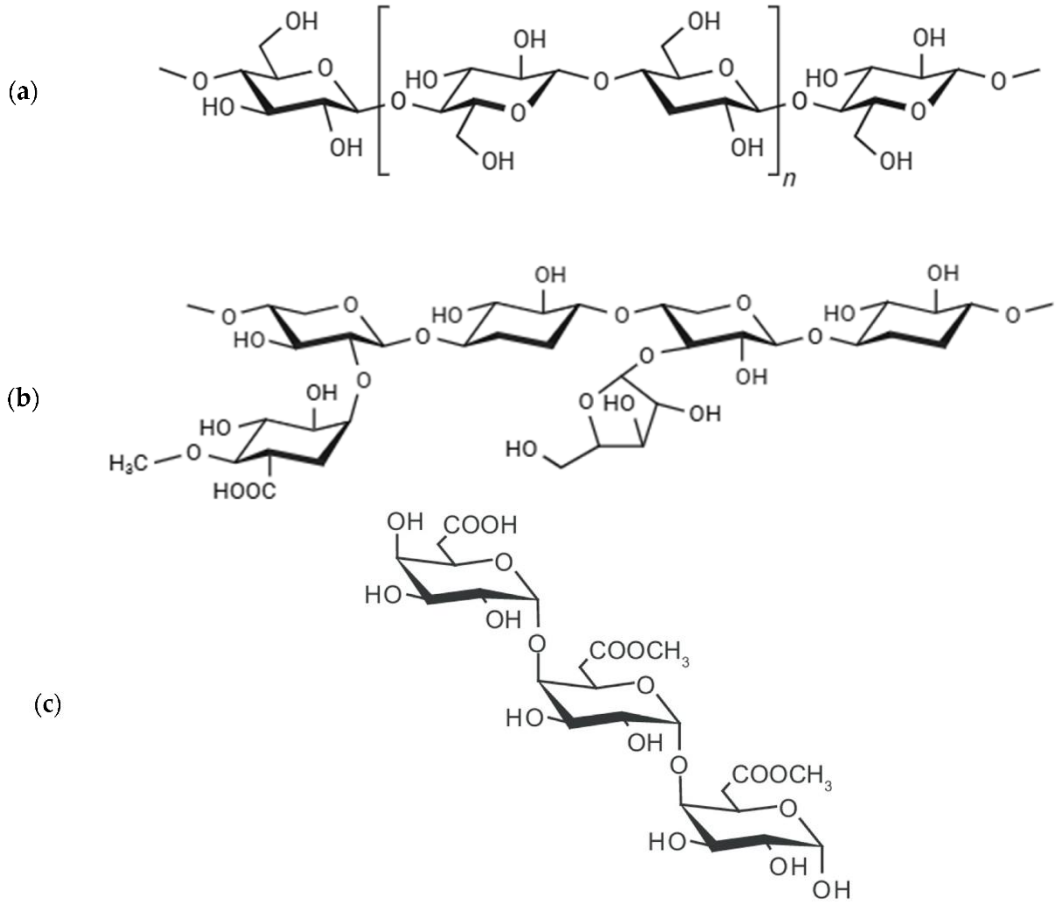

*Figure 3: (**a**). Structure of Cellulose present in Nannochloropsis and Scenedesmus. Created with BioRender.com. (**b**). Structure of Hemicellulose present in Scenedesmus. Created with BioRender.com. (**c**). Structure of Pectin present in Scenedesmus and Schizochytrium(**Ali et al .,2015**) .*

Among the major taxonomic groups, green algae (Chlorophyta) typically possess cell walls composed primarily of cellulose, often arranged in crystalline microfibrils embedded in an amorphous matrix of hemicellulose and pectin. This structure bears some resemblance to plant cell walls, although with notable differences in the specific polysaccharide composition and cross-linking patterns (Chew et al., 2023). For instance, *Chlorella* species have cell walls containing a rigid layer of glucosamine polymers similar to chitin, which contributes to their resistance to enzymatic degradation **(Shivakumar et al., 2024).**

Diatoms (Bacillariophyta) exhibit a unique cell wall structure known as the frustule, composed primarily of hydrated silicon dioxide (silica) arranged in intricate patterns. This siliceous structure provides exceptional mechanical

strength and resistance to degradation, necessitating specialized pretreatment approaches for efficient bioethanol production **(Wang et al., 2024).**

Cyanobacteria, including *Spirulina platensis*, possess cell walls that are structurally distinct from those of eukaryotic microalgae. The cell wall of *S. platensis* consists of four layers: (1) an outer membrane containing lipopolysaccharides, (2) a peptidoglycan layer, (3) an electron-dense layer, and (4) an inner cytoplasmic membrane (Bora et al., 2024). Notably, *S. platensis* lacks the recalcitrant components found in many eukaryotic microalgae, such as cellulose, algaenan, or silica, which contributes to its relatively easier degradability and suitability for bioethanol production **(Wang et al., 2024).**

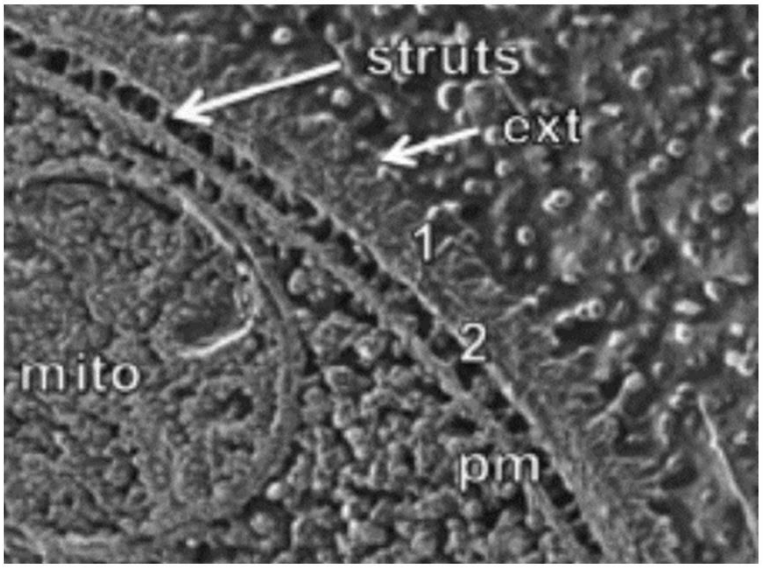

*Figure 4: Quick-Freeze Deep-Etch Electron Microscopy (QFDE-EM) image of the cell wall of Nannochloropsis. 1,2—Layers of cell wall, ext—extensions, pm—Plasma Membrane, mito—Mitochondria (**Scholz et al.,2014**)*

4.1.Pretreatment Methods:

Pretreatment methods for microalgal biomass aim to disrupt cell walls, increase the accessibility of intracellular carbohydrates, and enhance the efficiency of subsequent enzymatic hydrolysis and fermentation processes. These methods can be broadly categorized into physical, chemical, and biological approaches, each with distinct advantages, limitations, and applications depending on the target microalgal species and process requirements. Table 3 provides an overview of cell wall characteristics and effective pretreatment methods for selected microalgae.

Table 2: Overview of Microalgae Cell Wall Characteristics and Pretreatment Methods

Representative Species	Key Cell Wall Components	Effective Pretreatment Methods	Reference(s)
Chlorella vulgaris	Cellulose, Hemicellulose, Pectin, Glycoproteins, Algaenan (some strains)	Physical (Bead Milling, Ultrasound), Chemical (Acid, Ionic Liquids), Biological (Enzymes: Cellulase, Pectinase)	Wang et al. (2024), Shivakumar et al. (2024)
Scenedesmus obliquus	Cellulose, Hemicellulose, Pectin, Sporopollenin-like layer	Physical (Bead Milling), Chemical (Acid), Biological (Enzymes: Cellulase, Hemicellulase)	Chew et al. (2023), Bakare et al. (2022)
Spirulina platensis	Peptidoglycan, Lipopolysaccharides (Outer Membrane)	Physical (Homogenization, PEF), Chemical (Alkali), Biological (Enzymes: Lysozyme, Protease)	Bora et al. (2024), Markou et al. (2023)
Phaeodactylum tricornutum	Silica (Frustule), Organic layer (varies)	Chemical (Alkali, HF - carefully), Biological (Limited enzyme options for silica)	Hildebrand et al. (2012), Chew et al. (2023)
Nannochloropsis spp.	Cellulose, Algaenan	Physical (Homogenization), Chemical (Solvents, Ionic Liquids, Oxidative), Biological (Limited)	Shivakumar et al. (2024), Raghupathi & Ravi (2023)
Porphyridium cruentum	Sulfated polysaccharides (Carrageenan-like), Cellulose	Biological (Enzymes: Carrageenase, Cellulase), Chemical (Acid)	Chew et al. (2023)

4.1.1. Physical Methods: Breaking Down Barriers Mechanically:

Physical pretreatment methods rely on mechanical forces or physical phenomena to disrupt microalgal cell walls without the addition of chemicals or biological agents **(Krishnamoorthy et al., 2022).** These methods, such as high pressure homogenization, bead milling, and ultrasonication, apply shear forces, impact, or cavitation to break down the complex and often rigid cell wall structures **(Krishnamoorthy et al., 2022).** While generally non-selective in their action compared to enzymatic or chemical methods, physical techniques are widely applicable and have been proven effective for enhancing the extraction of intracellular components like lipids or carbohydrates from a diverse range of microalgal species for biofuel production **(Krishnamoorthy et al., 2022).**

a) Mechanical Milling (e.g., Bead Milling):

This method involves agitating the microalgal suspension with small, dense beads (e.g., glass, ceramic) within a chamber. The high-speed movement and collisions between the beads, microalgal cells, and the chamber walls generate intense shear forces and impacts, effectively rupturing the cell walls, Bead milling is recognized for its effectiveness across a variety of microalgal species, including those with robust cell walls, Recent innovations are primarily focused on optimizing parameters like bead size, material, loading ratio, and agitation speed to enhance disruption efficiency while significantly reducing the typically high energy consumption associated with this technique **(Wang et al., 2024; Shivakumar et al., 2024).**

b) Ultrasonication:

This technique utilizes high-frequency sound waves (typically >20 kHz) applied to the microalgal suspension, These sound waves create cycles of compression and rarefaction in the liquid, leading to the formation, growth, and rapid collapse of microscopic bubbles a phenomenon known as cavitation, The collapse of these bubbles generates localized extreme conditions, including intense shock waves, micro-jets, and high shear forces, which physically disrupt the microalgal cell walls, While effective, continuous ultrasonication can be energy-intensive and generate heat. Recent advancements include the use of pulsed ultrasonication (alternating on/off cycles), which has been shown to reduce overall energy consumption and minimize potential thermal degradation of target products **(Bora et al., 2024; Wang et al., 2024).**

c) High-Pressure Homogenization:

In HPH, the microalgal suspension is forced under very high pressure (often 100-500 MPa) through a specially designed narrow valve or orifice, As the suspension passes through this restriction, it experiences a rapid pressure drop, intense shear stress, turbulence, and high-velocity impact against surfaces, This combination of forces effectively disrupts even very rigid microalgal cell walls. HPH is known for its high disruption efficiency, Recent advances focus on optimizing valve geometry, operating pressure, and the number of passes to maximize cell rupture while improving the overall energy efficiency of the process **(Shivakumar et al., 2024; Bora et al., 2024).**

d) Microwave Treatment:

This method employs electromagnetic radiation in the microwave frequency range (typically 300 MHz to 300 GHz) to heat the microalgal

suspension, Microwaves interact primarily with polar molecules, particularly water, within the cells, causing rapid and volumetric heating through dipole rotation and ionic conduction, This rapid internal heating generates significant intracellular pressure and thermal stress, leading to the swelling and eventual rupture of the cell wall, It is considered a promising method, especially for species with high water content like *Spirulina*, Current research is exploring the use of specific, targeted frequencies and optimized power levels to enhance disruption efficiency and reduce energy requirements **(Wang et al., 2024).**

e) Pulsed Electric Field (PEF):

