MICROALGAL-BASED TECHNOLOGY FOR SPENT LUBRICANT OIL WASTE BIODEGRADATION

By

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PREFACE

The data described in this thesis were collected in Pietermaritzburg, Republic of South Africa from February 2019 to April 2023. Experimental work was carried out while registered at the Department of Microbiology, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, under the supervision of Prof Ademola Olaniran and Prof Gueguim kana

This thesis, submitted for the degree of Doctor of Philosophy in the College of Agriculture, Engineering, and Science, University of KwaZulu-Natal, School of Life Sciences, Pietermaritzburg campus, represents original work by the author and has not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others, it is duly acknowledged in the text.

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Date: July 2024

DECLARATION 1: PLAGIARISM

I, **Stella B. Eregie**, declare that:

The research reported in this thesis, except where otherwise indicated, is my original work;

This thesis has not been submitted for any degree or examination at any other university;

This thesis does not contain other persons’ data, pictures, graphs, or other information unless specifically acknowledged as being sourced from other persons;

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Where their exact words have been used, then their writing has been placed in italics and inside quotation marks and referenced;

This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the reference’s sections;

This dissertation is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

This work is based on the research supported by the National Research Foundation of South Africa and the opinions, findings and conclusions or recommendations expressed in any publication generated by the NRF supported research is that of the author(s), and that the NRF accepts no liability whatsoever in this regard.

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Stella B. Eregie

Date: 5 July 2024

DECLARATION 2: PUBLICATIONS

This thesis involves a compilation of manuscripts. Each chapter is an individual entity prepared as per the journals' specifications hence some repetition between chapters has been inevitable. The first author (student) conducted all experimental work, data collection and manuscript preparation, guided by the co-authors (mentors and supervisors).

Chapter 3

Stella B. Eregie, Isaac A. Sanusi, Gueguim E.B. Kana, Olaniran O. Ademola (2023). Effect of ultra-violet light radiation on *Scenedesmus vacuolatus* growth process kinetics, metabolic performance and preliminary biodegradation study. *Biodegradation*. 13:1-6. https://doi.org/10.1007/s10532-023-10029-2.

Chapter 4

Eregie SB, Jamal-Ally SF (2021). Comparison of biodegradative efficiency of wild-type versus mutagenised *Scenedesmus vacuolatus* of spent coolant waste: dehydrogenase activity and total petroleum degradation studies. *International Journal of Environmental Analytical Chemistry*. 25:1-27. https://doi.org/10.1080/03067319.2021.1965593.

Chapter 5

Stella B. Eregie, Isaac A. Sanusi, Gueguim E.B. Kana, Olaniran O. Ademola (2023). Synergistic effect of process parameters and nanoparticles on spent lubricant oil waste biodegradation by UV-exposed *Scenedesmus vacuolatus*: Process modelling, kinetics and degradation pathways. *Bioresource Technology Reports*. 1:24:101627. https://doi.org/10.1016/j.biteb.2023.101627.

Chapter 6

Stella B. Eregie, Isaac A. Sanusi, Ajit Kumar, Gueguim E.B. Kana and Olaniran O. Ademola (2024). Transcriptomic removal and mass balance of polycyclic aromatic hydrocarbons in waste spent coolant oil: Gene discovery, enzyme identification and metabolic pathway. *Bioresource Technology Reports*. (In-press).

CONFERENCE PRESENTATION

Stella B. Eregie and Sumaiya F. Jamal-Ally. Tracking of the extracellular biotransformation of UV treated *Scenedesmus vacuolatus* microalgae for biodegradation of spent coolant wastes. Postgraduate Research and Innovation Symposium (PRIS). Oral presentation held at University of KwaZulu-Natal, Pietermaritzburg, South Africa, 9 - 10 December 2020.

Stella B. Eregie and Sumaiya F. Jamal-Ally. Comparison of biodegradative efficiency of wild-type versus mutagenized *Scenedesmus vacuolatus* of spent coolant waste: dehydrogenase activity and total petroleum degradation studies. Postgraduate Research and Innovation Symposium (PRIS). Poster presentation held at University of KwaZulu-Natal, Pietermaritzburg, South Africa, 9 - 10 December 2021.

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Stella B. Eregie, Isaac A. Sanusi, Gueguim E.B. Kana, Olaniran O. Ademola. Ex-situ degradation strategy of petroleum hydrocarbon by UV-exposed *Scenedesmus vacuolatus*: A case study of Msunduzi River, Pietermaritzburg lubricant oil spillage. Postgraduate Research and Innovation Symposium (PRIS). Poster presentation held at University of KwaZulu-Natal, Pietermaritzburg, South Africa, 9 - 10 December 2023.

Stella B. Eregie, Isaac A. Sanusi, Gueguim E.B. Kana, Olaniran O. Ademola. Synergistic effect of process parameters and nanoparticles on spent lubricant oil waste biodegradation by UV-exposed *Scenedesmus vacuolatus*: Process modelling, kinetics and degradation pathways. World Climate Research Programme (WCRP) Open Science Conference, Poster presentation held in Kigali, Rwanda, 23-27 October 2023.

DEDICATION

I dedicate this thesis to my husband Mr Kenneth Eregie and children (Osarumen, Osayamen and Osamagbe) for their immeasurable love, unwavering support, and constant motivation throughout my study period.

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LIST OF ABBREVIATIONS

SOW – Spent oil waste

SCW – Spent coolant waste

HCs – Hydrocarbon compounds

Carbon dioxide – CO_2

Ultraviolet – UV

TPF – Triphenyl formazan

TPH – Total petroleum hydrocarbon

Chlorophyll – Chl

RSM – Response Surface methodology

ANN – Artificial neural network

R^2 – Coefficient of determination

DHA – Dehydrogenase activity

NPs – Nanoparticles

PAHs – Polycyclic aromatic hydrocarbon

ALE – Adaptive laboratory evolution

NTG – N-nitro-N-nitrosoguanidine

EMS – Ethylmethane sulfonate

MNG – Methylnitrosoguanidine

GMM – Genetically modified microorganism

DNA – Deoxyribonucleic acid

RNA – Ribonucleic acid

Seq – Sequence

BTEX – Benzene, Toluene, ethylbenzene

PCB – Polychlorinated biphenyls

Ovat – One variable at a time

RSM – Response surface methodology

ANN – Artificial neural network
NGS – Next generation sequence
qRT – PCR Quantitative real-time polymerase chain reaction
NADH – Nicotinamide adenine dinucleotide
V/V – Volume per volume
Min – Minutes
NM – Nanometer
ML – Milliliter
BG11 – Blue green 11
FAD – Flavin adenine dinucleotide flavin (FMN),
FMN – Flavin mononucleotide
NADPH – Nicotinamide adenine dinucleotide phosphate
BP – Biological process
MF – Molecular function
CC – Cellular component
DEGs – Differentially expressed genes

ABSTRACT

The consumption of petroleum-based products has led to several world's most devastating oil spillage, with more than 4 million tons of oil-based products spilt into the environment. This has resulted in the pollution of up to 320 km of the coastline and resulted in severe damages, killing thousands of marine organisms. A single clean-up effort could cost over 10 billion dollars which makes some oil spill incident an environmental catastrophe. The widespread use and improper disposal of petroleum-based products such as lubricant oil has increased the likelihood of unintended spills and, subsequent long-lasting, economic, environmental and health impacts. Hence, there is a need to develop a cost-effective, sustainable, and environmentally friendly clean-up approach to replace the limited conventional treatment methods. To this end, microalgae-based treatment has gained global attention as a promising alternative to these conventional techniques due to its viability, cost effectiveness, versatile metabolic capacity, sustainability, and eco-friendliness. Despite the excellent degradative capability of microalgae, their application in recalcitrant spent oil waste (SOW) pollutants treatment is scantily reported in literature. The SOW contains recalcitrant organic pollutants, and the present degradation efforts for their removal are limited, necessitating novel removal strategies. To this end, the use of ultraviolet (UV) light radiation to enhance the microalgae *Scenedesmus vacuolatus* metabolic performance and degradative abilities was evaluated in this study. In addition, the morphological, metabolic, enzymatic, and transcriptomic profile of the exposed *S. vacuolatus* were elucidated. Also, the key process parameters on SOW degradation were modelled and optimized.

Firstly, the effects of UV radiation (wavelength 254 nm and intensity 1.4 mW/cm^2) on *S. vacuolatus* at different time intervals (2, 4, 6, 12, 24 and 48 h) were established. The 24 h UV-exposure induced the most desirable degradative traits in *S. vacuolatus* and was selected for further studies. These desirable traits include improved chlorophyll (chl a = 1.89–fold, chl b = 2.02–fold), carotenoid (1.24–fold), carbohydrates (4.62–fold), proteins (1.44–fold) and lipid accumulations (1.40–fold). Additionally, the 24 h UV-exposed *S. vacuolatus* exhibited an enhanced substrate affinity (1/Ks) (0.959) and specific growth rate (μ) (0.024 h^{-1}) (1.50-fold, 2-fold, and 1.9-fold respectively). Preliminary assessment of spent coolant waste (SCW) biodegradation using the UV-exposed *S. vacuolatus* resulted in significant dehydrogenase activity (55%) and total petroleum hydrocarbon (TPH) degradation efficiency (100% after 5 weeks). Moreover, response surface methodology was used to investigate the effects of temperature (25 – 40°C), substrate concentration (5 – 25% v/v) and inoculum concentration (5 – 30% v/v) on TPH degradation of SCW. The optimized process showed 100% TPH degradation of SCW within 15 days of treatment lower than the period before optimization. Additionally, the sensitivity of the process parameters on the degradation process assessed using Artificial Neural Network (ANN) revealed high sensitivity of the degradation process to operational temperature. To further enhance the degradation process, nanomaterials with catalytic potentials was incorporated during the degradation process. This resulted in increased biomass concentration (2.48-fold), specific growth rate (1.62 times), growth constant (1.23 times), 1/Ks (14.29), and consequently, achieving 100% TPH degradation in 12 days. In addition, the biodegradation kinetics showed high biodegradation rate constant (K) and shorter half-life ($T_{1/2}$) of 6.65 m1 t^{-1} and 0.08 days, respectively, especially in the degradation of monoaromatics, and PAHs which are important environmental pollutants. The degradation kinetics was best elucidated by the second-order reaction, suggesting SCW concentration is inversely proportional to the degradation half-life ($T_{1/2}$).

Using the obtained metabolic by-products via GC-MS analysis, enzymatic and transcriptomic assessment, a total of 116 metabolic pathways were strongly affirmed in UV_Sv+SCW. Of the 116 metabolic pathways linked to SCW HCs degradation, the UV-exposed *Scenedesmus* was observed to use the naphthalene degradation pathway for the oxidation of the PAHs fraction of SCW. Transcripts of biotechnological importance implicated in SCW degradation include six CoA-linked acetaldehyde dehydrogenase, three coatomer subunit alpha-3 and one arginine deiminase. Of these transcripts, the key transcript CoA-linked acetaldehyde dehydrogenase (gene name COO60DRAFT_1702088) encoding alcohol dehydrogenase enzyme was specifically used by UV-exposed microalgae to breakdown the aromatic compounds. This study further underscores the suitability of UV radiation as a physical agent to improve the degradative capability of *S. vacuolatus* through genetic modification and photosynthetic biomolecular apparatus enhancement. Moreover, the positive effects of metallic oxide nanoparticles on *S. vacuolatus* metabolism for an improved hydrocarbon waste degradation resulting from significant improvement in *S. vacuolatus* substrate affinity, growth rate, process kinetics and efficiency were elucidated. Hence, this study demonstrates a highly effective method for the degradation of TPH in SCW through the application of UV-exposed *S. vacuolatus*. The significant enhancement in SCW hydrocarbon biodegradation achieved in this study introduces an innovative approach for the treatment of recalcitrant hydrocarbons, with potential applicability to various environmental pollution contexts. Additionally, the microalgae-based treatment developed herein offers a cost-effective alternative for the degradation of environmental contaminants. The insights gained from this study will contribute to the advancement of environmentally friendly and efficient remediation strategies for recalcitrant organic pollutants, fostering the development of sustainable and green environmental practices.

CHAPTER 1

1. General introduction

Spent oil waste (SOW), or used lubricant oil waste, is a petroleum-based lubricant oil generated from industrial processes, automotive engines, machinery, workshops, and mechanical processes (Pongsilp and Nimnoi, 2022; Soumeya et al., 2022). Currently, the generation of spent oil waste (SOW) has attracted global interest, particularly in nations with growing industrial activities and populations (Pongsilp and Nimnoi, 2022). In 2020, there was a notable rise of 0.9 million barrels per day in the production of spent oil waste, while the demand for lubricant oil reached unprecedented levels, hitting 100 million barrels per day (Pongsilp and Nimnoi, 2022). As a result of the increased industrial activities, the environment is being polluted with SOW from various sources. Of the well-known pollutants generated by industrial processes, SOW is considered the most harmful pollutants produced from industrial activities (Obi et al., 2022; Soumeya et al., 2022). Environmentalists are concerned about the productions of SOW since they are dumped into sewage pipes, water sources, and drainage systems without being treated first, causing devastating environmental pollution. In addition, SOW contamination has become a worldwide issue that has drawn the interest of many governments, organisations, and researchers (Pongsilp and Nimnoi, 2022; Soumeya et al., 2022).

The SOW contains a broad range of hydrocarbons (HCs), carbon-based compounds, and other harmful chemicals, including aromatics such as monoaromatic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs) (Medić et al., 2020). The monoaromatic and PAHs are of major concern and a threat to humans as well as the environment. Moreover, the untreated SOW pollutants can easily leach into water and soils. SOW in water produces an oily film, which limit the inflow of oxygen thereby endangering aquatic species (Sattar et al., 2022). On the other hand, when SOW leaches into the soil, plant roots have difficulty in absorbing and respiring oxygen, leading to death (Obi et al., 2022; Sattar et al., 2022). Additionally, ingesting the undegraded SOW endangers human and animal health causing irritation, neurodegenerative diseases, bone marrow damage and the risk of various type of cancer (Obi et al., 2022; Sattar et al., 2022).

Various physiochemical treatment techniques such as landfilling, incineration, solvent extraction, chemical decomposition and composting have been employed to reclaim SOW polluted site (Sutherland and Ralph, 2019). The physiochemical treatments require relatively high operational and maintenance cost (Pongsilp and Nimnoi, 2022). Moreover, these methods can also produce secondary pollutants that require additional treatment with specialized equipment, as many of the hazardous pollutants are combustible, highly volatile and extremely flammable. Also, the physiochemical treatments are not sustainable for restoring the contaminated sites as they incur additional treatment costs for the treatment of incomplete degraded pollutants. For instance, landfilling and composting of SOW result in: (1) incomplete degradation of some toxic compounds of SOW, leading to residual contamination such as leachate soil and ground water contamination; (2) generation of foul odors (Touliabah et al., 2022). Incineration of SOW emit volatile organic compounds, heavy metals, dioxins, methane and toxic greenhouse gases into the atmosphere, which negatively impact public health and air quality (Sutherland and Ralph, 2019; Touliabah et al., 2022). Similarly, solvent extraction and chemical decomposition is another method used to treat SOW, but when solvent extraction and chemical decomposition of SOW is not properly managed (which could be challenging), can release harmful pollutants into the atmosphere and discharge toxic chemicals into the water and land environments (Dell' Anno et al., 2021; Radziff et al., 2021). Furthermore, exposure to organic solvents or chemicals poses significant chemical health risks (El-Sheekh et al., 2013; Subashchandrabose et al., 2013). In the light of the various physiochemical treatment limitations, there is an urgent need to develop sustainable, eco-friendly and cost effective solutions, such as bio-based solutions to reduce the harmful effects of the SOW contaminants.