PEF involves applying short pulses (microseconds to milliseconds) of high-intensity electric fields (typically 10-80 kV/cm) across the microalgal suspension placed between two electrodes, The external electric field induces a critical transmembrane potential difference, causing the formation of temporary or permanent pores in the cell membrane through a process called electroporation, This permeabilization facilitates the release of intracellular contents, PEF is generally more effective at disrupting the cell membrane than the entire cell wall and is particularly suitable for species with less rigid walls, such as *Spirulina*, It is often highlighted for its relatively low energy consumption compared to mechanical methods and its non-thermal nature, which helps preserve sensitive intracellular compounds **(Bora et al., 2024).**

4.1.2.Chemical Methods: Dissolving and Degrading Cell Walls:

a) Acid Treatment (e.g., H_2SO_4, HCl):

This method utilizes dilute or concentrated acids to hydrolyze the glycosidic bonds within cell wall polysaccharides (like cellulose and hemicellulose), breaking them down into simpler sugars. The mechanism involves protonation of the glycosidic oxygen followed by cleavage of the C-O bond, Acid treatment is effective for disrupting the carbohydrate matrix of many microalgal species, making the sugars accessible for fermentation, However, harsh conditions (high temperature, high acid concentration) can lead to the degradation of sugars into inhibitory compounds such as furfural (from pentoses) and 5-hydroxymethylfurfural (HMF) (from hexoses), which can hinder subsequent fermentation processes. Therefore, careful optimization of reaction conditions (temperature, time, acid concentration) is crucial to maximize sugar release while minimizing inhibitor formation **(Wang et al., 2024; Bora et al., 2024).**

b) Alkaline Treatment (e.g., NaOH, KOH):

Alkaline agents work by disrupting the cell wall structure through several mechanisms, including saponification of ester linkages (present in lipids and

between polysaccharides and lignin-like compounds), solubilization of proteins, and swelling of cellulose fibers. This increases the porosity and surface area of the biomass, enhancing enzyme accessibility, Alkaline treatment is particularly effective for microalgae with significant protein content in their cell walls or matrix, such as Spirulina. It generally operates under milder conditions (lower temperatures) compared to acid treatment, which can reduce the formation of inhibitory compounds. Research focuses on optimizing alkali concentration and reaction time to minimize chemical usage and potential environmental impact while maximizing disruption efficiency **(Chew et al., 2023; Wang et al., 2024).**

c) Solvent Treatment (e.g., Ethanol, Acetone, Hexane):

Organic solvents are primarily used to extract lipids from microalgae but also contribute to cell disruption by permeabilizing or dissolving the lipid-based cell membrane and potentially disrupting hydrophobic interactions within the cell wall matrix. Methods like Soxhlet extraction or accelerated solvent extraction utilize solvents at elevated temperatures and pressures to enhance efficiency. This approach is particularly effective for lipid-rich microalgal species targeted for biodiesel production, Key research areas include the development and use of greener, less toxic solvents (like bio-based solvents), improving solvent recovery and recycling processes to reduce costs and environmental footprint, and integrating solvent extraction with other pretreatment methods **(Shivakumar et al., 2024; Bora et al., 2024).**

4.1.3. Enzymatic Hydrolysis:

This pretreatment method utilizes specific enzymes or enzyme cocktails to selectively break down the complex components of the microalgal cell wall, Unlike broad physical or chemical methods, enzymes act as highly specific biological catalysts, targeting particular chemical bonds within polysaccharides (e.g., cellulases, hemicellulases, pectinases), proteins (proteases), or peptidoglycans (lysozyme), The mechanism involves the enzyme binding to its specific substrate within the cell wall and catalyzing the hydrolysis (breaking) of targeted bonds, leading to the solubilization or degradation of the wall structure **(Shivakumar et al., 2024).**

A key advantage is that enzymatic hydrolysis operates under mild conditions (moderate temperature, near-neutral pH), which minimizes the degradation of valuable intracellular products (like sugars for fermentation) and avoids the formation of inhibitory compounds often generated by harsher methods **(Shivakumar et al., 2024).** The high selectivity ensures that only specific wall components are targeted, potentially leading to cleaner product streams. However, the effectiveness is highly dependent on tailoring the enzyme(s) to the specific

and often variable cell wall composition of the target microalga. For instance, lysozyme (which targets peptidoglycan) and proteases are particularly effective for disrupting the protein/peptidoglycan-rich walls of cyanobacteria like *Spirulina* **(Bora et al., 2024; Markou et al., 2023)**, whereas cellulase-based cocktails containing endoglucanases, exoglucanases, and β-glucosidases are commonly employed for the cellulose-rich walls of green algae **(Wang et al., 2024).**

The main challenges remain the relatively high cost of enzymes and potentially slower reaction rates compared to other methods. Current research focuses on discovering novel, more robust enzymes, developing cost-effective enzyme production strategies (including consolidated bioprocessing), enzyme immobilization and recycling techniques, and optimizing enzyme cocktails for synergistic activity to improve efficiency and reduce costs **(Shivakumar et al., 2024; Wang et al., 2024)**

Chapter 02: Materials & Methods

I. in Silico:

1.Docking :

Molecular docking is a powerful bioinformatics approach used to predict the conformation and interaction affinity between two molecules, typically a protein (receptor) and a small molecule (ligand). This method relies on simulation algorithms that explore the possible spatial orientations of the ligand within the active site of the protein to estimate the stability of the formed complex based on an energy scoring function.

In the context of this project, a molecular docking study was conducted to examine the interaction between the glucoamylase enzyme derived from *Aspergillus niger* (PDB identifier: 5GHL) and the disaccharide β-maltose (PubChem CID: 6255). The primary objective of this in silico modeling is to elucidate the molecular recognition between the enzyme and its substrate and to better understand the stabilizing interactions, such as hydrogen bonds, hydrophobic interactions, and salt bridges, which could influence the catalytic efficiency of glucoamylase in the hydrolysis of polysaccharides.

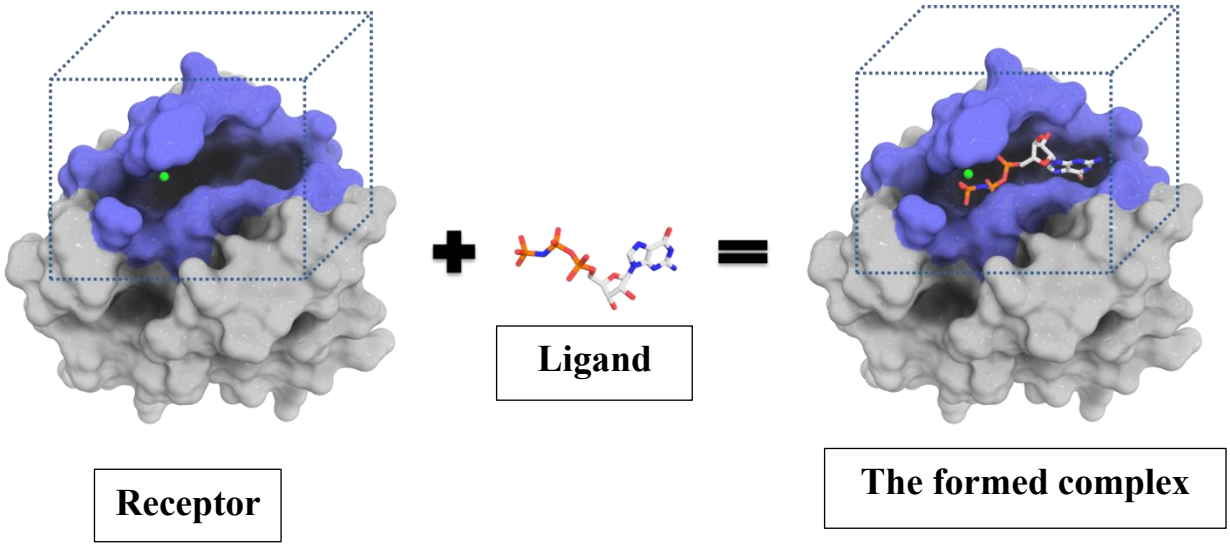

Figure 5: Three dimensional representation of the molecular docking process between Receptor and Ligand, illustrating the binding sites and key interactions between the molecules.

2. Material and Methods :

2.1 Data preparation

2.1.1-Preparation of the Receiver:

a-The three dimensional structure of **glucoamylase** was obtained from the **Protein Data Bank (PDB)** (accession code: **5GHL**).

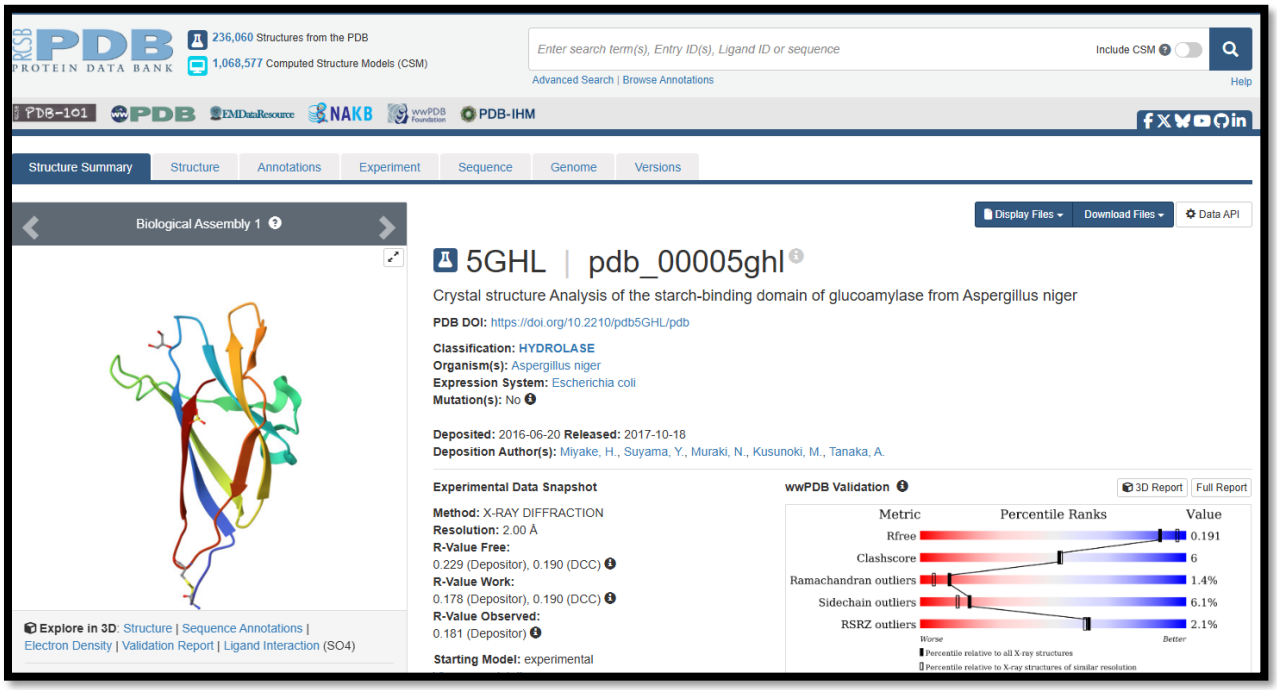

Figure 6: Three dimensional structure of glucoamylase enzyme extracted from the PDB database (accession code: 5GHL),

b- The protein structure was prepared by Tool: Use AutoDock Tools (ADT)

- Elimination of water molecules
- Add Hydrogenes
- repair missing atoms
- add charges

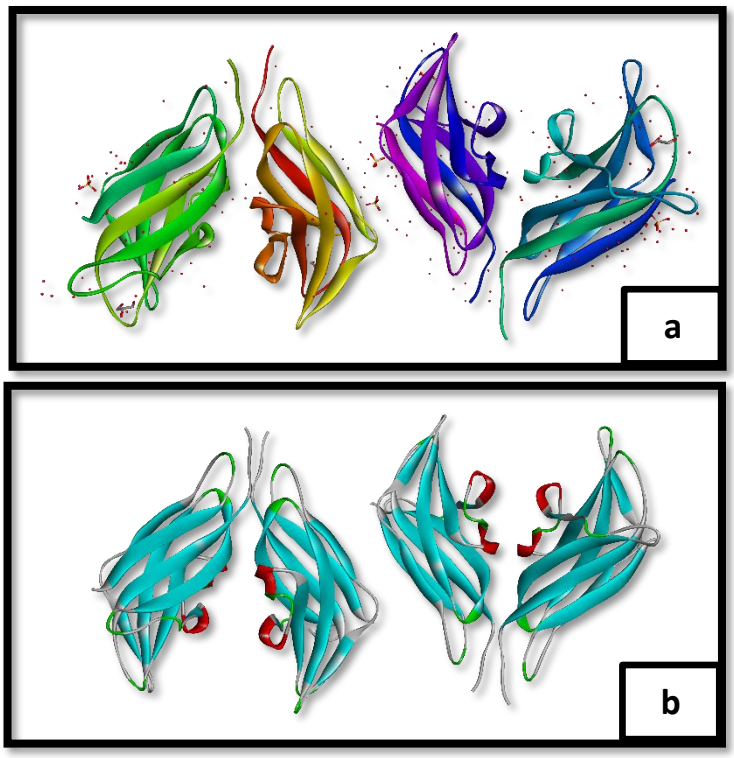

Figure 7 : Stages of protein structure preparation using AutoDock Tools, showing (a) the original structure, (b) after prepartion .