The use of microalgae as one of the bio-based approach have sparked a lot of scientific interest in the recent years as a promising technology for treating oil polluted areas (Touliabah et al., 2022). This is because of their (1) environmental friendliness, (2) high adaptability, biosorption and tolerance to organic pollutants, and (3) possession of catabolic genes that has potential to breakdown organic pollutants (Touliabah et al., 2022). Microalgae of the genus *Scenedesmus* represent a largely unexplored resource, with an estimated species >79. However, merely 10 of the *Scenedesmus* species have been economically cultivated on a large scale for commercial purposes such as food supplements and feed stocks, biofuels, cosmetics, pigments, etc. (Gonçalves, 2021; Ismail et al., 2020). Over the past few years, *Scenedesmus* species have been used in treatment of various industrial wastes such as crude oil, used lubricant oil, waste water and oil emulsions (Dell' Anno et al., 2021; Radziff et al., 2021). For instance, *Scenedesmus obliquus, S. quadricauda* and *S. Platydiscus* have been employed in the degradation of waste lubricant oil (Aldaby and Mawad, 2019; El-Sheekh et al., 2013; Subashchandrabose et al., 2013). Moreover, these microalgae have been found to possess excellent degradation capabilities of lubricant oil wastes (Sutherland and Ralph, 2019). In addition, these microalgae species were described to degrade HCs by; first, emulsification of the HCs, then emulsified HCs is adsorbed on the surface of the microalgae and ingested via endocytosis (either by active transport or passive transport) (Subashchandrabose et al., 2013). Due to the recalcitrant nature of organic contaminants to degradation, enhancing the degradative ability of microalgae to breakdown these contaminants are desirable and could lead to a more efficient degradation of recalcitrant contaminants. To improve microalgae ability for an efficient biodegradation process; (1) the microalgae strain should be able to grow and use the hydrocarbon pollutants as a carbon and energy source; (2) ability to withstand extreme conditions; (3) fast growth rate; (4) ease of harvest (Arora et al. 2020). Previous studies have shown that *S. vacuolatus* fulfill some of these criteria, making the microalgae the perfect candidates for strain improvement. Recently, strain improvement has attracted attention as one of the strategies for improving microorganism's capabilities (Trovão et al., 2022). There are three main strategies for microbial strain improvement: mutagenesis, adaptive laboratory evolution (ALE), and genetic engineering (Arora and Philippidis, 2021). Among these strategies, mutagenesis is often utilized because it has proven to be useful in the production of novel microalgae strains with biotechnologically significant properties. Mutagenesis involves the mechanism of inducing mutations in the cells of living microorganisms (Arora et al., 2020). To our knowledge, no research on mutagenesis of microalgae for enhanced biodegradation abilities has been reported. However, mutagenesis has been used on microalgae to enhance the production of bioactive compounds such as lipids, fatty acids, and biofuels (Arora et al., 2020; Arora and Philippidis, 2021). For example, mutagenesis was used to increase the production of lipid and fatty acids production in different microalgae including *C. reinhardtii, Pavlova lutheri, C. sorokiniana* and *Nannochloropsis oceanica, Crypthecodinium cohnii*, *Schizochytrium* sp., and *Aurantiochytrium* sp., (Arora et al., 2020; Trovão et al., 2022). Aside from increasing microalgae lipid production, UV mutagenesis has also been employed to improve *Scenedesmus* sp. resistance to industrial wastewater (Arora et al., 2020). Mutagenesis in microalgae has been accomplished through the use of both physical and chemical mutagens (Kumar et al., 2020; LaPanse et al., 2021). Chemical mutagen involves the use of chemicals to induce random mutation on organism genome by the substitution of guanine and cytosine to adenine and thymine (Trovão et al., 2022; Kumar et al., 2020). Alkylating agents such as ethyl methane sulfonate (EMS), N-nitro-N-nitrosoguanidine (NTG) and methylnitronitrosoguanidine (MNG) are examples of chemical mutagens. On the other hand, physical mutagens include various forms of irradiation, such as ultraviolet (UV) light, ion beams, nuclear, X- and y-rays. Mutagenesis by UV light radiation is the most widely used method on microalgae because its relatively simple, safe, and low cost, needing no depth of biochemical processes, and requires no complex equipment and training (Arora and Philippidis, 2021; Arora et al., 2020; Trovão et al., 2022). Additionally, strains produced by mutagenesis are not subject to the same stringent regulatory constraints as genetically modified microorganisms (GMM), necessitating no legal and regulatory approval prior to commercial deployment (Trovão et al., 2022). UV mutagenesis improves strain efficiency by targeting the organism's deoxyribonucleic acid (DNA) and causing random mutations on the genome by forming pyrimidine dimers and thymine dimers (Kumar et al., 2020; LaPanse et al., 2021).

1.1 Project Rationale

Contamination of the environment by SOW is rapidly increasing due to global increase in the usage of lubricant-based products (Hentati et al., 2022; Sattar et al., 2022). Environmental pollution with lubricant-based products has attracted much attention in recent decades. The presence of different types of lubricant oil producing, refinery and manufacturing industries has resulted in the high generation of SOW (Hentati et al., 2022; Pongsilp and Nimnoi, 2022). Of significant interest, is the uncontrolled and improper discharge of SOW from industries into the environment. For instance, the incineration of SOW on landfills pollutes the air by emitting greenhouse gases such as carbon dioxide (CO_2), nitrogen oxide, sulphur oxide, methane, etc., (Obi et al., 2022; Soumeya et al., 2022). The major issue of global warming has been reported to be caused by CO_2 build-up in the atmosphere while, acid rain is caused by nitrogen dioxides and sulphur dioxides in the atmosphere. Furthermore, land-filling of SOW has also been linked to the production of toxic by-products, which have the potential to contaminate ground and fresh water sources (Obi et al., 2022; Sattar et al., 2022). Several physicochemical treatment methods, including incineration, landfilling, re-refining, and recycling have been used for SOW treatment (Kajdas, 2014; Rathi and Yadav, 2019). Although these treatment techniques are effective and have been used to remove SOW contaminants, they are still faced with many challenges, such as: (1) incomplete decomposition of SOW; (2) high cost of operations; (3) production of toxic by-products; and (4) high energy consumption (Ibrahim et al., 2016; Ichor et al., 2016). As a result, the need and search for a cost-effective and environmentally friendly clean-up approach such as microalgae-based treatment that can efficiently degrade and mitigate the harmful effect of these oil waste pollutants is of great importance to the future sustainability of the environment. Globally, microalgae treatment has attracted extensive attention as a result of their degradative capabilities, less energy consumption, low-cost and eco-friendliness in converting environmental contaminants into valuable biomasses and metabolites (Dell' Anno et al., 2021). The potential of the microalgae *Scenedesmus* as a bioremediator for the removal of oil waste HCs to produce non-toxic by-products has been reported in various studies (Dell' Anno et al., 2021; Radziff et al., 2021; Touliabah et al., 2022). However, oil waste pollutants such as monoaromatics, polychlorinated and polycyclic aromatic hydrocarbons have proven to be recalcitrant and resistant to degradation. Additionally, the bioaccumulation, toxicity, carcinogenicity, and mutagenicity associated with these pollutants necessitate their bioremediation. Moreover, given the complexity of these oil waste pollutants, the wild-type microalgae can only degrade a limited number of the pollutants. Therefore, microalgae with enhanced metabolic and degradation capabilities would be required to effectively degrade these oil waste contaminants. This enhancement could be achieved through strain improvement utilizing ultraviolet (UV) light mutagenesis (Arora et al., 2020).

1.2 Aims

The aim of this work was to:

- Investigate and evaluate the impact of physical mutagenesis (ultraviolet light radiation) on *Scenedesmus vacuolatus* to enhance its metabolic performance and degradation ability.
- Compare the biodegradation efficiencies of the wild-type and the UV-exposed *S. vacuolatus*.
- Optimize the process parameters influencing the degradation of spent oil waste.
- Identify the catabolic genes, enzymes and metabolic pathways involved in spent oil waste degradation by *S. vacuolatus*.

1.3 Objectives

- To achieve this aim, the following specific objectives were carried out: Evaluation of the impact of ultraviolet light radiation on *S. vacuolatus* metabolic performance for spent oil waste biodegradation;

- Comparison of biodegradation efficiencies of the wild-type and the UV-exposed *S. vacuolatus* in removing spent oil wastes contaminants;
- Modelling and optimization of key operational process parameters of temperature, inoculum concentration and substrate concentration using response surface methodology for spent oil waste biodegradation;
- Analysis of genes expression by the wild-type and UV-exposed *S. vacuolatus* during spent oil waste degradation through a transcriptomic approach.

1.4 Thesis outline

This thesis comprises of seven chapters – Introduction, literature review, four experimental chapters and the conclusion. Each experimental chapter is independent, containing an introduction, materials and methods, results and discussion, conclusion and references. These chapters are presented according to the format of the journals where they have been published.

Chapter 1 is an introductory chapter and provides an overview of the relevant background, rationale, aims and objectives of the study. The chapter also provides relevant information about the layout of the thesis.

Chapter 2 focuses on the literature review and discusses spent oil wastes and their associated environmental risks. It also reviews the role of microalgae in the biodegradation of spent oil contaminants highlighting the enzymatic mechanisms and the metabolic pathways used by microalgae for aerobic degradation of spent oil waste pollutants.

Chapter 3 detailed the mutagenesis and metabolic potential of *S. vacuolatus* for spent oil waste biodegradation. The impact of the UV radiation on the *S. vacuolatus* morphology, growth, chlorophyll, protein, carbohydrate, biomass, substrate affinity, substrate versatility and the growth kinetics were assessed as well as elucidated.

Chapter 4 report on the comparison of the biodegradation efficiency of UV-exposed *S. vacuolatus* vs wild type for biodegradation of spent coolants. The biodegradation efficiency was determined based on the dehydrogenase activity and gas chromatography/mass spectrometry analyses output.

Chapter 5 focuses on the modelling and optimization of degradation key process parameters using response surface methodology. In addition, the impact of nanoparticles inclusion on the degradation process was undertaken. Moreover, the degradation kinetics to predict the UV-exposed *S. vacuolatus* behaviour based on substrate utilization and biodegradation rate was evaluated.

Chapter 6 outlines the transcriptomic study to identify genes and metabolic pathways employed by UV-exposed *S. vacuolatus* to degrade spent oil waste.

The final chapter, **Chapter 7**, provides a general overview of the major findings of this study, highlights the significant contributions and relevance to knowledge in the research area. The chapter further provides future perspectives and highlights some recommendations for future studies.

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CHAPTER 2

Literature review

2. Introduction

Globally, oil is the primary source of fuel and energy in many sectors, but its production and transport generate a considerable amount of used lubricant oil waste otherwise called spent oil waste (SOW) (Gertsen et al., 2023). Lubricant oils are made up of base material obtained from oil and additives. They consist of more or less 90% of oil base stocks and 10% additives. In the petrochemical and manufacturing industries, the use of additives is to enhance the physical and chemical quality of the lubricant oil. These are included to reduce friction, wear, tear, temperature and prevent oxidation, improve viscosity index, as well as act as anti-corrosive and cooling agent between the mechanical parts (Enerijiofi et al., 2020). These features together form a protecting film, called a tribofilm, over the work piece and workplace. The wide use of lubricant oils due to the above importance and functions has resulted in large release of SOW to the environment. The different sources of SOW include manufacturing industries, workshops, marine sectors (ship garages), automobiles, service filling stations, used oil loading and pumping stations, oil product depots, garages, rail ways, mining activities, agricultural activities etc, (Chunyan et al., 2023). Although, disposal of SOW is a global challenge, this is more severe in developing countries, where there are no effective environmental regulatory policies. The major problem facing the manufacturing industries in the world; is the proper disposal of spent oil wastes since inappropriate treatment can cause serious environmental contamination (Bhattacharya et al., 2015). According to Asgari et al. (2017), SOW are the most difficult oil waste to treat, mainly because of its high chemical content, which is toxic. Hence, the need for biodegradable lubricant oil is growing alongside the public interest for a pollution-free ecosystem.

Currently, the global generation of SOW is steadily increasing each year. In 2016, the global SOW generation reached 2.017 billion tons per year, and projections for 2050 indicated a 70% increase, reaching up to 3.4 billion tons per year (Kuttiyathil et al., 2020; Touliabah et al., 2022). Furthermore, there is no indication that this tendency will slow down, owning to the factors such as population increase, industrial expansion, and urbanization. From recent reports, the total amount of SOW that goes into the environment each year is approximately 200 billion tons (Gertsen et al., 2023; Stepanova et al., 2022). Similarly, it was estimated that South Africa produces an average of 120 million litres of SOW in a year. This is a large quantity of SOW that, if not disposed of properly, could make its way into the environment (Vrede, 2014). The total percentage of SOW that is successfully treated is that which is either recycled or burnt. While the major proportion of SOW enters the environment directly or indirectly, as effluents or spills, or by runoff or atmospheric deposition. This has made SOW to be considered a hazardous waste and should be treated accordingly. Currently, in South Africa SOW is utilized as a dust cure to prevent dust and this method of disposal is not environmentally safe (Nowak et al., 2019). Another method presently used in getting rid of SOW is by incineration (Nowak et al., 2019). This is more severe than the practice of dust cure and road oiling. The method of incineration of use SOW creates a larger health hazard than the discharge of metals to the atmosphere (Baghour, 2019). Previous studies have shown that in every 100 tons of spent oil incinerated, it releases an average of 1.4 tons of sulphur dioxide to the environment (Asgari et al., 2017; Sihag and Pathak, 2016). Spent oil waste contains harmful compounds that are known to be toxic and carcinogens (Touliabah et al., 2022). The persistence of these compounds in the environment depends mainly on their physiochemical characteristics (Touliabah et al., 2022; Davoodi et al. 2020; Sihag and Pathak, 2016). The accumulation of SOW poses a serious threat on humans, animals, plants, fresh water, and marine ecosystems (Touliabah et al., 2022). Recently, there has been increasing interest in the use of microbial biodegradation as a green technology, which uses microorganisms with capability to utilize pollutants and making them harmless (Touliabah et al., 2022; Michael-Igolima et al., 2022).