2.1.2-Preparing the Ligand:

The chemical structure of maltose was retrieved from databases PubChem (CID: 6255).

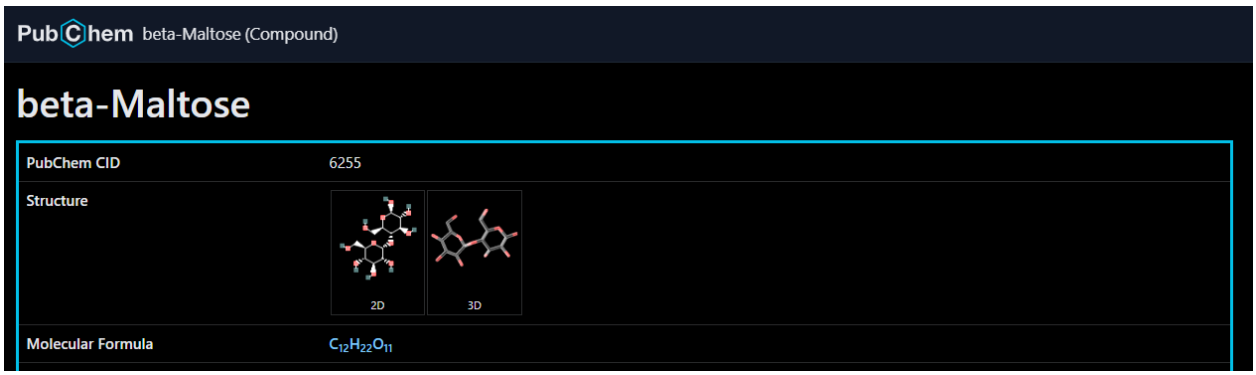

Figure 8: Chemical structure of maltose extracted from the PubChem database (CID: 6255),

2.1.3.Ligand Optimization :

Next Use OpenBabel to convert the SDF file to mol To optimize the ligand using Chem3D, first select all atoms, then go to Calculations MM2 Set up MM2 Atom Types and Charges, followed by Minimize Energy to optimize the structure, and finally select Molecular Dynamics to run a dynamic simulation and save , use OpenBalel again to convert the mol file to pdbqt.

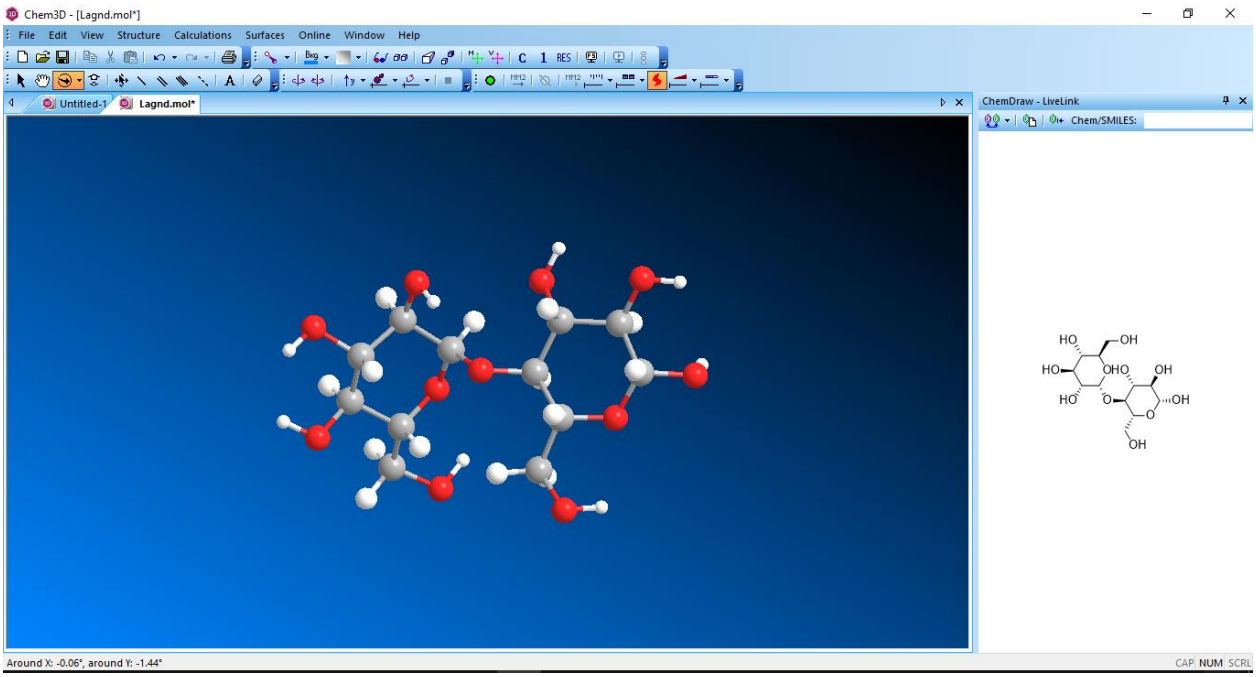

Figure 9: optimize the ligand using Chem3D

2.2. Defining the Binding Site and Grid Setup :

Define a grid covering the active site to guide the docking process by use Discovery Studio.

The active site of glucoamylase was identified through structural analysis and comparative modeling. A structural alignment with homologous enzymes and known substrate-bound complexes was performed using Discovery Studio tools.

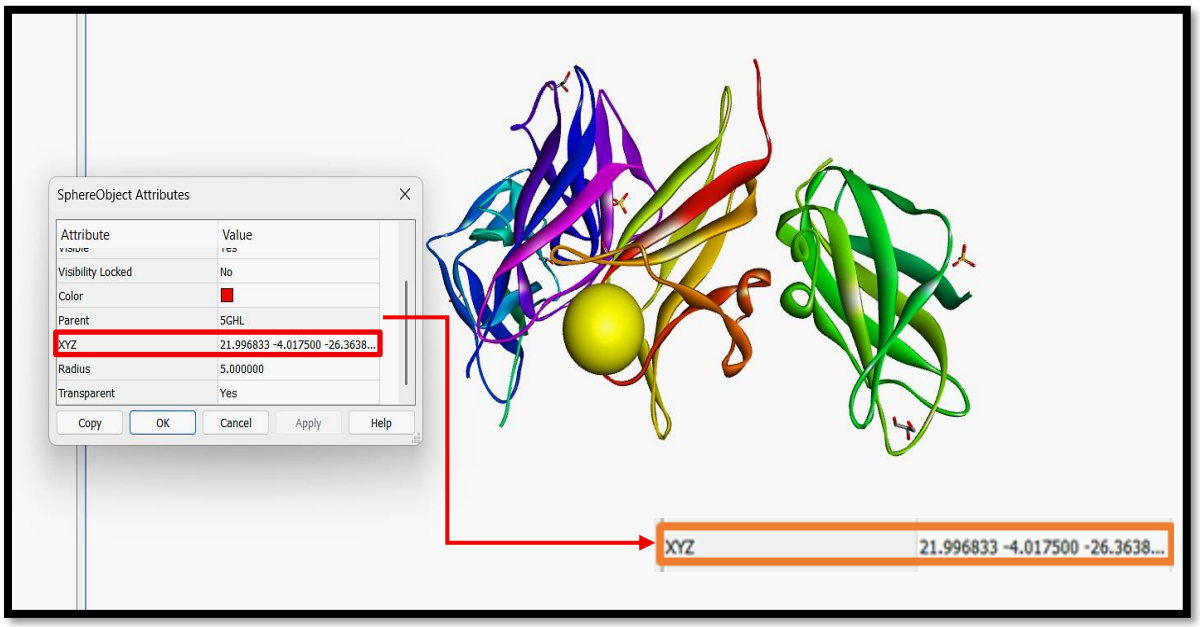

Figure 10: Determination of the active binding site in glucoamylase enzyme using Discovery Studio

2.3.Preparation of the config.txt Files:

Ensure that you have prepared the necessary files for docking:

Receptor (Protein): receptor.pdbqt
Ligand (Molecule): Ligand.pdbqt
out = out.pdbqt
center_x = 21.996833
center_y = -4.017500
center_z = -26.36380
size_x = 60
size_y = 60
size_z = 60
num_modes = 10
energy_range = 4

Table 3: Summary of AutoDock Vina Input Parameters in the Study of Maltose Glucoamylase Complex Formation

Parameter	Description
receptor = receptor.pdbqt	Specifies the input file for the target protein (receptor) in .pdbqt format. This file includes atomic coordinates, partial charges, and atom types required for docking.
ligand = ligand.pdbqt	Specifies the input file for the small molecule (ligand) in .pdbqt format. It contains the 3D structure, rotatable bonds, and partial charges.
out = out.pdbqt	Defines the output file where the docked poses (binding conformations) will be saved in .pdbqt format. Each pose represents a predicted binding orientation of the ligand within the receptor's binding site.
center_x, center_y, center_z	Define the center coordinates of the docking grid box in 3D space. These values are typically determined based on the location of the active site or catalytic residues in the protein. In this case: X = 21.996833 Y = -4.017500 Z = -26.36380
size_x, size_y, size_z	Set the dimensions of the grid box along the x, y, and z axes. A larger box allows the ligand to explore more space, but increases computational cost. Here, the box size is set to 60 × 60 × 60 , which ensures full coverage of the binding pocket and surrounding area.
num_modes = 10	Specifies the number of binding poses (conformations) to generate. The program will return up to 10 different orientations of the ligand within the binding site, ranked by predicted binding affinity.
energy_range = 4	Defines the energy window (in kcal/mol) within which all generated poses must fall relative to the best (lowest-energy) pose. Only poses with a binding energy within 4 kcal/mol of the best pose will be reported.

2.4.Docking Simulation

To run AutoDock Vina , place all required files(receptor.pdbqt, ligand.pdbqt, config.txt, vina.exe) in a single directory (folder). This helps the program find them easily .

- Open Command Prompt (CMD)
- Go to your folder: cd C:Users\Sud_Tec\Desktop\docking\
- Run Code = Vina.exe –config conf.txt –log log.txt

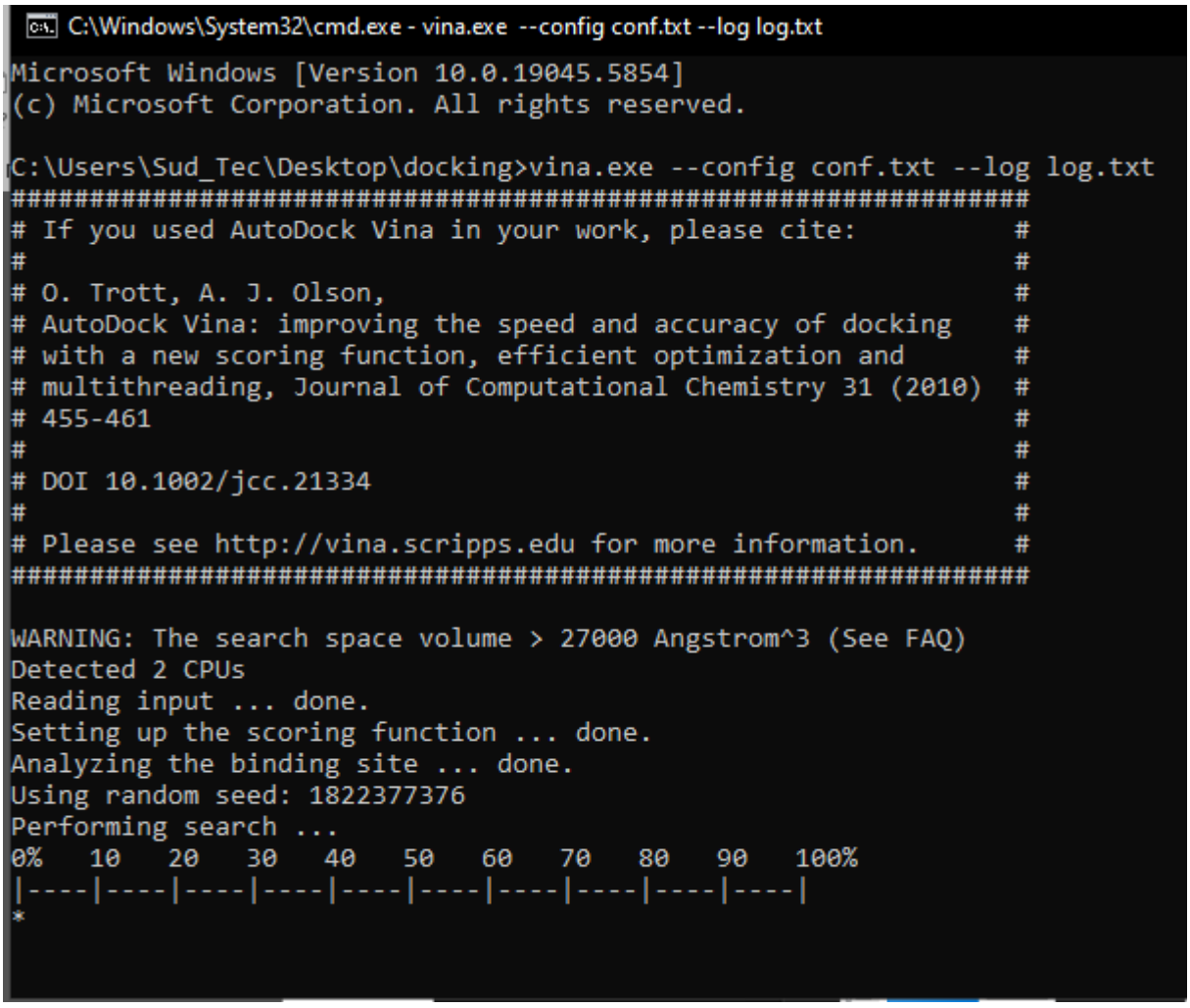

Figure 11: Docking Simulation using AutoDock vina.

II. in vitro

1.Microalgae Cultivation:

Spirulina was successfully isolated from the Tadjmout dam in the Laghouat province using a plankton net with a 20 µm mesh size. This microalgae, known for its spiral morphology and nutritional value, thrives in alkaline freshwater environments and serves as a bioindicator of water quality. The isolation process involved collecting water samples, concentrating microorganisms using the plankton net, and purifying the sample through laboratory techniques such as microscopy.

Water Samples Were Collected from Locations with Colors Ranging from Colorless, Bluish, and Brownish to Green, Specific to the Water's Coloring. The research was conducted in September 2024 at the Tadjmout Dam.

Approximately 300 ml of water samples were collected in three sterile 150 ml bottles from each location. The sterile bottles were submerged in the surface water at a distance of 20 to 25 cm from the surface, sufficiently far from the edges. The samples were transported to the laboratory in a cooler and kept awaiting analysis at 4°C.

To further refine the isolation process, a sterile syringe was used to carefully transfer individual cells or small clusters of *A. platensis* into flasks containing an appropriate culture medium specifically designed for cyanobacteria. This step ensured minimal contamination and optimal growth conditions. *A. platensis* was chosen for this study due to its abundance in the sample and the relative ease it offers during the isolation process, thanks to its distinct spiral shape and filamentous structure, which make it easily identifiable under a microscope.

Figure 12: Barrage infer-flux Tadjmout, Laghouat 2025.

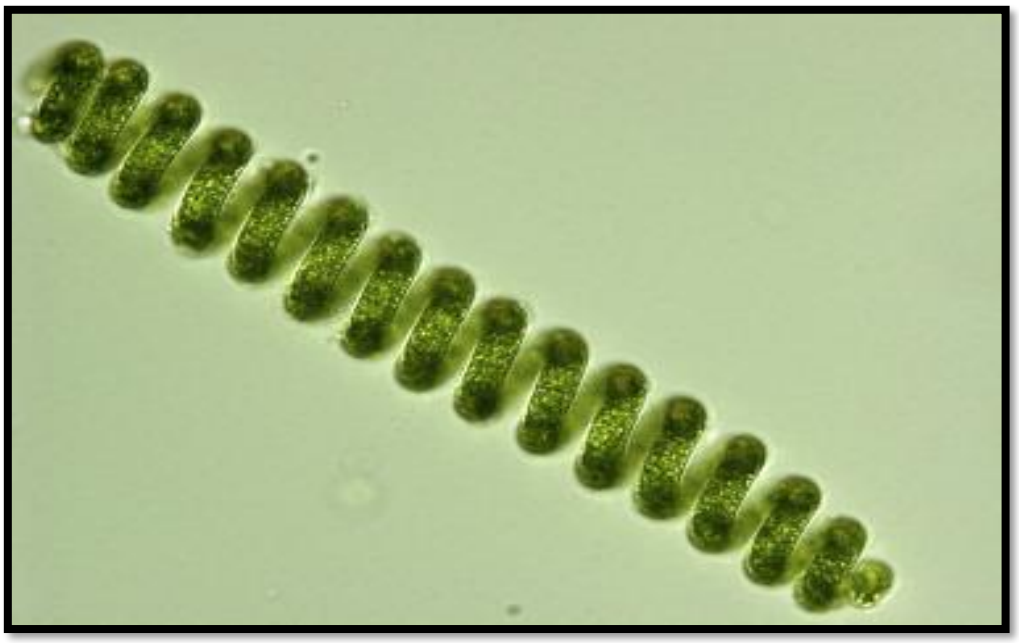

*Figure 13: A trichome of the cyanobacterium Arthrospira platensis. Trichome diameter ~12 µm. Courtesy of Dr. Amha Belay(**Borowitzka .,2016**) .*

2.Preparation of Culture Media :

2.1.Algal And Cyanobacterial Cultures Medium :

The strains of algal and cyanobacterial cultures are grown on a specific medium, the modified Zarrouk medium (Cyanobacteria can develop in various culture media such as BG11, BG12, Hiri medium, and Aiba Ogawa medium, among which BG11 (Blue-Green medium) is notable). We have selected the Zarrouk medium because it supports the growth of cyanobacteria and can meet their nutritional requirements. The recipe used in the laboratory is simplified, as it requires the preparation of only two solutions, solution A, and a mineral solution. Subsequently, it is sterilized in an autoclave at 120 °C for 20 minutes. The final pH is 9.

To establish the microbial population, the samples were enriched with Zarrouk liquid medium and incubated at ambient temperature (23 ± 2 °C) next to a window allowing adequate sunlight for a duration of three months. 180 mL of Zarrouk medium was added to 20 mL of the sample.

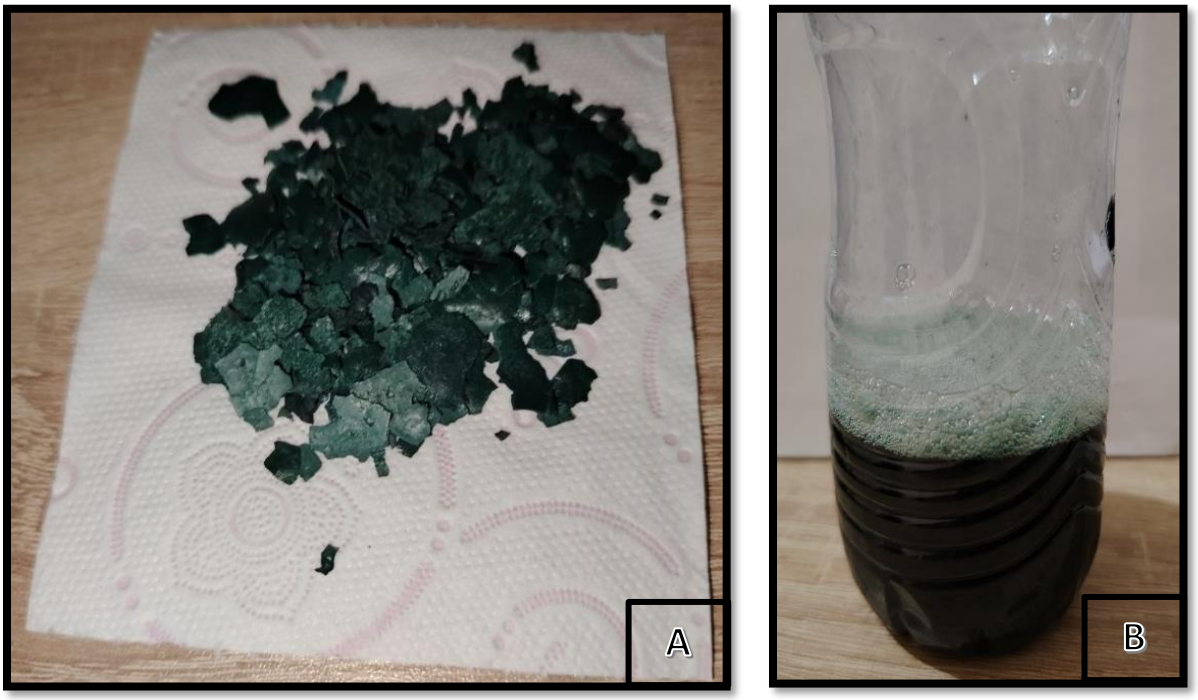

Figure 14*: **A** Dried Arhtrospira & **B** Arthrospira platensis in Zarrouk medium*

2.2.Sample Preparation And Extraction Of Microalgae Spirulina Sp:

The preparation of the microalgae *Spirulina sp.* sample for analysis begins with the collection of algal biomass. The collected biomass is thoroughly washed with distilled water to remove impurities and extraneous materials. Following this, the sample is dried under controlled conditions, and away from direct light exposure to prevent degradation of sensitive compounds. Once adequately dried, the algal biomass is finely ground into a homogeneous powder to ensure consistency in subsequent analyses. This finely ground material serves as the starting point for the preparation of the algal extract. The extraction process involves utilizing the dried and powdered algae to obtain a concentrated extract, which can then be used for various analytical or experimental purposes. This meticulous preparation ensures the purity, stability, and representativeness of the sample for accurate downstream applications.