Microbial biodegradation is one remediation technique attracting worldwide attention since it is a less expensive way of cleaning up oil waste pollutants compared to the traditional methods. The traditional method includes incineration, burying, evaporation, dispersion, excavation, oxidation, chemical decomposition etc., (El-Sheekh et al., 2021). These methods are energy intensive, expensive, inefficient, produce hazardous by-products (Satpati et al., 2023). Due to these disadvantages and with the increase in environmental and legal constraints on the discharge of SOW, a need for cost-effective alternative green technology is important. In this regard, microalgae biodegradation has emerged as a potential and innovative treatment to substitute the traditional methods, offering economic and eco-friendly green treatment. Over the years, the biodegradation of SOW has been researched using bacteria, fungi, yeasts, and higher plants with dearth of study on microalgae (Aldaby and Mawad, 2019; Dell' Anno et al., 2021; Touliabah et al., 2022). Microalgae utilization in biodegradation is yet to be fully explored. The intensity of research activities using microalgae have accelerated tremendously in the past few years. This is because of microalgae's potential application in biotechnology, as a source of renewable energy, bioactive compounds, food, feeds, pharmaceuticals and cosmetics (Fig. 2.1) (Aldaby and Mawad, 2019; Subashchandrabose et al., 2013). Microalgae are autotrophic organisms that play a crucial role in the ecosystem as primary producers. Microalgae, mostly green algae, belonging to the genus *Selenastrum*, *Scenedemus*, *Chlorella*, etc., have been reported to be efficient in the breakdown of organic pollutants present in various industrial waste such as crude oil, used lubricant oil, waste water and oil emulsions (Fig. 2.2) (Dell'Anno et al., 2021). Table 2.1 depicts few of the microalgae general involved in the degradation of HCs pollutants. Microalgae employ three distinct mechanisms for eliminating organic pollutants from ecosystems: firstly, through the adsorption of pollutants onto the cell surface; secondly, via the bioaccumulation of pollutants within the cells; and thirdly, by biotransforming the pollutants into harmless compounds, such as carbon dioxide and water. This review discusses spent oil wastes generation and its environmental impact. The review also highlights the use of microalgae biodegradation as a green technology for the treatment of spent oil waste contaminants. Of particular focus are the metabolic pathways used by microalgae for aerobic degradation and biotransformation of SOW pollutant, as well as the strategies to improve microbial biodegradation of SOW.

Table 2. 1: List of microalgae genera involved in pollutant biodegradation

Microorganism	Genus	Reference
Microalgae	*Scenedesmus*	Dubey et al. (2023)
	Chlorella	Samuel et al. (2020)
	Selenastrum	Baghour (2019)
	Chlamydomonas	Luo et al. (2020)
	Oscillatoria	Aldaby and Mawad (2019)
	Synechococcus	Hamouda et al. (2023)
	Nannochloropsis	Satpati et al. (2023)
	Chlorococcum	Alaidaroos (2023)
	Prototheca	Gouthami et al .(2023)
	Ochromonas	Haiping and Fanping (2023)
	Spirulina	Ezenweani and Kadiri (2023)
	Synechocystis	Wang et al. (2023)
	Tetraselmis	Barra and Greco (2023)
	Coelastrella	Vimali et al. (2021)
	Phormidium	Baghour (2019)

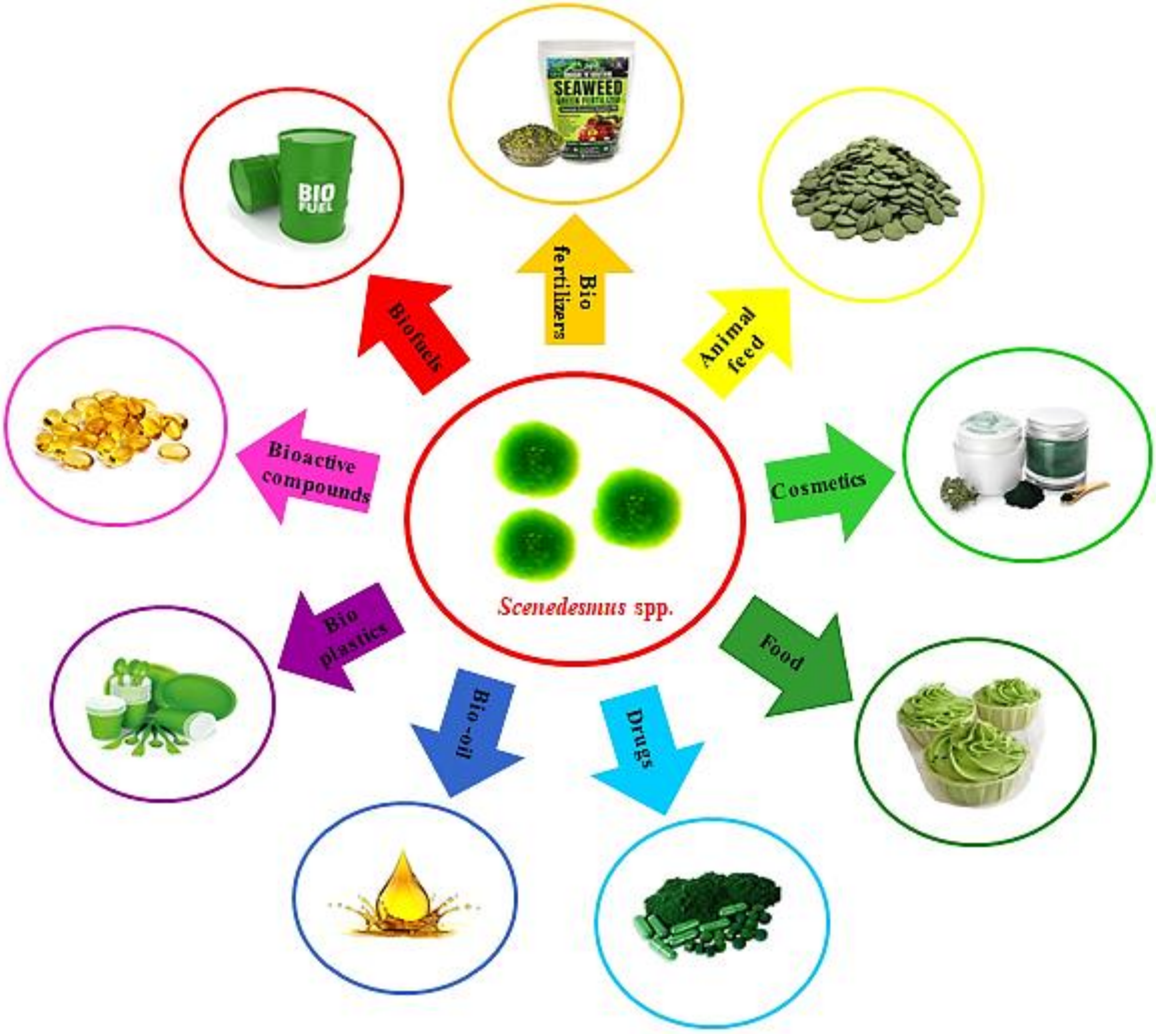

Fig. 2. 1: Commercial cultivation of microalgae strain *Scenedesmus*

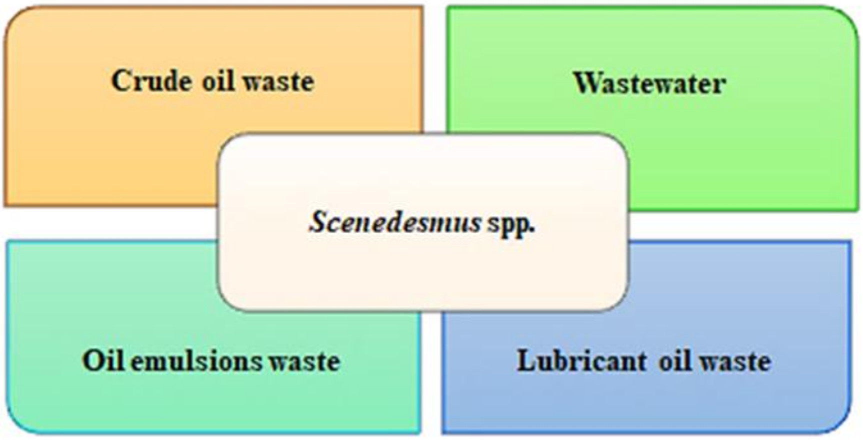

Fig. 2. 2: Different industrial waste degraded by the microalgae *Scenedesmus* spp.

2.1 Collection and disposal of spent oil waste

Industrial SOW are produced during routine operations such as machine servicing, product rolling, cutting, lubrication, cleaning, and cooling operations (Eregie and Jamal-Ally, 2019; Ngene et al., 2016). These SOW are collected, properly labelled, and stored before appropriately permitted off-site disposal (Kajdas, 2014). The most important subject now is how the collected spent oil are gotten rid of. In the past four decades, different traditional methods have been used for the disposal of SOW. These traditional methods include landfill dumping, dust cure, drainage system disposal, road oiling, and burning or incineration as fuel (Ngene et al., 2016). These methods are the main cause of environmental pollution. Presently, re-refining, and recycling of SOW into its original form are seen as the best method of disposal of SOW. These new technological methods are currently in use all over the world (Kajdas, 2014).

2.2 Composition of spent oil waste

Lubricant oil composition has been elucidated in detail over the years (McGenity et al., 2013; Pi et al., 2015). The primary components of spent oils are base oils, degraded additives, metal, oxidation products, fuel, water, dust, carbon soot, and rust. The component of each used oil component varies with different physiochemical properties, including viscosity, density, solubility, bioavailability, and toxicity (McGenity et al., 2013). The large variation in the composition of used oil relates to its crude oil source and additives (Lopes et al., 2010). The different types of additives used in lubricant oil include oxidation inhibitors, detergents, emulsifiers, anti-wear, anti-seize agents, friction modifiers, anti-corrosion agents, rust inhibitors, and anti-foaming agents (Asgari et al., 2017; Soni and Agarwal, 2014). Thus, this makes the composition of SOW, therefore difficult to generalize or characterize the exact chemical composition. This is because, during the use of lubricant oil, the lubricant oil picks up different contaminants due to the wearing out of the machine components. Hence, other contaminants include carbon, metal portions such as chlorine, sulphur, zinc, calcium, barium, phosphorus, lead, aluminium, iron, chromium, molybdenum, nickel, copper, steel, lead and arsenic, that are extremely hazardous (Abro et al., 2013). According to Ohanmu et al. (2019) and Pi et al. (2015), the chemical composition of spent oil consists of organic compounds i.e., aliphatic compounds such as n-alkanes, alicyclic compounds, aromatic compounds, polycyclic aromatic hydrocarbons (PAHs); heavy metals and other toxic compounds (Tuhuloula et al., 2018). The major carcinogenic agents in spent oil are the polycyclic aromatic compounds (PAHs) with 3-7 rings such as benzo(a)pyrene, benzo(a)anthracene and chrysene. These compounds, and many others like them, were reported to be found in spent oil waste, in the course of the machine runs or servicing and combustion process (Ohanmu et al., 2019).

2.3 Impact of spent oil wastes

2.3.1 Environmental Impact

There is a lot of reports about the severe damage caused by SOW spills on the ecosystems (Bhattacharya et al., 2015; Jafarinejad, 2017; Sihag and Pathak 2016; Soni and Agarwal, 2014). Soil pollution (Fig. 2.3) is the main problem caused by the improper disposal of spent oil. SOW wastes dumped in landfills sites have hazardous compounds that could leaked into the soil. These hazardous compounds could be bioaccumulated over time. Soil pollution does not only affect plant growth, but it is also unhealthy for humans and causes death in animals who consume plants growing in the polluted area or landfills (Jafarinejad, 2017). Polluted soil may turn a fertile piece of land barren or the produce harvested from the soil could turn out to be health hazards themselves (Ohanmu et al., 2019). Uquetan et al. (2017) and Jafarinejad (2017) reported that the presence of SOW in the soil adversely affected the physical, chemical, and microbiological properties, these in-turns affected the germination of crop seeds and impeded on the growth of cultivated crops. Similarly, Ohanmu et al. (2019) and Osuagwu and Olaifa (2018) reported the impact of lubricant oil waste accidents on vegetation and land. Their report shows that after forty years of several clean-ups, the soils are still contaminated, brownish leafless vegetation and barren lands are seen.

Air pollution, SOW that is incinerated at landfills sites, emits gaseous compounds that contaminate the atmosphere (Fig. 2.3) and destroys our ozone layer. Sulphur dioxide, carbon monoxide, dioxins, methane gas, carbon dioxide, nitrogen oxides, particulate matter, and volatile organic compounds produced during the burning of used oil wastes are toxic to humans with potential to cause health problems such respiratory diseases when inhaled. Likewise, the volatile organic compounds and particulate matter produced during incineration causes the irritation of the lungs, asthma, and bronchitis (Ohanmu et al., 2019). These greenhouse gases are the main source of global warming. The harmful effect of greenhouse gasses includes extreme weather changes such as heavy rainfall, strong storms, tornadoes, hurricanes, whirlwinds, windstorms, and extreme heat that the world including South Africa is experiencing today (Ngene et al., 2016). Recently, the greenhouse gases were reported to have facilitated global temperature rise by 1.4 degrees Fahrenheit (Ngene et al., 2016).

Water pollution results from improper disposal of SOW. This oil waste can also leach into groundwater during activities such as dust cure and road rolling. This water is used for domestic activities, recreational activities, agricultural activities, and consumption. Hazardous hydrocarbon compounds from SOW can also leach into streams and other water bodies. Illegal disposal or deposition of used oil waste in water bodies can cause the loss of aquatic creatures (Fig 2.3). It is also very harmful to humans who consume contaminated fishes. For instance, Lopes et al. (2010) reported the Exxon Valdez accidental spent oil land spill, with mussels and oysters. Their result revealed that even many years after the disaster, significant genotoxic damage still exists in mussels and oysters living in the impacted area. Moreover, Lopes and Bidoia (2009) reported that one litre of SOW could make freshwater undrinkable and could deplete the oxygen of a million litres of water, causing the suffocation of aquatic creatures and preventing plants from photosynthesizing. Also, Harper (2019), reported the accidental spill of 1.6 million litres of lubricant oil into the Msunduzi River in South Africa. According to his report aquatic creatures such as fishes and animals such as cows and goats died after drinking from contaminating the river.

2.3.2 Health impact

Improper disposal of SOW has been linked to be the cause of many diagnosed diseases (Ohanmu et al., 2019). The health problems associated with SOW may be through any or combinations of the following routes: contaminated food, water, and emission, from landfills and open dumpsites (Fig. 2.3) (Ohanmu et al., 2019). Allergies, skin irritations, gastrointestinal issues psychological disorders, respiratory problems, birth defects and death are the major human health problems associated with spent oil (Ohanmu et al., 2019). Polycyclic aromatics compounds are the most hazardous components of spent oil waste with chronic and carcinogenic effects (Romero et al., 2018). Akpor et al. (2014) and Alrumman et al. (2015) reported the chronic effects of naphthalene, a polycyclic aromatic hydrocarbon when consumed include hepatitis, kidneys failure, heart rate malfunction, lungs disease, and nervous system disorder. For animals and birds, the impact of SOW includes polluted habitat, destruction of food sources and reduced reproduction. Also, consumption of SOW contaminated food reduces the ability of animals to absorbs and break down food, affects their central nervous systems, lungs, and livers and thus, destroying their intestinal organs (Romero et al., 2018).