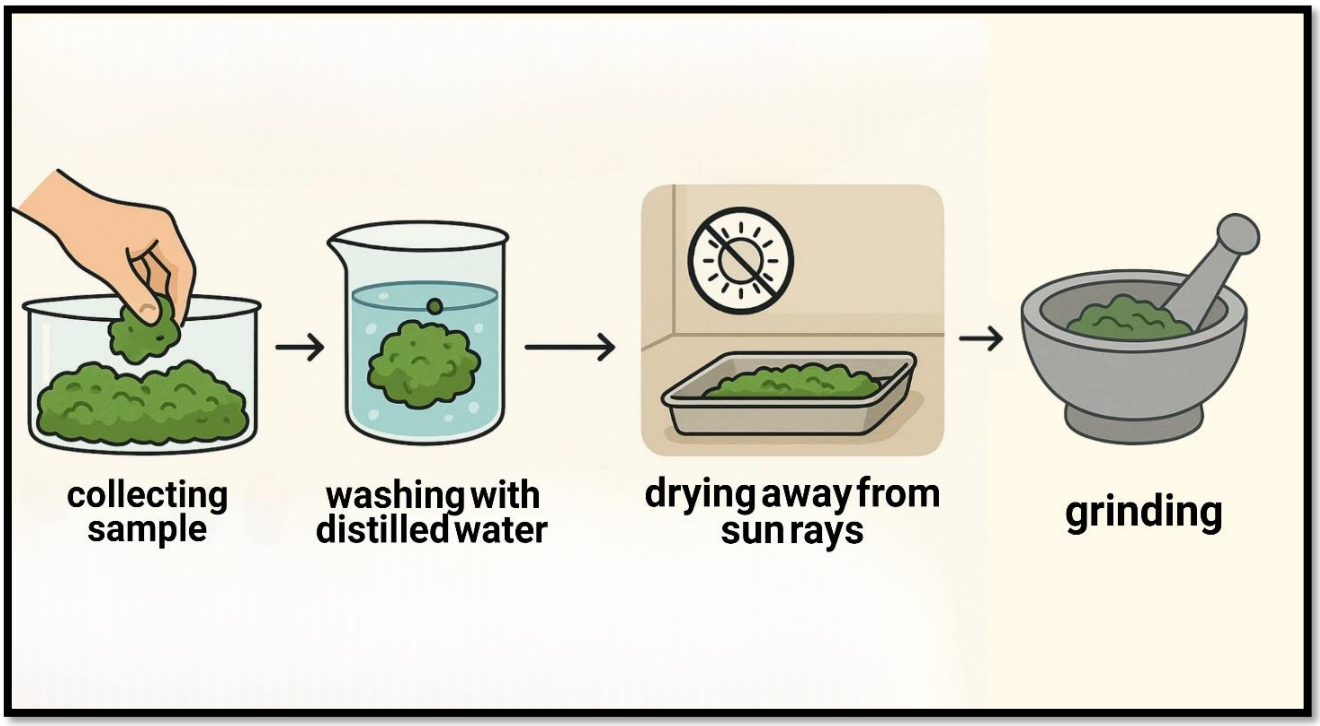

Figure 15: A schematic diagram of the sample preparation stages.

2.3. Preparation of *Aspergillus Niger* Growth Medium:

In this practical effort, a rigorous methodology was followed to develop a customized growth medium for fungal cultivation using PDA (Potato Dextrose Agar), which is considered one of the ideal media for cultivating fungal strains such as *Aspergillus niger*. The process began with selecting the necessary materials and equipment, including two electric heaters for heating the solution during preparation, magnetic stirrers for continuous mixing to achieve a homogeneous mixture, and two 1-liter Erlenmeyer flasks for accurately measuring and preparing the components. Additionally, a delicate balance was used to determine the exact quantities of dry components, while distilled water was employed to avoid any impurities that would impact the quality of the medium. For the chemical components, 200 grams of potatoes were utilized as a natural source rich in nutrients, 20 grams of glucose (dextrose) to provide a primary carbohydrate source, and 15 grams of agar to give the medium its solid consistency.

The preparation method started with chopping 200 grams of potatoes into thin slices, then boiling them in one liter of distilled water for 30 minutes using the electric heater. This procedure extracted the natural nutrients inherent in the potatoes. After boiling, we filtered the mixture using fine cloth or a sieve to collect the pure potato extract. In the next phase, 20 grams of glucose and 15 grams of agar were added to the filtered extract, and the total volume of the solution was adjusted by adding distilled water to reach one liter. A magnetic stirrer was used to thoroughly mix all the parts in the Erlenmeyer flask to make sure that the dry materials would dissolve completely and be evenly distributed.

Subsequently, the mixture was transported to an autoclave and sterilized at 121 degrees Celsius for 15 minutes, a key step to ensure the medium was free from any microorganisms or contaminants that could interfere with experimental results. Once the sterilizing process was complete, the liquid medium was carefully put into sterile Petri plates near a Bunsen burner to prevent any contamination. The plates were then kept in a quiet area until the medium set entirely. This medium provides an ideal environment for fungal development due to its richness in nutrients, such as the carbohydrates found in the potato extract and glucose. This method ensures the production of a high-quality PDA medium suitable for the accurate and reliable cultivation of fungi like ***Aspergillus niger***.

After the liquid medium was poured into sterile Petri dishes and left to solidify in a quiet, contamination-free setting, we prepared the sample to analyze the growth of ***Aspergillus niger***. Ten coffee beans were carefully placed upside down on the surface of the solidified agar medium, making sure they were uniformly spaced. Coffee beans were chosen because they are rich in carbohydrates, offering a good nutritional supply to encourage fungal development. The equal spacing of the beans enabled each colony to have adequate room to develop without overlapping or interfering with others, ensuring obvious and observable growth patterns.

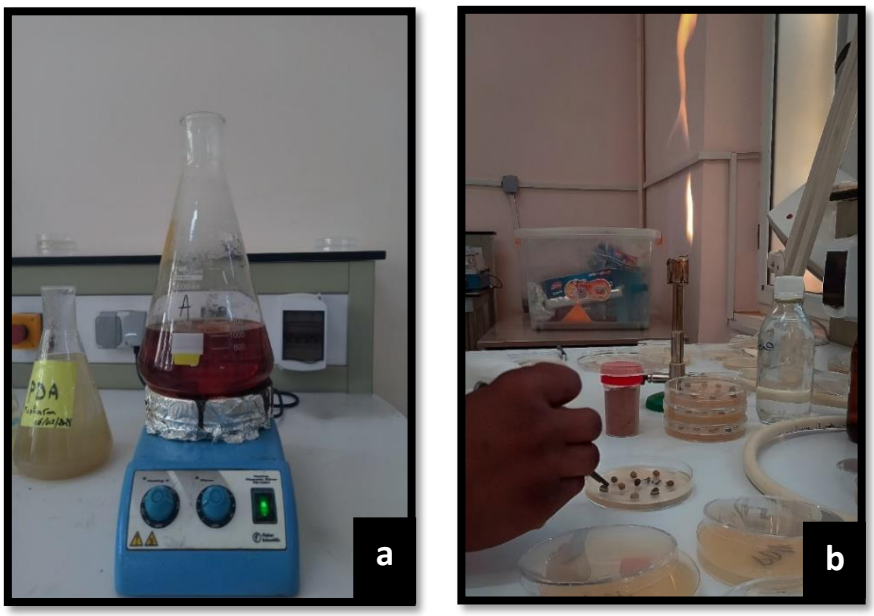

Figure 16**:** Two images illustrating the processes of mixing the culture medium components(**a**) and placing coffee beans in Petri dishes**(b)**.

Subsequently, the Petri dishes were transferred to an incubator and maintained at a constant temperature ranging between 25-30 degrees Celsius, which are the optimal conditions for the growth of ***Aspergillus niger***. The incubation period lasted between 3 to 5 days, during which the development of fungal colonies was closely monitored.

To ensure more accurate and diverse isolation of *Aspergillus niger* colonies, a soil sample was collected from a natural environment, known to be a rich source of fungi and other microorganisms. One gram of the soil sample was dissolved in 9 milliliters of sterile distilled water, with vigorous mixing to ensure a homogeneous distribution of components. Subsequently, a single drop of this suspension (at a concentration of 10^{-1}) was inoculated onto the surface of a Petri dish containing pre-prepared PDA (Potato Dextrose Agar) medium.

To equally disperse the sample across the surface of the growing medium, we utilized the spread plate technique using a sterilized tool. This ensured the homogeneous and ordered distribution of microbes over the whole surface of the medium. This technique is particularly effective in separating separate colonies from one another, simplifying their identification and tracking of growth.

We conducted the same technique again but used physiological saltwater instead of sterile distilled water. The same methods were followed: dissolving 1 gram of soil in 9 milliliters of physiological saline, thorough mixing, and taking a drop of the suspension at a concentration of 10^{-1} to place on another Petri plate

containing PDA media. The sample was subsequently disseminated using the spread plate technique.

After finishing the inoculation steps, the Petri dishes were transported to an incubator and incubated at a temperature ranging between 25-30 degrees Celsius for a period of 3 to 5 days. During this time, the dishes were checked every day for the appearance of fungal colonies. This method focuses on supplying two contrasting habitats (sterile distilled water and physiological saline) to boost the chances of development and colonization of ***Aspergillus niger*** and other fungi present in the soil sample.

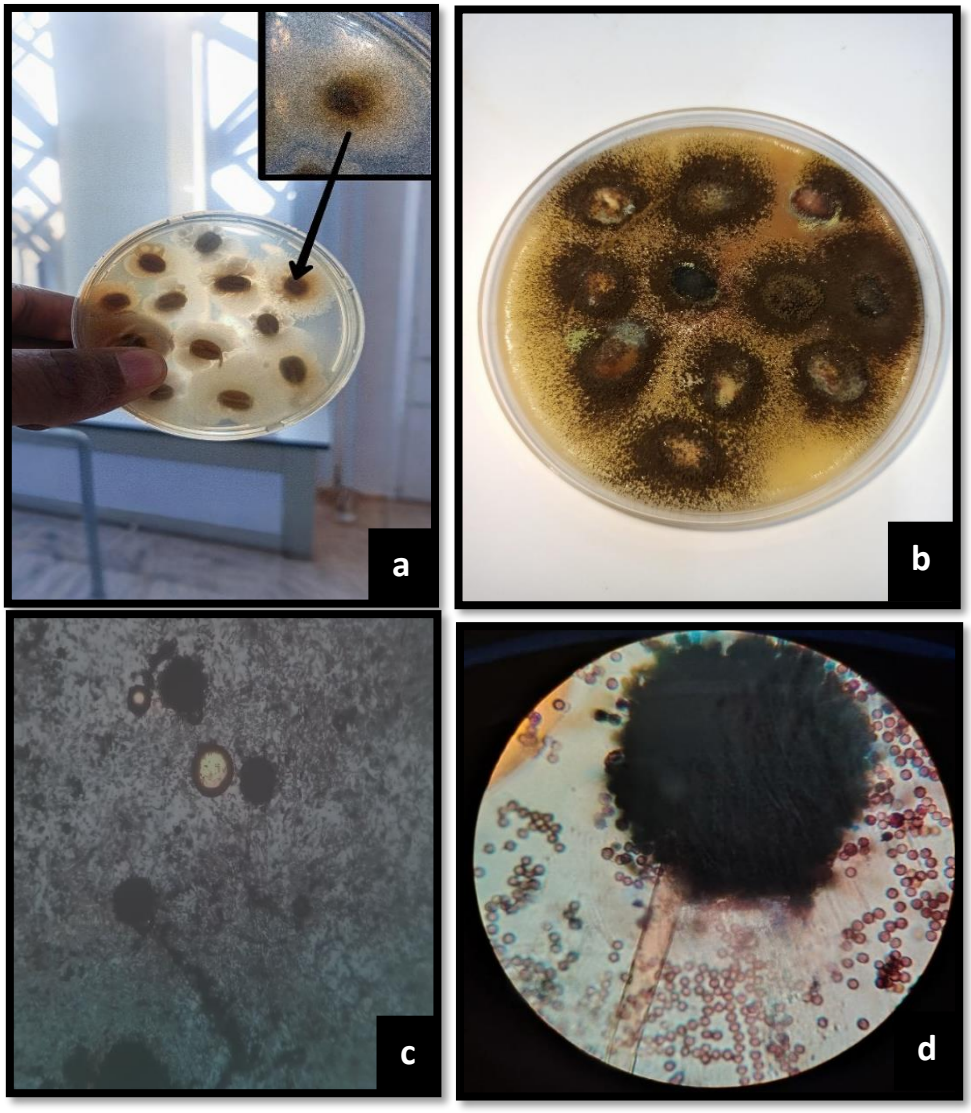

Figure 17: Appearance of fungal colonies in Petri dishes: ***(a)*** *after 3 days of incubation,* ***(b)*** *after 5 days of incubation* **,** *Aspergillus niger under microscope (****c*** *and* ***d****).*

To minimize any contamination during this sensitive operation, every step was carried out near a Bunsen burner, a key laboratory equipment used for sterilization and heating. The Bunsen burner produces a clean, concentrated flame that permits sterilizing tools (e.g., forceps or glassware) by passing them through the flame, so generating a limited sterile environment. This approach ensured safe working conditions and minimized the danger of contaminating the sample or medium with foreign microbes.

After cultivating Aspergillus fungi, we performed Single spore Isolation procedure to ensure proper isolation of the strain and maintained it for 5 to 7 days.

2.4.Preparation Of Czapek-Dox Medium (Liquid And Solid Fungal Medium):

The Czapek-Dox medium, a widely used artificial medium for fungal growth, particularly for *Aspergillus niger* , was prepared. This medium provides rich inorganic sources of carbon and nitrogen necessary to support the growth of these microorganisms. The preparation process began with dissolving a set of chemicals in one liter of distilled water to form the basic solution. These chemicals included: 30 grams of sucrose as the primary energy and carbon source, 2 grams of sodium nitrate ($NaNO_3$) to supply the required nitrogen for protein synthesis, and 1 gram of dipotassium phosphate (K_2HPO_4) which acts as a pH buffer and a source of phosphorus. Additionally, 0.5 grams of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) and 0.5 grams of potassium chloride (KCl) were added to provide essential minerals such as magnesium, sulfur, potassium, and chlorine. Furthermore, 0.01 grams of ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) was included to supply trace elements required for the fungi's metabolic processes.

After completely dissolving all components with continuous stirring to ensure a homogeneous mixture, we checked the pH, it was right at, so we didn't need to adjust it., as this level is optimal for the growth of many fungi. Subsequently, we split the mixture into two batches. To the first batch, we added 7.5 grams of agar powder (that's what makes it solid medium) for growing cultures in petri dishes, The other batch stayed liquid for later use.

To make sure both the liquid and solid media were completely sterile, we zapped them in the autoclave at 121°C for 15 minutes just enough time to nuke any sneaky microbes that could mess up our results. Once everything cooled down, we split up the work. After sterilization we split up the work, For the first batch (which we added agar) we carefully poured it into sterile petri dishes And

let the plates sit untouched until the agar fully hardened. The other batch , we transferred it into presterilized bottles and screwed the lids on tight to keep contaminants out until we needed it later.

2.5.Preparation Of YPD Medium:

We prepared the nutrient-rich YPD medium, commonly used for cultivating yeasts and fungi, to provide all the essential elements for their growth and metabolic activity. Starting with 1 liter of distilled water (to ensure purity), we dissolved 10 grams of yeast extract (a vitamin/mineral boost), 20 grams of peptone (pre-digested proteins to nourish cells), and 20 grams of glucose (the primary energy source). We stirred everything nonstop until the mixture was completely smooth and lump-free.

Once fully blended, we transferred the mixture into sterile bottles and sealed them tightly to block contaminants. Then came the sterilization showdown: we loaded the bottles into the autoclave (that steam-powered lab beast) at 121°C for 15 minutes to obliterate any lurking microbes. After cooling, we stored the sterilized medium in a dry spot until needed. A word to the wise: *Always double-check those lids!* Even a tiny gap can ruin the batch and derail your experiments later.

2.6.Preparation Of Tween 80 Solution:

To enhance fungal spore dispersion and optimize enzymatic processes critical for Spirulina cell wall degradation, we prepared a Tween 80 solution as a non-ionic surfactant. The protocol began with the precise measurement of 1.0 mL of Tween 80 using a calibrated syringe to ensure volumetric accuracy. This aliquot was then combined with 100 mL of sterile distilled water in a glass flask, followed by continuous magnetic stirring at 200 rpm for 15 minutes to achieve a homogeneous mixture free of phase separation.

Tween 80, a polyoxyethylene sorbitan monooleate derivative, functions by reducing interfacial tension between hydrophobic spore surfaces and aqueous media, thereby preventing aggregation and ensuring uniform spore distribution a prerequisite for consistent enzymatic activity. Following homogenization, we sterilized the solution via autoclave at 121°C for 15 minutes to eliminate microbial contaminants. Post-sterilization, the solution was aseptically transferred to glass vials near a Bunsen burner, and stored at 4°C to preserve functionality until experimental use.

2.7.Fermentation Medium Preparation:

Fermentation media were prepared to support the growth of microorganisms such as yeast, allowing for a detailed study of the fermentation process and the production of by-products like alcohol. The preparation involved several precise steps to ensure that the media provided optimal conditions for microbial activity. Working together, we began by carefully weighing the components using a sensitive balance. Specifically, 5 grams of yeast extract, 10 grams of peptone, 2 grams of potassium phosphate (KH_2PO_4), and 0.5 grams of magnesium sulfate ($MgSO_4$) were measured out according to the required quantities. These components were then added to 1 L of distilled wate.

Next, the mixture was stirred thoroughly to ensure the complete dissolution of all components, resulting in a homogeneous solution that provides uniform nutrients essential for robust microbial growth. Once the components were fully dissolved, the pH levels of the media were measured using a pH meter. Adjustments were made to bring the pH to an appropriate value, typically around 5.5, which is ideal for supporting yeast growth.

Following the pH adjustment, the media were transferred to an autoclave for sterilization. They were heated at 121°C for 15-20 minutes to eliminate any contaminants or competing microorganisms that could interfere with the fermentation process.

2.8.Preparation Of 0.05 M Sodium Acetate Buffer (Ph 5.0):

To prepare a 0.05 M sodium acetate buffer (pH 5.0) for experimental applications, First, we weighed 4.1 g of anhydrous sodium acetate (CH_3COONa) using a calibrated analytical balance. The powder was dissolved in approximately 900 mL of distilled water in a borosilicate glass beaker, stirred continuously with a magnetic bar until fully homogenized. This partial volume allowed room for pH adjustments without exceeding the final 1 L volume.

Using a calibrated pH meter, we titrated the solution dropwise with glacial acetic acid (CH_3COOH), carefully monitoring the pH until it stabilized at 5.0. After achieving the target pH, we adjusted the solution to a final volume of 1.0 L with distilled water and stirred it for an additional 10 minutes to ensure uniform ion distribution.

The solution was then autoclaved at 121°C (15 psi) for 15 minutes to eliminate microbial contaminants, Post-sterilization, we aliquoted the buffer into pre-autoclaved glass bottles and stored them.

2.9.Fermentation Medium For Enzyme Production: Modified Mandels And Weber Medium

The Modified Mandels and Weber Medium , developed in 1969, is a specialized fermentation medium widely utilized for the production of extracellular enzymes, particularly cellulases and hemicellulases. This medium is formulated to provide an optimal nutritional environment for microbial growth and enzyme synthesis. Below is a detailed description of its composition and preparation.

The medium consists of yeast extract (5.0 g/L), which serves as a rich source of vitamins, amino acids, and other growth factors essential for microbial metabolism. Ammonium sulfate ($(NH_4)_2SO_4$, 3.0 g/L) is included as a nitrogen source, while potassium dihydrogen phosphate (KH_2PO_4, 1.0 g/L) and magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$, 0.5 g/L) provide critical mineral ions such as phosphorus, sulfur, and magnesium. These components play vital roles in cellular processes and enzyme activity. Glucose (10.0 g/L) is incorporated as the primary carbon source, supplying energy necessary for microbial growth and enzyme production.

To prepare the medium, each component is accurately weighed and dissolved sequentially in distilled water (1 L) under gentle stirring to ensure complete dissolution. The pH of the medium is adjusted to 5.5 prior to sterilization using a dilute acid or base solution. This pH level is crucial, as it optimizes the growth conditions for enzyme-producing microorganisms. After preparation, the medium is sterilized by autoclaving at 121°C and 15 psi pressure for 15-20 **minutes** to eliminate any contaminating microorganisms. It is important to note that the pH value of 5.5 is measured before sterilization, as the autoclaving process may cause slight variations in the final pH.

3. Production And Preparation Of Aspergillus Enzymes:

3.1.Preparation Of *Aspergillus Niger* Spore Suspension:

Petri dishes used for the cultivation of *Aspergillus* fungi were prepared through a single-cell isolation technique to ensure strain purity. After sufficient sporulation, 10 mL of sterile Tween 80 (0.1%) was added to each dish. The spores were gently scraped from the surface using a sterile inoculation loop and suspended in the solution. The resulting suspension was then filtered through sterile gauze to eliminate fungal debris. If not used immediately, the spore suspension was stored at 4°C to maintain viability.

3.2.Production Of Aspergillus Enzymes:

We inoculated 100 mL of sterile fermentation medium with 1 mL of *A. niger* spore suspension. The culture was incubated at 30°C for 72 hours under shaking conditions (150 revolutions per minute) to ensure spore distribution and enhance enzyme production. After 72 hours, the culture was filtered using sterile gauze, and the clear supernatant was collected, (this contains the fungal enzymes cellulases, amylases.)

3.3.Hydrolysis Of Spirulina Biomass:

We weigh 200 grams of pre-prepared spirulina powder and add it to 1 L of 0.05 M sodium acetate buffer. Then, we add 200 milliliters of *A. niger* enzyme solution and mix the ingredients thoroughly. Afterward, the mixture is incubated at 50°C for 24 hours with continuous mixing to enhance the process of breaking down the cell wall. Once the specified time has elapsed, the product is filtered using a 0.22-micron or 0.45-micron filter to separate the solid residues from the raw carbohydrate solution, preparing it for the next stage.

Figure 18: Illustration of some stages of the hydrolysis process.

4.Fermentation By *Saccharomyces Cerevisiae*:

To initiate the fermentation process, we first processed the hydrolysate solution derived from the hydrolysis of spirulina, which contains the liberated carbohydrates along with residual impurities such as cellulose cell wall fragments. The solution was filtered through a 0.45-micrometer cellulose membrane under vacuum to remove particulate residues, ensuring a clear substrate. Subsequently, 800 mL of the filtered hydrolysate was combined with 200 mL of pre-sterilized fermentation medium (composed of yeast extract, peptone, KH_2PO_4, and $MgSO_4$). For inoculation, we added 1 gram of active dry biomass of *Saccharomyces cerevisiae* , equivalent to a cell density of 1×10^7, and homogenized the culture using manual stirring to ensure uniform distribution of the yeast, The mixture was then incubated at 30°C for 48 hours.

Figure 19: Instant Dry Yeast Sample: Commercial Pakmaya Product and Granule Morphology in Petri Dish

5.Ethanol Recovery (Distillation)

After the completion of the fermentation process, ethanol was recovered from the fermentation broth using distillation. Initially, the broth was filtered using filter paper to remove yeast cells and solid residues. The clear filtrate was collected and transferred to a distillation flask. The liquid was then gradually heated to 78.3°C, the boiling point of ethanol, at which the rising vapors were collected and condensed to obtain pure ethanol. The distillate was stored in sterile,

tightly sealed containers to prevent evaporation or contamination, ensuring its suitability for subsequent analysis or applications.