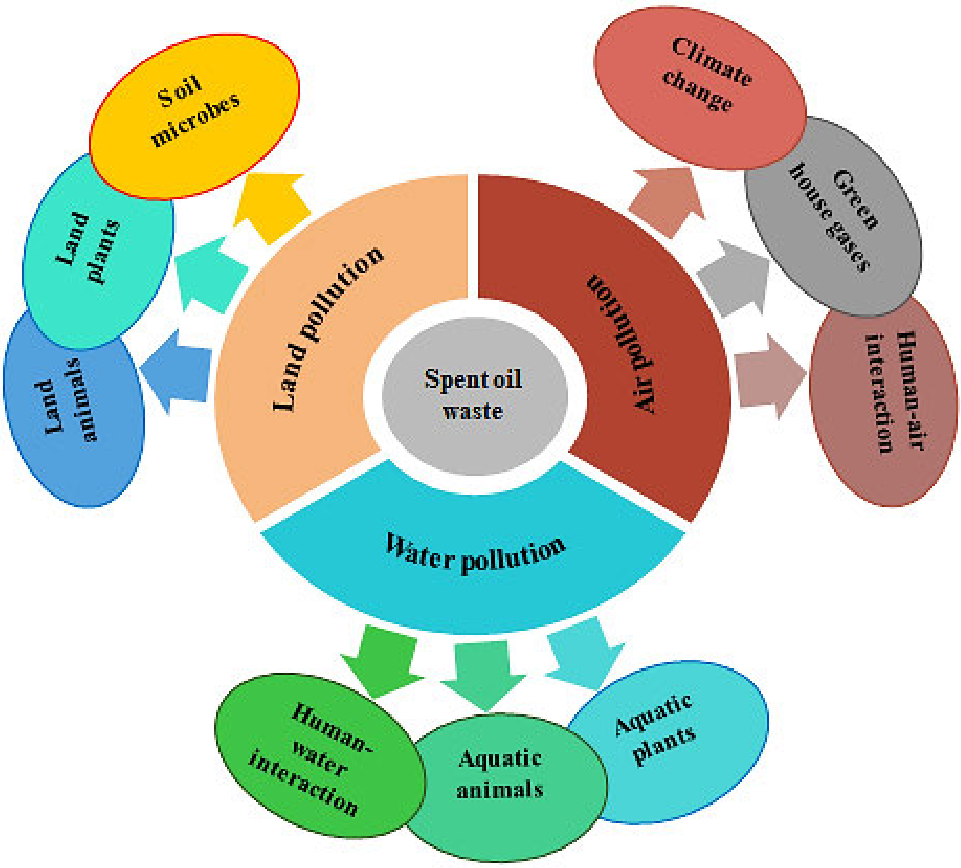

Fig. 2. 3: Spent oil waste impact on the environment and human health

2.4 Challenges associated with spent oil waste management

Over the years due to frequent environmental pollution in the world, the oil-based product industries have been in search of new formulation to produced biodegradable lubricant oil (Soni and Agarwal, 2014). On the other hand, SOW management scheme in several forms is present in different nations, but no standard or best practices exist. With the increase in energy costs, industrial sectors are experiencing an increase in their operational costs. The management of SOW collection and disposal has likewise become a problem in relation to cost, land utilization, inadequate infrastructure, disposal technologies, lack of public awareness, and poor legal regulations. The industrial sector that generates most SOW is mandated to get rid of these oil wastes in an appropriate way. The collection, transport, and disposal treatment technique of SOW requires huge capital investment. The collection and disposal is additional operational cost for the industries. Again, once the SOW is disposed of, the risk of environmental pollution is inevitable (Kajdas, 2014; Ngene et al., 2016).

2.5 Legislation and regulations on spent oil waste management

Laws and regulations form a substantial part in the management of SOW. Different nations have their laws and regulatory policies governing the collection and disposal of SOW in accordance with local needs and environmental hazards (Kajdas, 2014). However, the situation is different in some countries including South Africa, Kenya, Nigeria, India, Mozambique, Namibia etc., where there is no legislation, lack of regulatory policy, supervision, and implementation of an effective modern waste management plan for the collection and disposal of SOW (Kajdas, 2014). The government in collaboration with industrial sectors and private sectors needs to adopt an all-round strategy for the proper implementation of the Waste Act. Through the proper legal implementation of the Waste Act, the SOW industrial sectors will ensure proper compliance.

2.6 Current methods and drawbacks of spent oil waste management

The mismanagement of SOW and disposal alongside the environmental safety is the most crucial problem in the world today including South Africa. As previously indicated, millions of tons of SOW are produced annually worldwide, and a large amount of it is improperly disposed of into the environment (Kajdas, 2014; Sattar et al., 2022). This indiscriminate practice has been linked to be the principal cause of environmental contamination, which has attracted both governmental and global concern due to the severe threats it poses to both public health and the environment. Several approaches including refining, recycling, burning, landfilling, high temperature incineration and chemical decomposition have been employed to dispose SOW (Sattar et al., 2022; Touliabah et al., 2022). However, these approaches not only incur high cost but also give rise to additional environmental concern, such as the generation of toxic by-products (Sattar et al., 2022). For instance, incineration, as mentioned earlier, is the most common approach for SOW disposal. However, this approach ultimately results in the release of harmful gases and pollutants into the atmosphere. Also, the cost of disposal of SOW is on the rise as landfill sites becomes full and restricted, making the disposal procedure more difficult. In addition, refining and recycling of SOW is another major challenge. This is because refineries and recycling facilities are typically located distance from the source of SOW generation and could require a specialist and specialized equipment. This result in increased transportation cost and spills during transport. Given the drawbacks associated with these methods, the search for a cost-effective and environmentally friendly clean up strategy for the safe disposal of SOW holds paramount importance for the long-term sustainability of the environment.

The utilization of microorganisms such as microalgae has proven to be highly effective and successful for safe disposal of SOW from the environment (Dell'Anno et al., 2021; Satpati et al., 2023; Touliabah et al., 2022). Globally, the utilization of microalgae for SOW disposal has drawn substantial attention due to the significant advantages over the current traditional approaches. These include reduced costs, low energy usage, lower risk of environmental contamination, degradation efficiency and biomass production (Al-Hussieny et al., 2020; Dell'Anno et al., 2021). In microalgae treatment process, microalgae attach itself to SOW and degrade the oil waste by using it as a source of carbon and energy (Al-Hussieny et al., 2020; Dell'Anno et al., 2021). Interestingly, this green technological approach using microalgae could be a promising strategy for the treatment and disposal of SOW.

2.7 Biodegradation

Biodegradation is the breakdown of organic pollutant into simpler non-toxic products by living microorganisms (Joutey et al., 2013). This emerging technology is one alternative method of bioremediation which has proven to be an economical method for the effective treatment of industrial SOW, detoxify contaminated soils, water, sediments and reclamation of polluted sites (Joutey et al., 2013). Several scientific research on the use of various microorganisms such as bacteria, fungi, yeast and microalgae to degrade SOW HCs have been reported (Pandey et al., 2016; Soumeya et al., 2022; Subashchandrabose et al., 2013). These microorganisms may be indigenous or exogenous which uses the contaminants as a source of carbon and energy. Biodegradation technology is broadly classed into two categories in-situ or ex-situ.

2.7.1 In-situ biodegradation

In-situ biodegradation involve treating pollutants at the site of pollution which prevents the challenges of transporting the for oil waste from one treatment facility to another, reducing cost of treatment, preventing public/site personnel exposure and environmental contamination. This technique involves the breakdown of contaminants into the non-toxic forms using indigenous microorganism native to the contaminated site (Romantschuk et al., 2023). In-situ biodegradation technique have been effectively employed to treat oil wastes polluted sites, chlorinated solvents, dyes, heavy metals, and other organic wastes (Madison et al., 2023; Pyke et al., 2023). In-situ biodegradation treatment classified into intrinsic in situ biodegradation and engineered in situ biodegradation. In-situ biodegradation treatment is employed using the following approach such as bioverting, biosparging, bioaugmentation, and phytoremediation (Madison et al., 2023).

2.7.2 Ex-situ biodegradation

Ex-situ biodegradation involves the removal of the contaminated wastes to be treated elsewhere (Azubuike et al., 2016; Angelucci and Tomei, 2016). In ex situ biodegradation, the contaminants are treated with a consortium of microorganisms in a controlled environment. Ex-situ biodegradation has been successfully implemented to clean-up variety of organic contaminants such as spent oil contaminants, gas condensates, crude oil, chlorinated compounds, aromatic compounds, heavy metals, pesticides, herbicides, explosive compounds, metalloids, and radionuclides (Folch et al., 2013; Frascari et al., 2015; Kuppusamy et al., 2016). The main advantage of ex-situ biodegradation compared to in situ biodegradation is that there is proper control of the operational process and is timesaving (Azubuike et al., 2016; Angelucci and Tomei, 2016). Ex-situ biodegradation treatment is carried out using the following approach such as bioreactor, biopile landfarming, and windrows.

2.8 Factors affecting biodegradation

2.8.1 Chemical composition and contaminant concentration

Biodegradation varies according to the chemical composition and concentration of hydrocarbons (Srivastava et al., 2014). Spent oils are complex mixtures of hydrocarbons contaminants that degrade at different rates depending on their chemical structure. Typically, the biodegradation rate increases with decreasing molecular weight and chemical structure of the hydrocarbon (Srivastava et al., 2014). Most hydrocarbon contaminants are grouped into four fractions: saturates aromatics, resins, and asphaltene. The saturated compounds are the n-alkanes with a carbon length ranging from C10 - C25 and easily degraded by microorganisms. The long-chain n-alkanes ranging from C25- C40 are hydrophobic solids and are readily degraded. Whilst the short-chain n-alkanes ranging from C16 – C20 are hard to degrade due to their poor water solubility and bioavailability. Branched-chain alkanes and cycloalkanes are also degraded slowly compared to their corresponding straight alkanes. Aromatic compounds are recalcitrant hydrocarbons that have great resistance to degradation (Sihag et al., 2014). Previous studies have shown that the rate of microbial uptake and biodegradation of aromatic hydrocarbons is dependent on the solvent solubility of the hydrocarbons (Eregie and Jamal-Ally, 2019; Srivastava et al., 2014). High solubility may become detrimental to the degrading microorganisms. Usually, contaminant concentration greater than 10% v/v leads to a decrease in microbial activity and biodegradation rate (Sihag et al., 2014). The concentration of contaminants directly affects microbial activity. High concentrations of contaminants have a toxic impact on the microbial population, while low concentrations of contaminants prevent microbial degradation (Adams et al., 2015; Sihag et al., 2014).

2.8.2 Temperature

Temperature plays very important roles in the biodegradation of hydrocarbon (HC) pollutants by its direct effect on the chemistry and physiology of the HC and microbial activities (Sihag et al., 2014). Additionally, temperature influences the solubility of hydrocarbons, with higher temperatures leading to increased HC solubility which ultimately increases the bioavailability of hydrocarbon molecule. Also, oxygen solubility decreases with increasing temperature, which reduces the metabolic activity of aerobic microorganisms (Adams et al., 2015). Biodegradation of hydrocarbon occurs over a broad temperature range. Most studies tend to focus on mesophilic temperatures rather than the efficiency of transformations at very low or high temperatures (Adams et al., 2015). The viscosity and solubility of lubricant oil increases with low temperature, therefore, causes the lubricant oil to become toxic and recalcitrant to microorganisms (Adams et al., 2015). Some hydrocarbons are more soluble at lower temperatures (e.g., short-chain alkanes), and some low-molecular-weight aromatics are more soluble at a higher temperature (Sihag et al., 2014). Although hydrocarbon biodegradation occurs over a wide range of temperatures, the rate of biodegradation generally decreases with decreasing temperature and increases with increasing temperature (15 to 40°C) (Adams et al., 2015; Ezeonu et al., 2012). This is because enzyme activity increases with temperature, facilitating the required biochemical reactions essential for the biodegradation process to occur.

2.8.3 Oxygen

Aerobic conditions are generally considered necessary for extensive degradation of oil contaminants in the environment since major degradative pathways for both alkanes and aromatics involve oxygen (Karigar and Rao, 2011; Thapa et al., 2012). Oxygen availability is dependent on the ability of oxygen to move or diffuse through the contaminants as well as on the uptake rate by microorganisms. The primary rate-limiting factor in aerobic biodegradation is the delivery of oxygen. Previous studies have shown that the addition of oxygen increases degradation rates, and oxygen depletion leads to a reduction in biodegradation activities (Karigar and Rao, 2011; Thapa et al., 2012). Kao et al. (2008) reported that under the aerobic condition, benzene, toluene, ethylbenzene, and xylenes (BTEX) compounds degraded faster compared to the anaerobic environment. Anaerobic hydrocarbon degradation has been shown in some studies to occur only at negligible rates. However, recent studies have shown that anaerobic hydrocarbon metabolism may be an important process in certain conditions (Karigar and Rao, 2011; Thapa et al., 2012). The biodegradation of BTEX compounds, has been demonstrated to occur under a variety of anaerobic conditions (Takáčová et al., 2015). Studies have also demonstrated that in some marine sediments, polycyclic aromatic hydrocarbons (PAHs) and alkanes can be degraded under sulphate-reducing conditions at similar rates to those under aerobic conditions (Pandey et al., 2016; Subashchandrabose et al., 2013).

2.8.4 Nutrient availability

Microorganisms need nutrients for growth and metabolic processes (Adams et al., 2015). Microorganisms require mineral nutrients such as carbon, nitrogen, phosphorus, potassium, sulphur, magnesium, etc., (Adams et al., 2015). In contaminated sites, where the carbon levels are often high due to the nature of the pollutant, available nutrients could become rapidly depleted during microbial metabolism (Sihag et al., 2014). Many studies have shown that the types and quantities of nutrients present in the system play a much more important role in limiting the rate of hydrocarbon degradation (Adams et al., 2015; Sihag et al., 2014). Higher concentrations of nutrient (oil) were reported to cause the toxicity of the growth medium and exert toxic stress on microorganisms making them unable to carry out their metabolic activities whilst lower nutrient concentration leads to competition among microorganisms due to insufficient nutrient source (Sihag et al., 2014). Nutrients especially, nitrogen, phosphorus, and in some cases, iron are very important ingredients for successful biodegradation of hydrocarbon pollutants. However, some of these nutrients in excess or limited amounts could become limiting factors, thus affecting the biodegradation process (Sihag et al., 2014). Nutrient supplementation can be used as a tool to increase biodegradation rates (Sihag et al., 2014). Moreover, the ability of a microorganism to grow in any system depends on the organism’s ability to utilize available nutrients. The amount of nutrients required to degrade a certain amount of oil is not fully understood (Sihag et al., 2014). Further work in this regard would benefit future bioremediation trials.