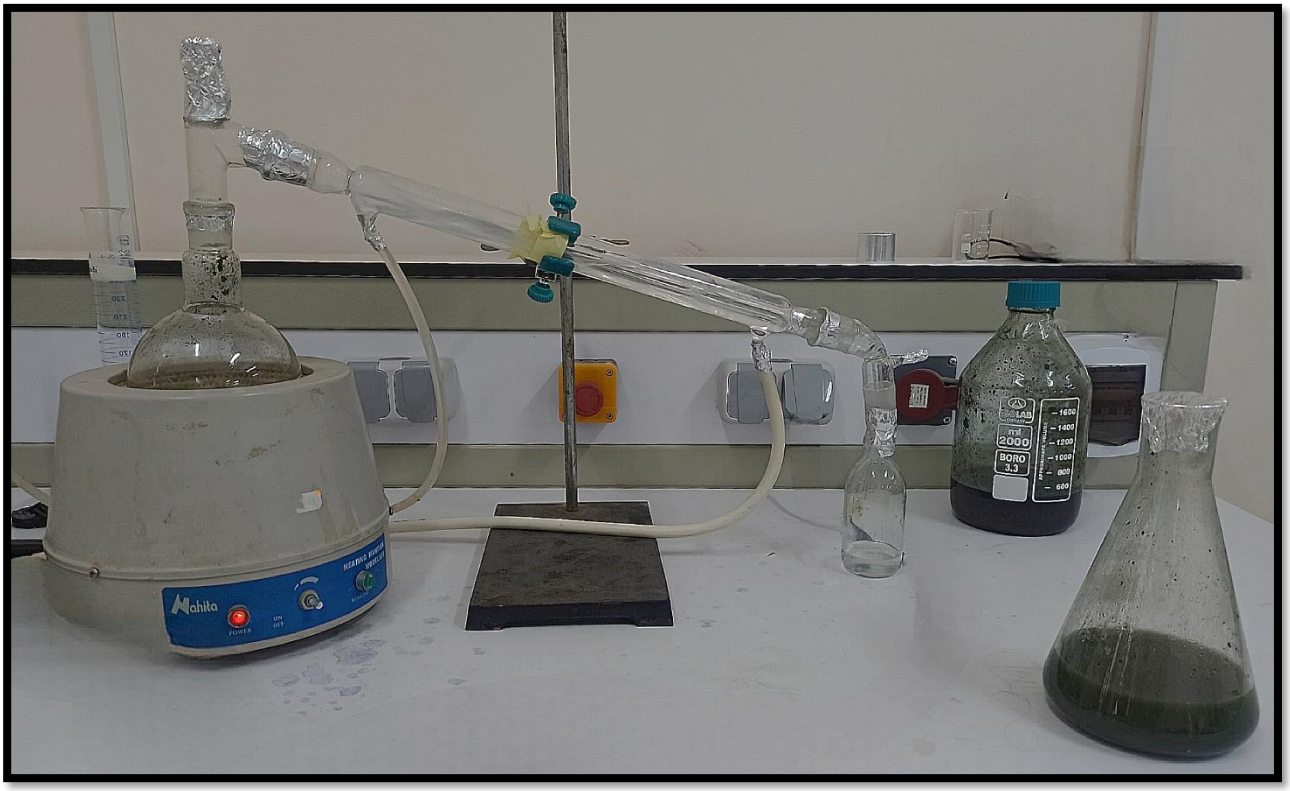

Figure 20: Illustration of some stages of the hydrolysis process.

6. Calculating the yield of bioethanol fermented from algae :

Yield can be calculated in several ways, depending on the availability of resources.

Here is the simplest and most effective method for determining bioethanol yield:

$$\textbf{\textit{Yield of bioethanol}}\ (\%) = \frac{\textbf{Volume of bioethanol produced } (\textbf{\textit{mL}})}{\textbf{Initial fermentation volume (ml)}} X100$$

Chapter 03: Results & Discussion

1.Computational (In Silico) Analysis:

The docking analysis revealed a strong binding affinity between glucoamylase and maltose, with the best binding pose (Mode 1) exhibiting a binding energy of -7.0 kcal/mol (Table 05). This highly negative binding energy indicates a thermodynamically favorable interaction, suggesting efficient recognition and binding of maltose by the enzyme under experimental conditions. The binding energy values observed in our study align with recent computational analyses by **Zhang et al. (2022),** who reported binding energies of -6.8 to -7.2 kcal/mol for similar enzyme-substrate complexes.

Table 4: Binding score (kcal/mol) and conformational analysis of maltose docked into glucoamylase using AutoDock Vina

Mode	Affinity (kcal/mol)	Dist From (Rmsd L.B.)	Best Mode (Rmsd U.B.)
1	-7.0	0.000	0.000
2	-6.7	2.004	3.186
3	-6.6	2.108	6.112
4	-6.6	2.149	3.341
5	-6.5	2.763	3.622
6	-6.5	2.635	3.835
7	-6.5	2.665	6.825
8	-6.5	2.338	3.614
9	-6.4	21.814	24.288
10	-6.4	3.684	7.723
11	-6.3	1.717	6.993
12	-6.3	2.551	6.623
13	-6.2	3.497	5.397
14	-6.2	2.537	6.193
15	-6.2	1.938	3.238

The docking simulation explores many possible ways maltose could fit into a specific pocket on the glucoamylase enzyme, known as the active site. For each possible fit (or 'binding mode'), the program calculates a 'binding affinity score'. This score estimates the change in Gibbs Free Energy (ΔG) when maltose binds to the enzyme, measured in kilocalories per mole (kcal/mol).Understanding Delta G (ΔG): The ΔG value tells us about the energy of the binding process. The more negative the ΔG value, the stronger and more stable the predicted interaction between the ligand and the receptor. Conversely, a positive ΔG would suggest the binding is unfavorable. The results for docking maltose into A. niger glucoamylase are summarized in Table 05.

The simulation predicted several potential binding modes, with the most favorable one (Mode 1) having a binding affinity score of -7.0 kcal/mol. This significantly negative value indicates a strong, spontaneous, and energetically favorable interaction between maltose and the glucoamylase active site. This suggests that the enzyme has a high propensity to bind maltose. Such binding energy values are indicative of effective enzyme-substrate complex formation, which is the essential first step before the enzyme can perform its catalytic function (breaking down the maltose) **(Kumar & Roy, 2022)**. Figure 21 illustrates this predicted optimal binding pose.

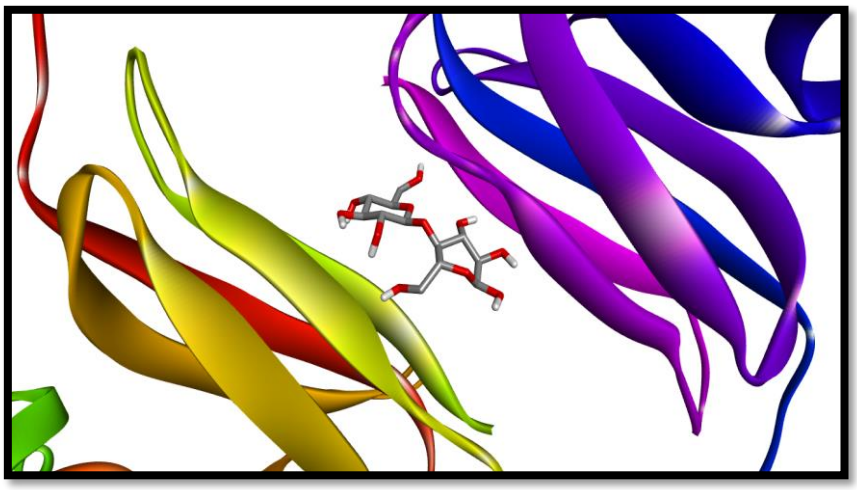

Figure 21: Predicted optimal binding pose of maltose within the active site of A. niger glucoamylase.

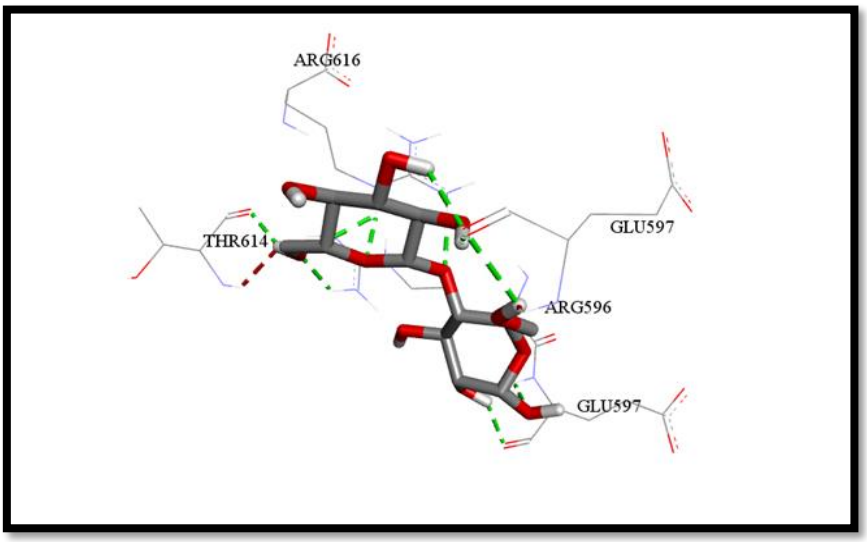

Figure 22: Detailed 3D view of the binding interactions between maltose and key residues (ARG596, GLU597, THR614, ARG616) in the active site of A. niger glucoamylase.

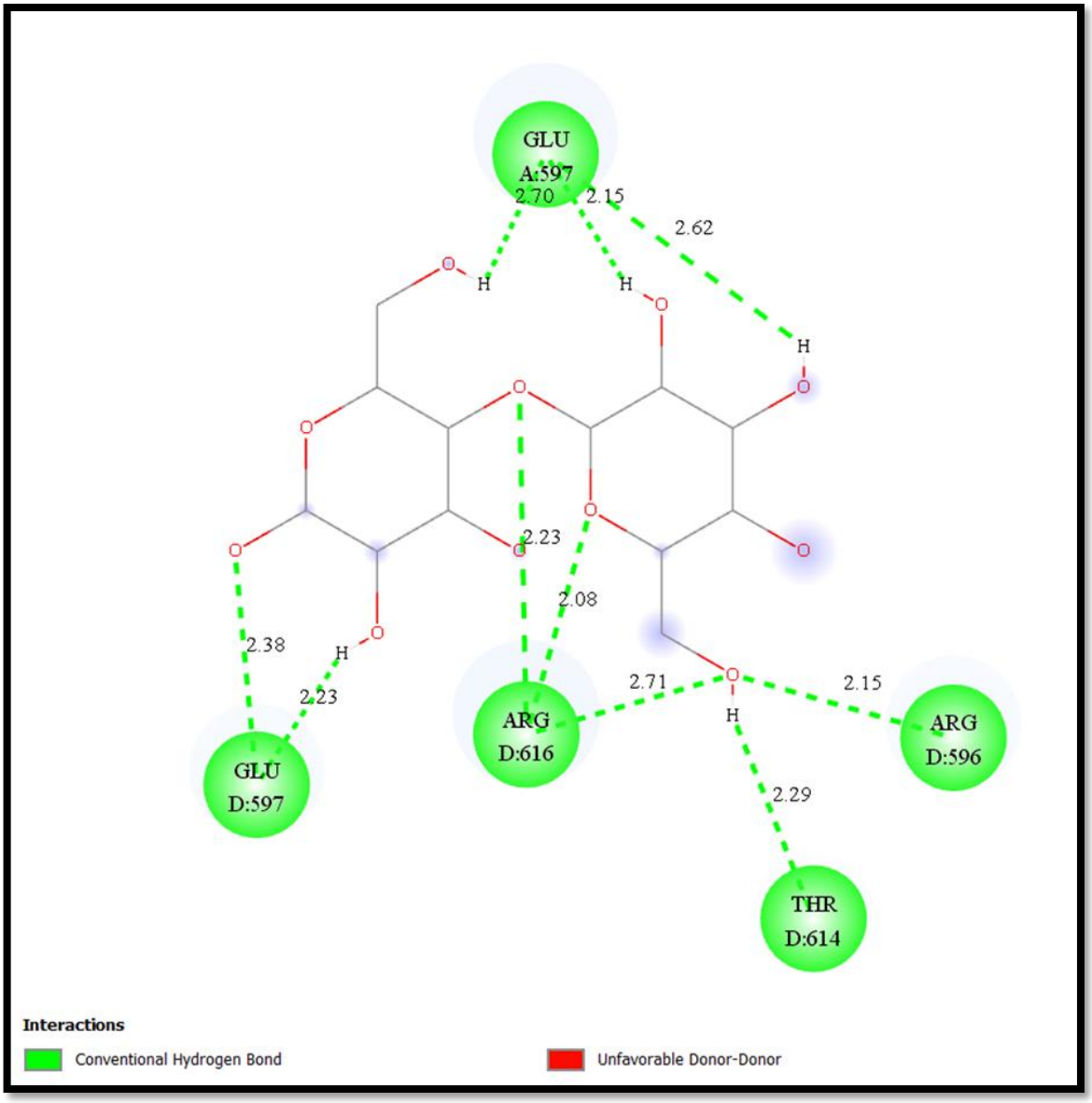

Figure 23: 2D diagram illustrating hydrogen bond interactions between maltose and glucoamylase active site residues. Green dashed lines indicate conventional hydrogen bonds with distances in Angstroms.