2.8.5 pH

Like temperature, pH also plays a vital role in determining the ability of microorganisms to grow and degrade hydrocarbon in the environments (Bhattacharya et al., 2015). Most commonly, microorganisms grow optimally within a narrow pH range between 6.7 and 7.5 during hydrocarbon degradation. The metabolic activities of microorganisms in a system can often be directly related to the pH (acidity or alkalinity) of the system under study. Studies have indicated that microorganisms naturally change the pH of their environment via the production of by-products that are either acidic or basic (Ezeonu et al., 2012; Sihag et al., 2014). The production of organic acid metabolites tends to lower the pH of the microbial environment, whilst the release of basic organic metabolites increases the pH of the microbial environment. An increase or decrease in the pH thus, indicate a significant influence on the microbial activity, potentially affecting various metabolic processes. Higher rates of biodegradation are observed at neutral pH conditions. Extreme pH, as can be noted in some environments, was reported to affect microorganism’s capability to disintegrate hydrocarbons (Sihag et al., 2014).

2.8.6 Microorganisms

The composition of microorganisms in a given environment plays a crucial role in determining the rate and efficiency of biodegradation. This is attributed to the diverse metabolic capabilities exhibited by different microbial species (Yemashova et al., 2007). Furthermore, the metabolic proficiency of microorganisms varies based on the nature of pollutants present. Consequently, a specific microorganism might be more effective in degrading one type of HC while proving ineffective against another. Due to this variability, a pivotal aspect of any bioremediation study involves isolating and identifying microbes capable of degrading specific contaminants. Moreover, the size of the microbial population is another factor impacting the biodegradation rate. Studies have demonstrated that an increase in microbial population correlates with a higher rate of hydrocarbon degradation. This suggests that the biodegradation rate is proportionate to the size of the microbial population (Yemashova et al., 2007).

2.9 Microalgae biodegradation of spent oil waste pollutants

2.9.1 Microalgae biodegradation of Alkanes

Alkanes are organic compounds composed of carbon and hydrogen atoms connected by single bonds (Ichor et al., 2016). Alkanes constitute a significant portion (>50%) of oil waste, depending on the lubricant oil. As saturated hydrocarbons, alkanes are chemically inert due to their apolar nature. They can be categorized as linear (straight chain-alkanes), cyclic (cyclo-alkanes), or branched (iso-alkanes). Typically, straight or long-chain alkanes and short-chain alkanes are more easily degraded, followed by branched and cyclic alkanes, as indicated by research (Ichor et al., 2016; Wang et al., 2023). Furthermore, alkanes with fewer carbon atoms tend to volatilize more readily, while those with more carbon atoms are less volatile. Alkanes are highly insoluble in water, with solubility decreasing as molecular weight increases (Wang et al., 2023). Despite being organic compounds, alkanes exhibit lower reactivity. However, their inert nature causes ecological problem when discharged into the environment. Few microalgae strains have been reported have been identified as capable of degrading alkanes, utilizing them as a source of carbon and energy (Dell' Anno et al., 2021). For instance, *Nitzschia linearis* has been reported to effectively metabolize and degrade alkanes (Hammed et al., 2016). Similarly, *Dicrateria* sp., *Skeletonema costatum*, and *Phaeodactylum tricornutum* demonstrated high utilization and degradation of both long and short-chain alkanes (Pi et al., 2015). Additionally, the genus *Prototheca* was observed to degrade both long and short-chain alkanes (Dell' Anno et al., 2021). Likewise, Ichor et al. (2016) reported the complete degradation of decane, pentacosane, hexacosane, octacosane and nonacosane by *Nostoc punctiforme* and *Spirulina platensis*. Their findings revealed that these alkanes were degraded by 100%.

2.9.2 Microalgae biodegradation of monoaromatics and polycyclic aromatic hydrocarbon

The monoaromatics and polycyclic aromatic hydrocarbon (PAHs) are the most dangerous environmental pollutants, they consist of two or more fused benzene rings in linear, angular, or bunch arrangements chemical structures (Satpati et al., 2023). They are characterized as a persistent HC compound in the environment due to their low water solubility, low volatility, and resistance to biodegradation (Aldaby and Mawad, 2019; Ghasemi et al., 2011; Subashchandrabose et al., 2013). They are released into the ecosystem via human and industrial activities such as automobile exhausts, agricultural and waste incineration, forest fires, coal, oil spillage, consumption of fossil fuels and petroleum products (Ghasemi et al., 2011). Removal of monoaromatics and PAHs from the environment is very important because most of them are known be carcinogens and mutagens (Ghasemi et al., 2011; Subashchandrabose et al., 2013; Vijayaraghavan and Shanthakumar, 2015). Traditional strategies have been employed to remove these compounds from contaminated sites. Still, these strategies have been linked with some disadvantages, such as high operating costs, low efficiency, and groundwater contamination due to the formation of toxic by-products. Microalgae strains such as *Scenedesmus platydiscus*, *Chlorella vulgaris*, *S. quadricauda*, *Prototheca zopfii*, *Chlorella sp., and Selenastrum capricornutum*, *Parachlorella kessleri* have been reported to degrade PAHs via bioaccumulation and biotransformation (Satpati et al., 2023). For instance, Takáčová et al. (2014) reported that the green microalgae (*Parachlorella kessleri*) degraded benzene, toluene, ethylbenzene and xylene (BTEX) compounds. The result showed the complete removal of BTEX after 72 hrs, respectively. Similarly, Ghasemi et al. (2011) observed that *S. capricornutum* oxidized benzylaminopurine. The oxidation of benzylaminopurine resulted in the formation ofcis-11,12-dihydroxybenzo(a)pyrene, cis-7,8-dihydro-7,8-dihydroxybenzo(a)pyrene and cis-4,5-dihydro-4,5-dihydroxybenzo(a)pyrene via dioxygenase system. Aldaby and Mawad (2019) reported the complete metabolism and biodegradation of anthracene, pyrene by three green microalgae strains (*Oscillatoria* sp., *Chlorella* sp., and *Agmenellum quadruplicatum*). Likewise, Subashchandrabose et al. (2013) reported the biodegradation of phenanthrene, fluoranthene, and pyrene by *S. capricornutum*. Their findings revealed that phenanthrene was degraded by 96%, fluoranthene by 100%, and pyrene by 100% within four days. Also, this microalgae strains biotransformed phenanthrene to 1-, 2-, 3-, and 9-hydroxyphenanthrene, fluoranthene to monohydroxylated fluoranthene, and pyrene to monohydroxylated and dihydroxylated pyrenes. The presence these metabolites indicates the involvement of enzymes monooxygenase and dioxygenases. In another study, the degradation of naphthalene by *C. angulosa* was assessed. It was found that *C. angulosa* was able to oxidize naphthalene to 1-naphthol, 4-hydrox-4-tetralone, cis-naphthalene dihydrodiol, and trans-naphthalene (Ghasemi et al., 2011). In addition, Subashchandrabose et al. (2013) reported on the efficiency of *Ankistrodesmus* sp. to degrade naphthalene, dibenzofuran, and dibenzo-p-dioxin. It was found that the strain was capable of degrading naphthalene, dibenzofuran, and dibenzo-p-dioxin simultaneously. Furthermore, the efficiency of *A. quadruplicatum* to degrade phenanthrene was reported. The study shows that the microalgae metabolizes and biotransformed phenanthrene to phenanthrene trans 9, 10-dihydrodiol, trans-9,10-dihydroxy-9,10-dihydrophenanthrene and 1-methoxyphenanthrene. In another related study by Subashchandrabose et al. (2017), the oxidation of anthracene, pyrene, benzo(a)anthracene, benzo(a)pyrene by *A. fertilissima* and *Chlamydomonas reinhardtii* CC-503 was reported. *S. capricornutum* has also been demonstrated to biotransform benzo[a]pyrene to sulphate ester and glucoside conjugates via a dioxygenase pathway (Patel et al., 2020). Though these studies have demonstrated that microalgae could play a vital role in the oxidation of monoaromatics and PAHs, however, it is noteworthy that their role in monoaromatics and PAHs biotransformation has received comparatively less attention compared to bacteria and fungi.

2.9.3 Microalgae biodegradation of polychlorinated biphenyls (PCBs)

PCBs are a class of chlorinated hydrocarbons listed as persistent organic pollutants, they are highly lipophilic, toxic, and with stable chemical properties (Touliabah et al., 2022). The high toxicity and bioaccumulation of PCBs in the food chain pose a hazardous threat to the ecosystem (Zhang et al., 2015). High concentrations of PCBs can result in neurotoxicity, carcinogenesis, developmental and reproductive toxicity, dermal toxicity, endocrine effects, and hepatotoxicity (Zhang et al., 2015). Increasing public awareness and concern has impelled researchers to identify ways to remove these hazardous oil contaminants from the environment. Many studies on the chemical, physical, and biological treatments have been conducted to remediate these hazardous contaminants (Wang et al., 2014). Among these treatments, microbial degradation was found useful (Kaleem et al., 2023). Many microorganisms with the capability of degrading of PCBs have also been reported (Kaleem et al., 2023). Most biodegradation studies have focused primarily on the use of bacteria and fungi (Dixit and Singh, 2015; Subashchandrabose et al., 2013). Lately, there have been studies showing the capability of microalgae cyanobacteria degrading PCBs (Touliabah et al. 2022). Microalgae *S. obliquus* and *C. fusca var. vacuolata* were reported to degrade dichlorophenols (Papazi and Kotzabasis, 2013). *Cyanobacterium Synechocystis* was reported to degrade pesticide chlorpyrifos and organochlorine pesticide lindane (Zhang et al., 2015). The genus *Anabaena* was also reported to degrade more than 90% of the organochlorine insecticide endosulfan after eight days (Zhang et al., 2015). In a recent study, Lauze and Hable (2017) confirmed the accumulation of PCBs in *Fucus vesiculosus* tissue after twenty-four hours of incubation.

2.9.4 Microalgae biodegradation of phenolic compounds

Phenol compounds are the primary hazardous pollutant found in the wastewater of many industries (Abujayyab and Al-Zuhair, 2017; Baldiris-Navarro et al., 2018). Phenol compounds consist of a hydroxyl group attached to a benzene ring. They are colourless compounds, soluble in both organic solvents and water, which enters the environment naturally or artificially as a major pollutant. These compounds form the basic structural unit used in several industries and produced from the combustion of coal wood and municipal solid waste, oil refineries and pharmaceutical, and coke industries (Al–Muhtaseb and Khraisheh, 2015). In the environment, the sources of phenol include forest, plants, and rangeland fires (Abujayyab and Al-Zuhair, 2017; Baldiris-Navarro et al., 2018). Phenol has been described to cause harmful effects on aquatic life and ecosystems and has been detected in surface water, groundwater, drinking water, rainwater, waste sites, sediments, and industrial runoff. Phenol could be fatal by ingestion, inhalation, or skin absorption since it rapidly permeates the skin and may cause serious irritation to the eyes and the respiratory tract. They are listed among the priority organic pollutants by the United States Environmental Protection Agency (USEPA) (Baldiris-Navarro et al., 2018). Phenol compounds are also carcinogenic to humans and animals (Abujayyab and Al-Zuhair, 2017). Various techniques have been employed to remove phenols from wastewater, including thermal decomposition, adsorption, advanced oxidation method, and electrocoagulation. But these treatments are limited because they are usually complex, expensive and produce hazardous by-products (Abujayyab and Al-Zuhair, 2017; Baldiris-Navarro et al., 2018). Biological methods, employing microorganisms have also been undertaking for the removal of phenol from wastewater, which showed very promising results (Baldiris-Navarro et al., 2018). Nevertheless, the release of these microorganisms could cause diseases in plants and animals and may also have the potential to cause diseases to humans (Baldiris-Navarro et al., 2018). It would be advantageous, therefore, to use another type of microorganism, such as microalgae, which are less harmful and environmental-friendly.

In the last decade, the use of microalgae has been used for the degradation of phenol compounds. *Chlorella* sp., *Scenedesmus* sp., *S. capricornutum, Tetraselmis marina*, *Ochromonas danica*, *Lyngbya gracilis*, *Nostoc punctiforme*, *Oscillatoria animalis*, and *Phormidium foveolamm* were reported to have the capacity to degrade phenolic compounds (Surkatti and Al-Zuhair, 2018). Al-Dahhan et al. (2018) reported that *Chlorella* sp., and *Scenedesmus* sp., degraded a variety of phenolic compounds, such as phenol, bisphenol-A, 4-nitrophenol,4-chlorophenol, 2,4- dinitrophenol completely, and 2,4- dimethylphenol. *C. vulgaris* and *Coenochloris pyrenoidosa* were found to degrade p-chlorophenol, and *Anabaena cylindrical* was also found to degrade 2,4-dinitrophenol completely (Baldiris-

Navarro et al., 2018). Zhou et al. (2013) reported *Scenedesmus* sp., having the ability to degrade acylated phenols and bisphenol-A. Similarly, Sanchez-Aponte et al. (2019) carried out a study on *C. reinhardtii,* their results showed that this microalgal strain oxidized 2, 4-DNP to 2-amino-4-nitrophenol (2-ANP) from phenolic effluents. Although, phenolic compounds and other component in SOW are biodegradable, but the process is often quite slow and can take months. Consequently, process parameters are regarded as limiting variables impacting biodegradation process. Therefore, the determination of the process set points is an essential step in obtaining maximum and faster degradation of organic compounds. The significance of process parameter in microalgae biodegradation is related to their impact on microalgae growth, enzymatic and metabolic activities. Although, these process parameters play crucial roles during hydrocarbon biodegradation, however, extreme process conditions can negatively impact the degradation process. Moreover, extreme conditions could adversely affect the viability and metabolic activity of microbes. Therefore, there is a need to optimize the process parameters for maximum biodegradation of pollutants using reliable and efficient optimization approach.

2.10 Optimization of microalgae-based hydrocarbon waste biodegradation

Process optimization is a crucial step in total petroleum hydrocarbon degradation of HC pollutants. Given the numerous factors influencing HCs biodegradation, process optimization enables the determination of optimal conditions of the process parameters (Rehman et al., 2022; Sabour et al., 2022). Several approaches employed for optimizing biodegradation processes. For example, one variable at a time (OVAT) approach investigates a single factor at a time while disregarding the simultaneous influence of other factors (Abuhena et al., 2022). Response Surface Methodology (RSM) is a statistical and mathematical approach widely employed in the design and optimization of biological processes (Abdulrasheed et al., 2020; Goveas et al., 2020). The optimization of a degradation process by employing RSM gives an understanding of the complex relationships between input variables and their impact on the response of interest. Furthermore, it provides insights into the critical factors influencing the biodegradation process and optimizes process conditions. RSM not only aids in understanding the underlying relationships between inputs variables but its also cost effective and ultimately enhance the overall biodegradation efficiency (Abdulrasheed et al., 2020; Goveas et al., 2020). Artificial neural network (ANN) is another statistical tool used in modelling biological processes (Ezemagu et al., 2021). ANN provides a valuable approach for modelling biodegradation processes by effectively capturing intricate relationships between input variables and biodegradation response (Abuhena et al., 2022). This application of ANN contributes to more accurate predictions and a better understanding of how various factors influence the biodegradation of HCs. The advantage of using ANNs in modelling biodegradation process lies in their ability to analyze complex and dynamic relationships, which traditional statistical models may struggle to capture (Rehman et al., 2022). RSM and ANN has been effectively employed for the optimization of biodegradation of lubricant oil waste (Chouaibi et al., 2020; Ezemagu et al., 2021; Goveas et al., 2020; Rehman et al., 2022; Sabour et al., 2022)

2.11 Process kinetics of hydrocarbon biodegradation

Microalgae have been effective and successful in degrading oil pollutants. However, evaluating the process kinetics of the various degradation processes is of utmost importance. Process kinetics is the study of the rate at which a biological process occurs over time (Pi et al., 2023; Sousa et al., 2021). By investigating the kinetics of a biodegradation process, it provides insights into microbial behaviour, contaminants degradation and reaction mechanisms (Abonyi et al., 2022). Numerous kinetic models have been used to elucidate microbial growth and degradation processes (Abonyi et al., 2022; Ani and chukwuma, 2020). These models include the Monod, Logistic, and modified Gompertz models, etc., (Pi et al., 2023). Numerous kinetic models have been used to elucidate microbial growth and degradation processes (Pi et al., 2023; Sousa et al., 2021). These models include the Monod, Logistic, and modified Gompertz models, etc., (Pi et al., 2023). Monod model is the most commonly used kinetic model that elucidates the biomass growth of a microorganism in correlation to a limiting substrate (Abonyi et al., 2022; Ani and chukwuma, 2020; Pi et al., 2023; Sousa et al., 2021). Several studies have reported the growth kinetic of microalgae in oil waste (Abonyi et al., 2022; Ani and chukwuma, 2020; Sousa et al., 2021). For instance, Pi et al. (2023) reported the growth kinetics of microalgae in waste motor oil using the Monod model. Similarly, Karim et al. (2022) observed the growth kinetic of *Chlorella* sp in oil mill effluents employing the Monod model. Subsequently, Monod was use to model the growth kinetics of *Chlorella* sp in crude petroleum oil–water emulsions (Kuttiyathil and Mohamed, 2021). The Logistic model also depicts the modification in microbial cells in relation to growth rate, biomass concentration and time (Pi et al., 2023). This model is also employed to predict microbial growth in response to varying environmental conditions and understanding the factors that regulate their growth. Studies have used the logistic model for waste oil biodegradation processes (Karim et al., 2022; Kuttiyathil and Mohamed, 2021; Pi et al., 2023). Additionally, the modified Gompertz model is another mathematical model used to describe microbial growth over time (Sousa et al., 2021). It is an extension of the original Gompertz model and is particularly useful for capturing more complex growth patterns exhibited by microorganisms. It is used to analyze microbial growth data, estimate parameters such as the maximum specific growth rate and provides insights into the factors influencing their growth and survival (Sousa et al., 2021). Like other models, the modified Gompertz model considers factors such as the lag phase, exponential growth phase, and stationary phase in microbial growth (Sousa et al., 2021). This model is routinely employed in microalgae-based bioremediation of oil wastes and wastewater (Sousa et al., 2021).

2.12 Improving microbial degradation ability by non-genetic approach

2.12.1 Mutagenesis

Mutagenesis as a non-genetic approach is attracting a lot of attention as one of the strategies for improving biodegradation rates and reclaiming contaminated sites. Mutagenesis is the process of inducing mutations in the cells of living microorganisms (Arora et al., 2020). Thus far, there is a dearth of knowledge on the use of mutagenized microalgae to enhance the biodegradation of environment pollutants. Nonetheless, mutagenesis has been successfully applied to microalgae to boost the production of bioactive compounds such as lipids, fatty acids, and biofuels (Arora et al., 2020; Arora and Philippidis, 2021). For instance, mutagenesis has been employed to increase lipid and fatty acid production in various microalgae species such as *C. reinhardtii*, *Pavlova lutheri*, *C. sorokiniana*, *Nannochloropsis oceanica*, *Crypthecodinium cohnii*, *Schizochytrium sp.*, and *Aurantiochytrium sp.* (Arora et al., 2020; Trovão et al., 2022).

Additionally, mutagenesis has been used to enhance *Scenedesmus* sp. resistance to industrial wastewater (Arora et al., 2020). Mutagenesis in microalgae has been achieved via both physical and chemical mutagens (Kumar et al., 2020; LaPanse et al., 2021; Thurakit et al., 2018). Table 2.2 lists several microalgae that have been improved through physical and chemical mutagens. Physical mutagens include various forms of irradiation, such as ultraviolet (UV) light, ion beams, nuclear, X- and y-rays. While, ethyl methane sulfonate (EMS), N0-nitro-N-nitrosoguanidine (NTG) and methylnitronitrosoguanidine (MNG) are chemical mutagen used to induce random mutation on microorganisms. For this study, UV light mutagenesis is of particular interest due to its simplicity, safety, low cost, minimal biochemical process requirements, and no complex equipment and training needed (Arora and Philippidis, 2021; Arora et al., 2020; Trovão et al., 2022). Furthermore, strains developed via UV mutagenesis are not subjected to stringent regulatory constraints imposed on genetically modified microorganisms (GMM), eliminating the need for legal and regulatory approval before commercial use (Trovão et al., 2022). UV mutagenesis increases and improves strain efficiency by targeting the organism's DNA leading to random mutations in the genome through the formation of pyrimidine and thymine dimers. (Kumar et al., 2020; LaPanse et al., 2021). In addition to enhancing microalgae degradation capabilities through UV mutagenesis, the use of catalyst such as nanoparticles has also been projected as a viable approach to improve the efficiency of microalgae in biodegradation of waste pollutants. Nanoparticles have been reported to enhance microbial growth and facilitate the biodegradation of waste pollutants.

Table 2. 2: Mutagenesis of microalgae by physical and chemical mutagens

Microalgae strains	Physical mutagens	Chemical mutagens	Reference
Pavlova lutheri, Chlorella sp.	UV	-	Liu et al. (2015)
C. reinhardtii	UV	EMS	Bleisch et al. (2022)
C. sorokiniana	UV	-	Trovão et al. (2022)
Nannochloropsis oculate		EMS, MNG	Hlavová et al. (2015)
*Nannochloroposis s*p.			Arora et al. 2020
Haematococcus pluvialis		NTG	Fu et al. (2016)
Dunaliella bardawil	UV	-	Fu et al. (2016)
C. vulgaris	UV	EMS	Arora et al. 2020
N. salina		EMS	Arora et al. 2020
C. minutissima		EMS	Arora et al. 2020
C. pyrenoidosa	UV	-	Liu et al. (2015)
Chlorella sp.	UV	EMS, MNG	Hlavová et al. (2015),
Schizochytrium sp.	UV	NTG	Lian et al. (2010)
Scenedesmus obliquus	UV	-	De Jaeger et al. (2014)
Brotryococcus braunii	UV	-	Thurakit et al. (2018)
Scenedesmus sp.	UV	-	De Jaeger et al. (2014))

2.12.2 Nanocatalysts

Despite the success of microalgae-based degradation treatment, achieving complete degradation of oil waste pollutants could take weeks (Vázquez-Núñez et al., 2020; Remya et al., 2022). Thus, the need to enhance microalgae degradation capability is crucial for faster biodegradation of oil pollutants. The inclusion of nanoparticles (NP) in microalgae-based degradation of HCs to improve it degradative abilities is a growing area of research. In recent years, the use of NPs has geared huge interest among researchers as a strategy to improve biodegradation processes due to its unique characteristics such as high surface area to volume ratio, stability, magnetic and catalytic properties (Borji et al., 2020). Inclusion of NPs has the potential to provide a greener approach for management and cleaning up lubricant oil waste contaminated sites (Borji et al., 2020; Punnoose et al., 2021). Nanoparticles are comprised of particles with at least one dimension measuring between 1.0–100 nm (Remya et al., 2022). The applications of nanoparticles in the remediation of oil waste pollutants such as PAHs, phenol and Polychlorinated biphenyls (PCB) have been reported (Table 2.3) (Remya et al., 2022; Singh et al., 2020). NPs were observed to enhance the biodegradation performance of microorganisms by stimulating enzyme synthesis involved in pollutant degradation (Remya et al., 2022; Singh et al., 2020). Furthermore, NPs were found to boost microbial growth, improve yield of biomass and other products such as carotenoids, proteins, and lipids (Remya et al., 2022; Singh et al., 2020). In spite of these reports, there is scarcity of studies on the impact of NPs on microalgae biodegradation of spent lubricant oil waste. Among the NPs used in environmental bioremediation, iron NP has gained tremendous attention in bioremediation research (Singh et al., 2020). Table 2.3 present a list of oil waste pollutants that have been removed by different NPs. Though nanoparticles (NPs) have been reported to enhance microbial growth, improve biomass yield, and facilitate the biodegradation of oil waste pollutants, there is a need to identify the key enzymes, genes, and metabolic pathways involved in these processes.

Table 2. 3: The use of nanomaterials in hydrocarbon pollutant

Nanoparticles	Oil waste pollutant degraded	References
Zero valent iron (nZVI)	Decarbominated diphenyl ether; Chlorinated hydrocarbons	(Vázquez-Núñez et al., 2020)
Fe (III)-doped TiO_2	Phenol	(Borji et al., 2014)
Iron (III) oxide (Fe_2O_3)	PAHs, PCBs	(El-hoshoudy et al., 2019)
Iron (Fe)	Anthracene, pyrene, benzo(a)pyrene	(Ehmedan et al., 2021) (Parthipan et al., 2022)
Iron sulphide (FeS)	Pyrene	(Li et al., 2021)
Cobalt and manganese (Co and Mn)	PAHs, 9,10 dihydrophenanthrene	Kumari and Singh (2016)
Zinc oxide (ZnO)	Benzo[ghi]perylene	(Mandal et al., 2018)
Silica	Benzo(a)pyrene	(Ehmedan et al., 2021)
Carbon Quantum Dots Iron (II, III) oxide (CQD.Fe_3O_4)	Phenanthrene	(Firoozbakht et al., 2022)
Iron (II, III) (Fe_3O_4)	Phenol, pyrene	(Baragaño et al., 2020)

2.13 Transcriptomics

Although microalgae have been efficient in the biodegradation of several oil waste pollutants, the HCs catabolic-related genes and its genetic regulation mechanisms in microalgae are still scarcely reported (Tripathi et al., 2021). With the recent advancement in molecular biology, novel sequencing approaches such as transcriptomics, genomics, proteomics and metabolomics have been made possible to study and analyze the molecular mechanisms of microorganism metabolic processes during HCs degradation. Of these, transcriptomics is one of the earliest and most widely used techniques due to its efficiency, high throughput, enhanced precision, high-sensitivity, high resolution, and low cost. Also, there has been a substantial increase in the genomic sequence databases due to these qualities (Tyagi et al., 2022). Transcriptomic sequencing or RNA-Sequencing (RNA-Seq) is a next-generation sequencing (NGS) approach used to study the transcriptome of model and non-model microorganisms (Bogaert et al., 2019). Transcriptomic sequencing can be used to elucidate the key catabolic genes expressed, gene structure, enzymes and cellular processes involved in the biodegradation and biotransformation of HCs, as well as provide an in depth understanding of the major pathways involved in the HC degradation (Xi et al., 2021). Additionally, transcriptomic sequencing is an essential step in the expression of genes, and understanding this process is of great interest for molecular and cellular biology in several research areas. In the field of environmental and bioremediation sciences, transcriptomic sequencing is a useful technique for obtaining a better understanding of the microbial expression and regulation of genes in response to the presence of different HCs. Through transcriptomic sequencing, it is possible to explore the molecular mechanisms related to oil waste HC degradation by microalgae. This knowledge can then be used for the development of oil waste bioremediation approaches. Recently, there has been a remarkable growth in transcriptomics research, due to the advancements in sequencing technologies (Wang et al., 2022) Transcriptomics has been used to study different species of microalgae based on their biomass, fatty acids, lipids and biofuels productions. Among the microalgae studied are *S. obliquus, Skeletonema marinoi, Dunaliella tertiolecta, Nannochloropsis granulate, Neochloris oleoabundans, Chlorella* sp, *Chlamydomonas reinhardtii*, and others. Moreover, their transcriptomes were analyzed using various high-throughput methods, including de novo assembled-transcriptome, total transcript amplification, quantitative reverse transcription polymerase chain reaction (qRT-PCR), and RNA-Seq, de novo assembled-transcriptome (Tripathi et al., 2021; Xi et al., 2021; Wang et al., 2022).

2.14 Enzymes involved in the biodegradation of spent oil waste pollutants

2.14.1 Oxidoreductase

Oxidoreductase comprises of the largest class of enzymes that play in major role in the oxidation-reduction reaction (Cardenas-Moreno et al., 2023). It is one of the enzymes involved in the breaking down of oil pollutants by microorganisms (Dave and Das, 2021). Microbes obtain energy via biochemical reactions carried out by this enzyme to attach chemical bonds and to allow the transfer of electrons from a reduced chemical compound. According to Cardenas-Moreno et al. (2023) the oxidoreductase plays a substantial part in the breakdown of various phenolic hydrocarbons. The authors also stated that oxidoreductases could degrade toxic xenobiotics, such as aniline compounds, via polymerization or copolymerization with other substrates. Over the year, oxidoreductase has been exploited in the decolorization and degradation of azo dyes (Karigar and Rao, 2011). Many microalgae species have been reported to use oxidoreductase enzyme to degrade radioactive metals from a soluble form to a non-toxic insoluble form. Some of the microalgae species decreases the radioactive metals indirectly via an intermediate electron, which lead to a redox reaction.

2.14.2 Oxygenase

Oxygenase is an enzyme that belongs to the oxidoreductase group of enzymes known for their role in the oxidation of hydrocarbon pollutants (Cheng et al., 2022). Oxygenase catalyze the insertion of one or two oxygen atoms into the substrates. Two classes of oxygenase have been identified based on the number of oxygen atom used in the oxidation reaction: monooxygenases and dioxygenases (Chikere et al., 2011). Monooxygenases incorporate one atom of the oxygen into the substrate, whereas dioxygenases add both atoms of the oxygen (Chikere et al., 2011). Monooxygenases and dioxygenases usually found in microorganisms catalyze several different types of oxygen insertion reactions. These classes of enzymes need co-substrates to carry out their functions. They play an important role in the degradation of oil contaminants by increasing their reactivity. Also, during the biodegradation process, the introduction of oxygen molecules brings about the cleavage of the aromatic rings. Karigar and Rao (2011) reported the involvement of the oxygenase enzyme in the biodegradation of halogenated HCs. Likewise, in another study, oxygenase was observed to play a role in the dehalogenation of n-alkanes and alkenes (Karigar and Rao, 2011).

2.14.3 Monooxygenase

Monooxygenases are another group of enzymes that insert one atom of oxygen molecule into their substrate in many metabolic pathways (Naramittanakul et al., 2023). Monooxygenases are grouped into two classes: flavin-dependent monooxygenases and P540 monooxygenases. Flavin-dependent monooxygenases contain flavin and require nicotinamide adenine dinucleotide (NADH) as coenzymes to carry out hydrocarbon oxidation degradation. Most monooxygenases require coenzyme, which needs molecular oxygen for their activities and identified and characterized that do not require any coenzyme for their activities. P540 monooxygenases are heme-containing oxygenase found in microorganisms. Monooxygenases act as biocatalysts in the bioremediation process and synthetic chemistry due to their high region selectivity and stereo selectivity on a wide range of substrates. They also play a vital role in the process of dehalogenation, hydroxylation, biotransformation, and biodegradation of oil contaminants (Xu et al., 2023). These processes have been explored in recent years for important applications in biodegradation and biotransformation of organic compounds (Karigar and Rao, 2011). In the presence of oxygen, monooxygenase catalyzes oxidative dehalogenation reactions, whereas, under low oxygen levels, reductive dechlorination takes place.