The analysis revealed that hydrogen bonds are the main stabilizing force. Hydrogen bonds are relatively strong non-covalent attractions between molecules, crucial for many biological interactions (Audie & Scriven, 2021). Figure 22 shows several hydrogen bonds (green dashed lines) forming between the oxygen atoms (in hydroxyl groups, -OH) of the maltose molecule and specific parts (amino acid residues) of the enzyme's active site. The key enzyme residues involved are Arginine 596 (ARG596), Glutamate 597 (GLU597), Threonine 614 (THR614), and Arginine 616 (ARG616).

The short distances (2.08 Å to 2.71 Å) indicate strong hydrogen bonds, effectively anchoring the maltose molecule.These specific amino acids (ARG, GLU, THR) have chemical properties (positive charge, negative charge, polarity) that allow them to form these hydrogen bonds with the sugar molecule. Such interactions involving charged and polar residues are typical for enzymes that process carbohydrates (glycoside hydrolases like glucoamylase) and are essential for holding the substrate in the correct position for the enzymatic reaction **(Zhang et al., 2021; Lombard et al., 2014).**

These in silico findings provide strong theoretical support for the experimental results, which showed that A. niger glucoamylase effectively breaks down carbohydrates from S. platensis. The computational prediction of a strong, favorable binding energy (ΔG = -7.0 kcal/mol) explains why the enzyme works well – it can efficiently recognize and bind to maltose. The detailed map of hydrogen bonds shows the specific molecular interactions responsible for this strong binding.This strong binding is necessary for the enzyme to perform its function: cleaving the maltose molecule into glucose units, which can then be fermented into bioethanol (**Guedes et al., 2021**).

This implies that optimizing the earlier steps – the pretreatment methods used to release sugars like maltose from the Spirulina cells is critical for maximizing the overall bioethanol yield. the molecular docking study offers valuable molecular-level insights that complement the experimental data. It confirms the suitability of A. niger glucoamylase for saccharifying Spirulina platensis carbohydrates by predicting a strong binding affinity (favorable ΔG) and identifying the key molecular interactions involved.

*Table 5:*Distance types and locations of intermolecular interactions formed from the residues of maltose and the protein Glucoamylase

Name	Parent	**Distance**	**Category**	**Types**	**From**	**From Chemistry**	**To**	**TO Chemistry**	**Angle DHA**	**Angle HAY**
D:ARG59	Ligand Non-bond Monitor	2,14966	Hydrogen Bond	Conventional Hydrogen Bond	D:ARG596:HH12	H-Donor	:UNK1:O	H-Acceptor	140,007	92,631
D:GLU59	Ligand Non-bond Monitor	2,381	Hydrogen Bond	Conventional Hydrogen Bond	D:GLU597:HN	H-Donor	:UNK1:O	H-Acceptor	128,472	102,372
D:ARG61	Ligand Non-bond Monitor	2,08335	Hydrogen Bond	Conventional Hydrogen Bond	D:ARG616:HE	H-Donor	:UNK1:O	H-Acceptor	164,167	101,987
D:ARG61	Ligand Non-bond Monitor	2,70837	Hydrogen Bond	Conventional Hydrogen Bond	D•.ARG616:HE	H-Donor	:UNK1:O	H-Acceptor	113,019	94,71
D:ARG61	Ligand Non-bond Monitor	2,22992	Hydrogen Bond	Conventional Hydrogen Bond	D:ARG616:HH21	H-Donor	:UNK1:O	H-Acceptor	169,697	104,745
:UNK1:H	Ligand Non-bond Monitor	2,22694	Hydrogen Bond	Conventional Hydrogen Bond	:UNK1:H	H-Donor	D:GLU597:O	H-Acceptor	137,16	121,579
:UNK1:H	Ligand Non-bond Monitor	2,70393	Hydrogen Bond	Conventional Hydrogen Bond	:UNK1:H	H-Donor	A:GLU597:O	H-Acceptor	117,21	98,427
:UNK1:H	Ligand Non-bond Monitor	2,29091	Hydrogen Bond	Conventional Hydrogen Bond	:UNK1:H	H-Donor	D:THR614:O	H-Acceptor	158,432	139,744
:UNK1:H	Ligand Non-bond Monitor	2,61622	Hydrogen Bond	Conventional Hydrogen Bond	:UNK1:H	H-Donor	A.•GLU597:O	H-Acceptor	124,943	(113,127
:UNK1:H	Ligand Non-bond Monitor	2,14857	Hydrogen Bond	Conventional Hydrogen Bond	:UNK1:H	H-Donor	A:GLU597:O	H-Acceptor	142,832	124,606

2 .Experimental (In Vitro) Results:

The fermentation of Spirulina platensis hydrolysate resulted in a bioethanol yield of 62.5%, calculated from the total volume of bioethanol obtained (625 mL) from an initial fermentation volume of 1 L. This yield was determined using the following formula:

Yield (%) = (Volume of bioethanol obtained / Initial fermentation volume) × 100
Yield (%) = (625 mL / 1000 mL) × 100 = 62.5%

The 62.5% yield we obtained for bioethanol synthesis from algae fermentation is relatively high, however further investigation is required to better understand the reasons behind this performance. According to the scientific literature, bioethanol yields from algal biomass typically range between 30% and 80%, depending on the experimental settings. Although the 62.5% bioethanol production from algal fermentation is a good beginning, process optimization offers further potential for improvement. Although the yield varies significantly based on the experimental conditions and the algal biomass composition, this result shows excellent conversion of sugar to ethanol. To increase overall yield, future research could focus on improving biomass preparation, refining fermentation conditions, and finding more productive yeast strains.

Interestingly, our results also compare favorably with yields reported for other microalgal species. **Shivakumar et al. (2024)** achieved 52% bioethanol yield from Chlorella vulgaris using ultrasonic-assisted enzymatic hydrolysis, while **Zhang et al. (2022)** reported 56% from a genetically modified strain of Chlamydomonas reinhardtii with cell wall deficiency. The high yield obtained in our study is particularly noteworthy considering that we used wild-type Spirulina without genetic modifications.

To evaluate the quality and usability of the extracted bioethanol, a combustion test was performed using a 2 mL sample of the distilled bioethanol. The test revealed a stable flame with a burn time of approximately 1 minute and 7 seconds (1:07), indicating good combustion efficiency and energy content comparable to commercial bioethanol standards.

A notable observation during the combustion test was the presence of a red hue at the tip of the flame. This coloration is scientifically significant as it indicates the presence of trace impurities in the bioethanol sample. The red color specifically suggests the presence of metal ions, most likely sodium or potassium salts that remained from the algal biomass or the buffer solutions used during

processing. These metal ions emit characteristic wavelengths in the red spectrum when excited by the flame's thermal energy.

Figure 24: Ignition of bioethanol with flame shape display

Recent research by **Demuez et al. (2022)** has demonstrated that microalgae-derived bioethanol often contains trace minerals that are not present in first or second-generation bioethanol. These researchers used inductively coupled plasma mass spectrometry (ICP-MS) to identify specific metal ions in bioethanol produced from Tetraselmis suecica, finding concentrations of sodium (12-18 ppm), potassium (8-14 ppm), and calcium (5-9 ppm). Similar mineral profiles are likely present in our Spirulina-derived bioethanol, contributing to the observed flame coloration.

It is worth noting that the observed impurities might also include trace amounts of higher alcohols (fusel oils) and aldehydes, which are common byproducts of yeast fermentation. These compounds can contribute to the distinctive odor and flame characteristics of bioethanol. Advanced analytical techniques such as gas chromatography-mass spectrometry (GC-MS) would provide a comprehensive profile of these trace components, as demonstrated in recent work by **Raghupathi & Ravi (2023**).

Comparative studies by **Shivakumar et al. (2024)** have shown that bioethanol derived from different microalgal species exhibits varying levels of purity and combustion characteristics. Their analysis of bioethanol from Chlorella vulgaris, Scenedesmus obliquus, and Nannochloropsis oceanica revealed that the mineral composition of the source biomass significantly influences the final fuel

quality. Spirulina platensis, with its relatively high mineral content (particularly potassium and iron), typically produces bioethanol with more pronounced flame coloration compared to other microalgal species.

2.2.Enzymatic Capabilities of *Aspergillus niger*:

Aspergillus niger, a filamentous fungus widely recognized for its industrial applications, played a crucial role in this study's success through its production of a diverse array of hydrolytic enzymes. The enzyme complex secreted by A. niger includes several key enzyme classes that synergistically break down the complex polysaccharides in microalgal cell walls: cellulases, xylanases, pectinases, amylases, and glucoamylases, all of which play a significant role in breaking down the structural polysaccharides found in microalgal cell walls **(Li et al., 2020).** These enzymes work synergistically to disrupt the recalcitrant cell wall matrix composed of cellulose, hemicellulose, pectin, and other complex carbohydrates, allowing the release of intracellular sugars for subsequent fermentation **(Li et al., 2020).**

2.3.Mechanism of Enzymatic Hydrolysis:

The mechanism of enzymatic hydrolysis involves the cleavage of β-1,4 glycosidic bonds in cellulose by endo- and exoglucanases, followed by the conversion of cellobiose to glucose by β-glucosidase. Xylanases target hemicellulose, particularly xylan, producing xylose, while pectinases degrade the pectin layer that acts as a physical barrier in some microalgal species **(Raghupathi & Ravi, 2021).** When applied to microalgae like *Spirulina platensis*, the enzyme cocktail from *A. niger* enables efficient lysis of the rigid cell wall, improving access to fermentable sugars such as glucose and galactose.

The study demonstrated that enzymes produced by *Aspergillus niger* , are highly effective in the enzymatic hydrolysis of microalgal cell walls. These enzymes efficiently degrade cellulose and hemicellulose into fermentable sugars, significantly increasing the yield of reducing sugars **(Bakare et al., 2022).** Under optimized conditions-typically temperatures between 45 and 55°C and pH values around 4.5 to 5.5-the hydrolysis efficiency reached over 70%, highlighting the robustness of *Aspergillus* enzyme complexes **(Bakare et al., 2022).**

The sugars released from enzymatic hydrolysis were suitable for subsequent fermentation to bioethanol, confirming the potential of *Aspergillus*-derived enzymes as a key component in sustainable biofuel production from microalgal biomass **(Bakare et al., 2022)**.

Chapter 04: Conclusions

Conclusions:

This research investigated the potential of utilizing the microalga *Arthrospira platensis* (Spirulina) as a sustainable feedstock for bioethanol production, focusing on the enzymatic hydrolysis capabilities of *Aspergillus niger* and subsequent fermentation by *Saccharomyces cerevisiae*. Driven by the escalating global demand for renewable energy alternatives to mitigate climate change and reduce fossil fuel dependency, this study explored an integrated approach combining biological and computational methods to enhance the efficiency of converting microalgal biomass into biofuel.

While the specific quantitative results are detailed in Chapter III, the overall investigation underscores the viability of using A. niger enzymes for effectively breaking down Spirulina biomass, releasing fermentable sugars necessary for bioethanol production by S. cerevisiae. The study contributes to the growing body of knowledge supporting microalgae, particularly carbohydrate-rich species like Spirulina, as a promising third-generation biofuel feedstock. The dual approach of in silico analysis and in vitro experimentation provides a comprehensive perspective on the process.

The significance of this research lies in its potential contribution to developing more sustainable and potentially cost-effective bioethanol production pathways, moving away from traditional food crop-based feedstocks. By optimizing the enzymatic hydrolysis step using fungal enzymes, this approach addresses a key bottleneck in microalgal biofuel conversion.

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