2.14.4 Dioxygenases

Dioxygenases are a group of enzymes that incorporate two atoms of molecular oxygen into their substrates using a variety of reaction mechanisms (Mishra et al., 2020). The cleavage of aromatic rings is the principal characteristic of dioxygenases which helps in the biodegradation of aromatic hydrocarbons (Pandolfo et al., 2023). The dioxygenases are divided into two groups according to their mode of ring scission. The Intradiol enzymes use a non-haem Fe(III) as a coenzyme to cleave the aromatic ring between two hydroxyl groups (ortho-cleavage), while estradiol enzymes use a non-haem Fe(II) as a coenzyme to cleave the aromatic ring between a hydroxylated carbon and an adjacent non-hydroxylated carbon (meta-cleavage) (Ichiyama et al., 2015). Dioxygenase enzymes are found in both eukaryotic and prokaryotic microorganisms, which help in the biotransformation oil contaminants. In biodegradation, dioxygenase was stated to oxidize aromatic hydrocarbon into aliphatic hydrocarbon (Ichiyama et al., 2015; Yesankar et al., 2023). Interestingly, *Selanastum capricornutum* studied by Subashchandrabose et al. (2013) was reported to degrade benzo(a)pyrene (BaP) via a dioxygenase enzyme.

2.14.5 Cytochrome P540

Cytochrome P540 (CYP540) is a well-known heme protein which forms one of the largest gene super families (Lin et al., 2022). CYP450 belong to the oxygenase group of enzymes, which catalyze the oxidation of various organic compounds (Behrendorff, 2021). Based on their catalytic characteristics, CYP540s are usually referred to as monooxygenases. These enzymes, participate in different oxidative reactions such as hydroxylation, dealkylation, oxidation, dehalogenation, peroxidation, and epoxidation of numerous organic compounds (Behrendorff, 2021; Hu et al., 2020). For example, CYP540 oxidizes a substrate by breaking the bond between C–H and then forms a stronger bond between O–H (Daccò et al., 2020). CYP540 enzyme is divided into 10 classes on the basis of their protein and amino acid sequence, among which class II CYP540 has been identified in green microalgae. Class II systems are composed of the CYP450 reductase (CPR) that contains prosthetic groups flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), that uses molecular oxygen and NADH or NADPH as a cofactor producing carbon substrate and oxidized products (Lin et al., 2022). They also use ferredoxin and ferredoxin reductase as a source of electrons for catalytic processes. CYP540 have been reported to play a key role in oxidizing various organic pollutants (Behrendorff, 2021; Hu et al., 2020). For instance, CYP540 has been found to break down PAHs such as naphthalene, chrysene, phenanthrene, fluoranthene, and pyrene to phenols and quinones as by-products (Behrendorff, 2021). Moreover, CYP540 have also demonstrated the potential degradation of polyhalogenated aromatics (Kumar and Kapur, 2016). Furthermore, the efficient oxidation of PCBs and alkanes by cytochrome P540 have also been reported (Daccò et al., 2020).

2.14.6 Dehydrogenases

Dehydrogenases are oxidoreductase enzymes that catalyze the oxidation of a compound via the transfer of hydrogen to an acceptor (Sui et al., 2023; Pandey et al., 2023). Dehydrogenases are classified according to the donor groups such as alcohol, aldehyde and the acceptor. Acceptors include nicotinamide adenine dinucleotide (NAD+ or NADH), nicotinamide adenine dinucleotide phosphate (NADP+ or NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and cytochromes. Alcohol dehydrogenase catalyzes the oxidation of alcohol to aldehyde or ketone with the reduction of $NAD(P)^+$ to NAD(P)H (Sui et al., 2023). Similarly, aldehyde dehydrogenases catalyzes the NAD(P)+-dependent oxidation of the aldehyde to a carboxylic acid (Lara-Moreno et al., 2023). Likewise, naphthalene dihydrodiol dehydrogenase and benzyl alcohol dehydrogenase have been reported in the metabolism and degradation of aromatics compounds (Pandey et al., 2023).

2.15 Enzymatic mechanisms used by microalgae for biodegradation

Studies have shown that microalgae species such as *Chlorella*, *Chlamydomonas*, *Spirulina*, *Scenedesmus*, *Nostoc*, *Oscillatoria*, *Desmodesmus*, *Arthrospira*, *Nodularia*, *Cyanothece*, *Ulva lactuca*, *Kappaphycus alvarezii* could degrade oil contaminants (Hammed et al., 2016; He et al., 2016). To date, no catabolic pathways have been confirmed regarding the biodegradation of oil contaminants and the enzyme activities with regards to the degradation process are yet to be understood and known.

2.15.1 Alkane catabolic mechanism

In general, long-chains and short-chain alkanes are the most easily degraded, followed by branched and cyclic alkanes (Ichor et al., 2016; Wang et al., 2023). Alkanes containing fewer carbons are prone to volatilization, while alkanes containing more carbons are less volatile (Patowary et al., 2017). Over the years, studies have shown that alkane degradation by microalgae is an oxidative process carried out by different enzymatic reactions (Olajire and Essien, 2014). The initial step of alkane degradation usually starts with the oxidation of a methyl group hydrocarbon via enzymes called alkane hydroxylases to form an alcohol. The alcohol formed is subsequently oxidized by alcohol dehydrogenase and aldehyde dehydrogenase to the corresponding aldehyde and fatty acid, respectively (Wang et al., 2023). The fatty acids are further oxidized via baeyer-villiger monooxygenase system to acetyl-CoA and finally to carbon dioxide and water (Ichor et al., 2016; Wang et al., 2023). In another route, the alkane methyl group is broken down into secondary alcohol, which is converted into a corresponding ketone, then oxidized via baeyer-villiger monooxygenase to an ester. The ester is further hydrolyzed by an esterase enzyme to form a fatty acid ester (Olajire and Essien, 2014; Patowary et al., 2017). Fig 2.4 shows a typical metabolic degradation of alkanes (heptadecane and nonadecane).

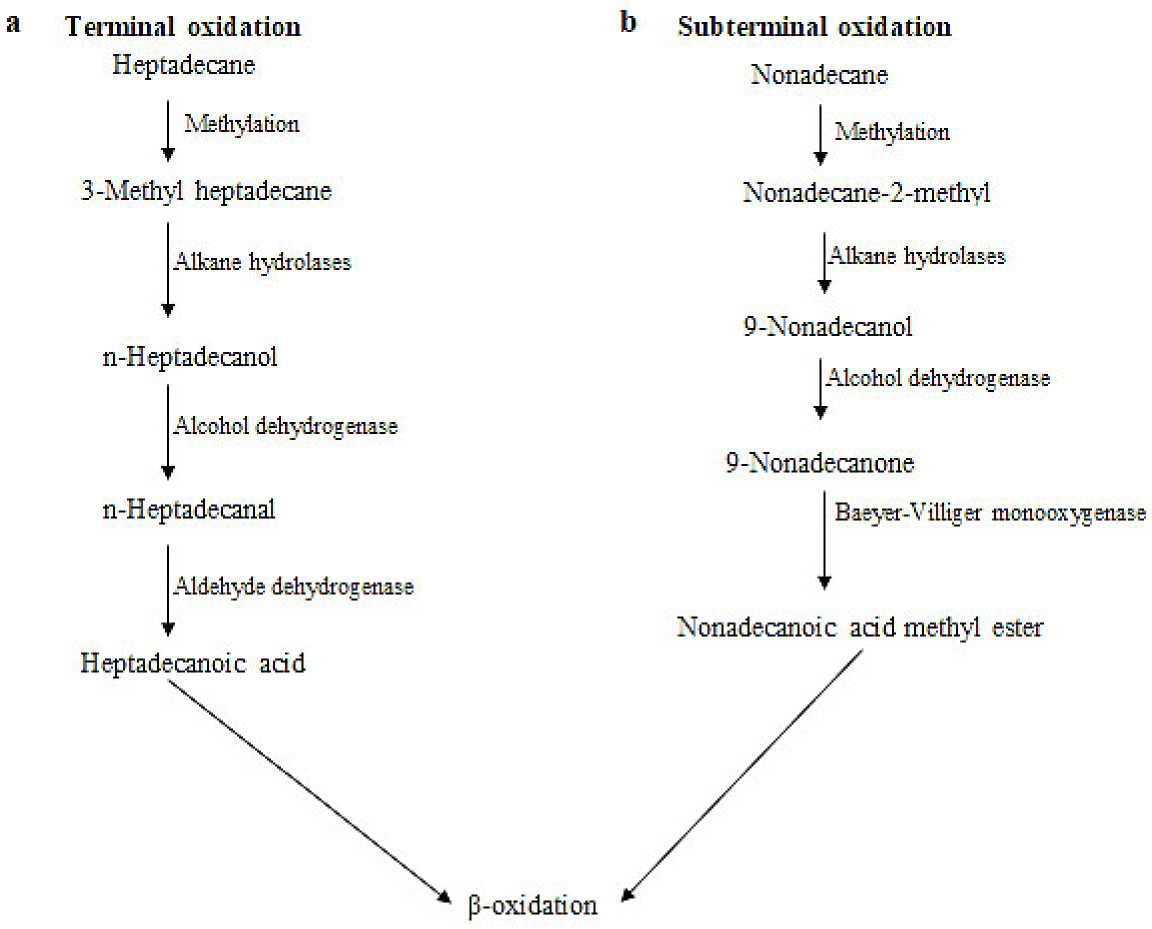

Fig. 2. 4: The proposed pathway for the degradation of alkanes (heptadecane and nonadecane) by microalgae. (a) Terminal oxidation pathway; (b) Subterminal oxidation.

2.15.2 Aromatic hydrocarbon catabolic mechanism

Different microalgae have been reported to biodegrade PAHs, in which biodegradation of naphthalene, benzo(a)pyrene, pyrene, phenanthrene, anthracene and fluorene have been the most researched. To date, not much is known about the metabolic pathway of the different PAH degradation (Padhi and Gokhale, 2017; Żyszka-Haberecht et al., 2019; Patel et al., 2020). In the aerobic degradation of aromatic HC, oxygen acts as the electron acceptor and as a co-substrate for the hydroxylation of the ring cleavage of the aromatic ring (Subashchandrabose et al., 2013). The catabolism of PAHs through an oxygenase-mediated process involves the monooxygenase and dioxygenase systems. Generally, the initial phase in the catabolism of PAHs occurs by hydroxylation of the aromatic ring through a dioxygenase enzyme system, to form a cis-dihydrodiol. This cis-dihydrodiol is then cleaved by intradiol dioxygenases or extradiol dioxygenases via ortho-cleavage or meta-cleavage pathway, to form catechol that is further transformed to tricarboxylic acid cycle (Ghasemi et al., 2011; Subashchandrabose et al., 2013). Żyszka-Haberecht et al. (2019) reported the catabolic degradation of naphthalene. Naphthalene was broken down into cis-naphthalene dihydrodiol by naphthalene dioxygenase. The cis-naphthalene dihydrodiol was further oxidized to 1,2-dihydroxynaphthalene via dehydrogenation by dehydrogenase, which was then metabolized into 2-hydroxychromene 2-carboxylate by ring-opening reaction mediated by dioxygenase. The final products were further oxidized into compounds such as acetyl-CoA and succinyl-CoA which entered the tricarboxylic acid cycle. Similarly, Subashchandrabose et al. (2013) reported the oxidation of benzo(a)-pyrene by *Selenastrum capricornutum*, via dioxygenase enzyme system to cis-dihydrodiols which were then converted to sulphate ester as well as α and β-glucoside conjugates. Moreover, Patel et al. (2020) reported the oxidation of phenanthrene which resulted in formation of cis-3,4-dihydroxy-3,4-dihydrophenanthrene by phenanthrene dioxygenase through hydroxylation. Then the cis-3,4-dihydroxy-3,4-dihydrophenanthrene was broken down into 3,4-dihydroxyphenanthrene after undergoing dehydrogenation. Afterwards, metabolized to 1-hydroxy 2-naphthoic acid and finally oxidized via salicylaldehyde and salicylic acid to catechol. Fig 2.5 shows a typical degradation of aromatic compounds (benzene and naphthalene).

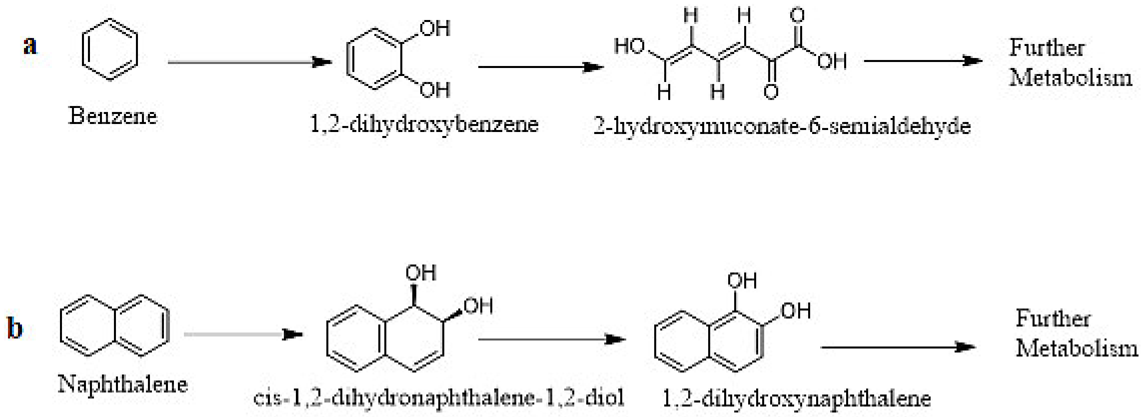

Fig. 2. 5: The proposed pathway for the degradation of aromatic compounds. (a) Benzene degradation; (b) Naphthalene degradation.

2.16 Conclusion and future prospects

The potential of microalgae in biodegrading spent oil waste contaminants has been highlighted. While microalgae has been successful in degrading HC pollutants using different approaches, however, the complete removal of persistent and recalcitrant pollutants remains a significant challenge. Therefore, further research is essential to enhance the degradation capabilities of microalgae. One strategy to address this challenge is through genetic modification to improve microalgae's biodegradation capability and overall metabolic performance. Additionally, optimizing the microalgae degradation process is crucial due to their sensitivity to growth and environmental conditions. This optimization process could provide insights into the factors influencing the microalgae biodegradation process and their interrelatedness. Furthermore, additional investigations are needed to analyze the enzymes and genes involved in the degradation of spent oil pollutants. This analysis will offer a comprehensive understanding of the key pathways, catabolic genes, and cellular processes involved in the biodegradation and biotransformation of spent oil pollutants. Implementing these strategies could make microalgae a viable option for effectively eliminating spent oil pollutants from the environment.

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CHAPTER 3

Effect of ultra-violet light radiation on *Scenedesmus vacuolatus* growth kinetics, metabolic performance, and preliminary biodegradation study

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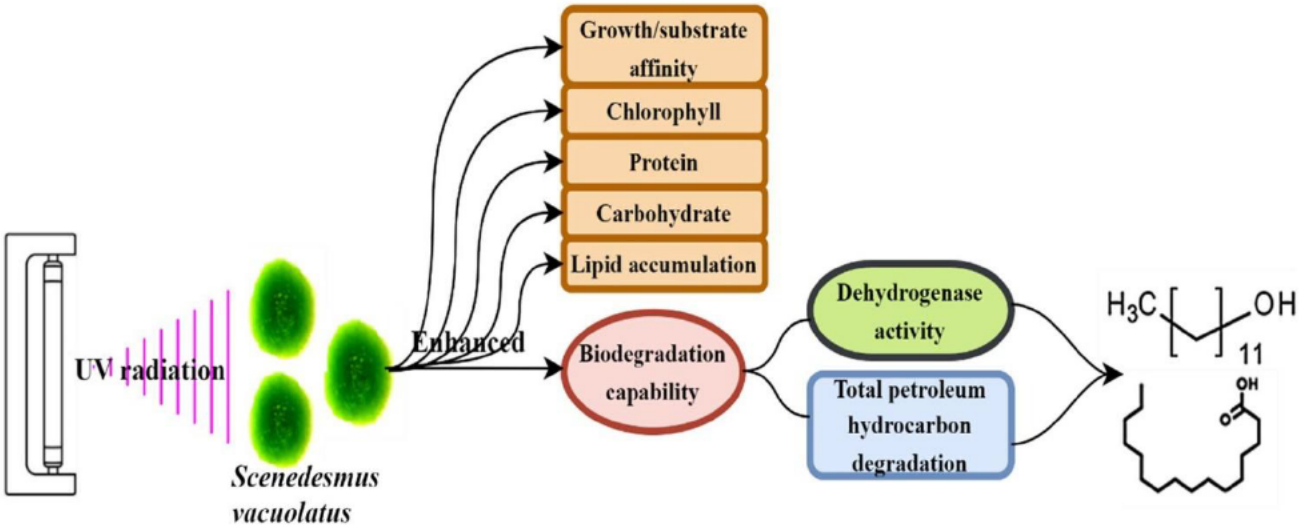
Graphical abstract



Keywords UV radiation · *Scenedesmus vacuolatus* · Growth kinetics · Substrate affinity · Biodegradation

Introduction

The illegal disposal of spent oil waste into the environment has sparked a lot of concern worldwide due to its impact on human health, plants, animals, and microorganisms (Rathi and Yadav 2019; Saha et al. 2019). The urgent need and search for a cost-effective and environmentally friendly clean-up approach that can mitigate the harmful effect of these oil waste pollutants is of great importance to the future sustainability of the environment. Various strategies such as landfilling, high-temperature incineration, chemical decomposition and recycling have been used to treat spent oil waste (Kajdas 2014). These methods are not only too expensive to provide a satisfactory solution but create another environmental concern, such as generating more greenhouse gases (dioxins, carbon monoxide, nitrogen dioxide, sulphur dioxide). These gases contaminate the atmosphere and destroy the ozone layer resulting in severe damage to the environment (Asgari et al. 2017; Sihag and Pathak 2016). The use of microbes such as microalgae to degrade lubricant oil waste has gained global attention due to its sustainability and environmental benefits. The use of microalgae to degrade lubricant oil waste has numerous advantages over existing traditional and chemical treatment methods. These include the ability to thrive in a variety of polluted environments with extreme conditions, production of enzymes for quick metabolism of pollutants, conversion of organic pollutants into useful environmentally friendly compounds, cost-effectiveness, relatively safe and ecologically sustainable. Microalgae treatment's investment and economic cost are 5–20 times less than traditional and chemical treatment (Touliabah et al. 2022). Studies using different green microalgae such as the species (sp) of *Chlamydomonas*, *Selenastrum, Scenedesmus Cyanobacteria* and *Chlorella* to treat lubricant oil waste have been reported (Dell' Anno et al. 2021; Radziff et al. 2021; Touliabah et al. 2022). *Scenedesmus* species (sp) has been recognized as one of the most effective microalgae in oil waste biodegradation due to their strong photosynthetic activity and cell surface area (Gonçalves 2021; Ismail et al. 2020).

Scenedesmus belong to the class of *Chlorophyceae* (Akgül et al. 2017). They are among the few photosynthetic microalgae that have been mass-produced for food, feed and high-value natural products globally (Sarwa and Verma 2017). It is a microalgae with unique characteristics: (a) rapid growth rate, (b) high accumulation of biomass and easy to harvest, (c) high source of natural products such as oils, pigments, protein, fatty acids and lipids, (d) it can be cultivated in low-cost medium and the presence of recalcitrant organic pollutants (e) high carbon fixation ability, (f) its ability to grow at extreme conditions such as high temperature and pH (Baghour 2019; El-Sheekh et al. 2013). Even though microorganisms can resist a wide range of recalcitrant organic pollutants under environmental pollution stress, this approach becomes inefficient in the presence of some recalcitrant organic pollutants such as spent lubricant oil waste. With the recent advancement in molecular biology and genetic engineering, novel approaches to solving the degradation of xenobiotic pollutants have been made possible (Fajardo et al. 2020; Kumar et al. 2020). Despite the depth of research on *Scenedesmus* for environmental pollutant degradation, there is a dearth of knowledge on improving their biodegradative potential by improving cellular metabolic performance. A study on strain improvement of *Scenedesmus vacuolatus* for improving spent lubricant oil waste degradation is desirable, cheaper and sustainable. Strain improvement through mutagenesis has been reported to be an effective method to enhance microalgae metabolic performance and degradative capabilities (Arora et al. 2020). Mutagenesis in microalgae is commonly carried out by physical or chemical mutagens. Physical mutagens involve the exposure of microalgae to radiation such as ultraviolet (UV) radiation, gamma-ray, and X-rays, whereas the chemical mutagens involve the use of chemicals including ethyl methanesulfonate, hydroxylamine, nitrous acid and methylnitronitrosoguanidine (Hlavova et al. 2015; Thurakit et al. 2018). In this study, mutagenesis by UV light radiation is of significant interest because it is considered efficient, simple, rapid, relatively safe and does not involve any specialized training for its implementation (Lim and Schenk 2017). Furthermore, microalgae strains obtained by mutagenesis are not subject to the same stringent regulatory constraints as genetically modified microorganisms, necessitating no legal or regulatory approval before commercial use. The UV radiation induces random mutation in the microorganism deoxyribonucleic acid via base pair deletion, addition and substitution resulting in new sets of pyrimidines dimer and thymine dimers (Choi et al. 2017; Fu et al. 2016; Moha-León et al. 2019; Sydney et al. 2018). Owing to this UV mutagenesis method's simplicity, it has been employed to obtain microalgae strains with improved metabolic and physiological processes (Arora et al. 2020). For instance, an increase in growth, biomass concentration, carbohydrates, protein, lipids and chlorophyll contents were observed with *C. vulgaris* after UV radiation exposure (Sarayloo et al. 2018). Likewise, the exposure of *Nannochloropsis oculata* to UV radiation resulted in a higher growth rate and production of several lipids and fatty acids (Moha-León et al. 2019). The authors also reported on the release of defense biomolecules by *Nannochloropsis oculate* to protect itself from the effects of the UV radiation. Many microalgae species have been reported to have a variety of protective mechanisms against UV radiation, including DNA repair, production of antioxidants and UV-absorbing compounds such as mycosporine-like amino acids (MAAs) and scytonemin (Pessoa 2012; Rastogi et al. 2020). However, with the abundance of literature on the effect of UV radiation on microalgae, no research on the impact of UV radiation on *Scenedesmus vacuolatus* has been reported. A study on UV mutagenesis of *S. vacuolatus* for improved spent oil degradation could provide insight into *S. vacuolatus* with improved metabolic and degradative performance. Therefore, this study investigates the effect of the physical mutagen UV radiation on *S. vacuolatus* cellular structure, metabolic performances and growth for improved spent oil degradation. Thereafter, the UV exposed *S. vacuolatus* was assessed for biodegradation of waste lubricant oil.

Materials and methods

Culture medium composition

Blue-green 11(BG11) medium containing three different solutions (solution A, solution B and solution C) was used in this study. Solution (A) contains the following in (g/L) 2.86 g H_3BO_3, 1.81 g $MnCl_2{\cdot}4H_2O$, 0.22 g $ZnSO_4{\cdot}7H_2O$, 0.39 g $Na_2MoO_4{\cdot}2H_2O$, 0.08 g $CuSO_4{\cdot}5H_2O$ and 0.05 g $CO(NO_3)_2{\cdot}6H_2O$. Solution (B) in (500 mL) contains 2.0 g K_2HPO_4, 3.75 g $MgSO_4{\cdot}7H_2O$, 1.80 g $CaCl_2.2H_2O$, 0.30 g Citric acid, 0.30 g Ammonium Ferric Citrate green, 0.05 g $EDTANa_2$, and 1.0 g $NaCO_2$. Solution (C) in (g/L) contains 15.0 g $NaNO_3$. To make 1L BG11 broth medium, 1 mL of solution (A), 10 mL of solution (B) and 100 mL of solution (C) were taken from each sock solution. For the solid BG11 agar medium, 15.0 g of bacteriological agar was added to the 1L BG11 medium. The pH was adjusted to 7.0 using 1 M

NaOH or HCl prior to sterilization by autoclave at 121 °C for 15 min.

S. vacuolatus UV exposure

Scenedesmus vacuolatus isolated from spent lubricant oil waste (Eregie and Jamal 2019) was used for the UV mutagenesis in this study. Kultschar et al. (2019) method was modified for the UV mutagenesis experiment. Colonies of *S. vacuolatus* were inoculated on BG11 agar plate and allowed to grow for 14 days under standard conditions. The obtained *Scenedesmus vacuolatus* colonies were exposed to UV light (UV-C) radiation at a distance of 15 cm and at a wavelength of 254 nm with an intensity of 1.4 mW/cm^2 for different time duration of 2, 4, 6, 12, 24 and 48 h. After the UV radiation exposure, colonies of the exposed cells were re-incubated under a 24 h darkness at 25 °C. The re-incubated cells were further subcultured onto a fresh BG11 agar plate under a 12 h daylight/darkness cycle at 25 °C for 2 weeks. The UV-exposed colonies at different time intervals were randomly selected based on the colony's visibility for characterization and preliminary biodegradation study.

Microscopy morphological characterization

Morphological characterization of both wild-type and UV-exposed microalgae was carried out using stereo microscopy (SM) (LEICA MZ16, with camera LEICA DFC450C), bright field microscopy (BFM) (OLYMPUS A×70) and scanning electron microscopy (SEM) (ZEISS EVO L515ss). The SM at different magnification was used to view directly the microalgae colonies grown on BG11 agar to capture the morphological features. While for BFM, a drop of microalgae aliquot grown in BG11 medium was pipetted on a clean glass slide, then one drop of formaldehyde to fix the microalgal cells for five minutes before the BFM analysis. The SEM was used for further morphological characterization of the microalgal cells. Samples were prepared by pipetting a drop of the microalgal sample securely onto an aluminium SEM stub with double-sided carbon tape. This was allowed to dry completely. The samples were sputter-coated with a gold coat using Quorum Q150RES before the SEM viewing.

Growth condition

The wild-type and UV-exposed *S. vacuolatus* (10% v/v) separately were inoculated into 100 mL BG11 medium in 250 mL Erlenmeyer flasks. The detailed BG11 growth medium constituent is described in section "Culture medium composition". The culture flasks were incubated at 25 °C for 21 days and sampling were carried out at regular interval (Eregie and Jamal-Ally 2019, 2021). Samples were thereafter analyzed for biomass concentration, cellular biomolecules (chlorophyll, carotenoid, protein, carbohydrate and lipid contents), growth rate, substrate versatility and substrate affinity.

Substrate versatility study

Substrate versatility of the UV-exposed *S. vacuolatus* using different substrate types was determined by replacing the BG11 medium as the only carbon source with sodium carbonate Na_2CO_3, glucose and glycerol differently. Growth was monitored at 25 °C for 21 days and sample for analysis was taken every 3 days.

Preliminary biodegradation of spent coolant waste setup

To evaluate the degradation efficiency of UV exposed *S. vacuolatus* using spent coolant waste (SCW) as substrate, biodegradation analysis was carried out. The experimental setup was carried out in BG 11 medium with a working volume of 100 mL containing 10 mL of the wild or UV exposed *S. vacuolatus* separately and 10% (v/v) of SCW as the only carbon source. The flasks were agitated on a shaker incubator at 25 °C for five weeks under white fluorescent light (12 h daylight followed by 12 h of darkness cycle). The control experiment was done with BG11 medium having SCW without inoculum. Samples were taken each week from different flasks and analyzed for dehydrogenase activity and total petroleum hydrocarbon. Total petroleum hydrocarbon and dehydrogenase activity were determined using previously established protocols by Irawan et al. (2018) and Ding et al. (2013), respectively.

Analytical methods

Microalgal biomass concentration (cell dry weight) was obtained by measuring the optical density at 680 nm using SpectroVis plus Spectrophotometer (USA). The cell dry weight was then determined using a standard calibration curve, a correlation dependence of biomass dry weight as a function of optical density (Savvidou et al. 2021).

The chlorophyll composition was estimated using the method described by Tomar and Jajoo (2021). 5 mL of microalgae culture was taken and centrifuged at 5000 rpm for 5 min. The supernatant was discarded, and 5 mL of 99.9% methanol was added to the microalgae pellet, mixed properly and incubated at 90 °C for 5 min. Then the sample was centrifuged at 10,000 rpm for 5 min. The supernatant was collected, and the optical density was measured at 666, 653, and 470 nm for chlorophyll A (Chl *a*), chlorophyll B (Chl *b*) and carotenoid (Cx), respectively. The actual chlorophyll estimation was afterward calculated as follows:

$$\begin{aligned}&\mathrm{Chl}a(\mu\mathrm{g/mL}) - 15 : 65\mathrm{A}_{666} - 7 : 340\mathrm{A}_{653}\\&\mathrm{Chl}b(\mu\mathrm{g/mL}) = 27 : 05\mathrm{A}_{653} - 11 : 21\mathrm{A}_{666}\\&\mathrm{Cx} + \mathrm{c}\,(\mu\mathrm{g/mL}) = \left(1000\mathrm{A}_{470} - 2 : 86\,\mathrm{Chl}a - 129 : 2\,\mathrm{Chl}b\right)/245\end{aligned} \tag{1}$$

The total protein content was obtained using the previously described protocol by Ganapathy (2017) and Sanusi et al. (2020). The protein concentration was then extrapolated by a predetermined standard calibration curve using bovine serum albumin as the standard (Bradford method 1976).

Carbohydrate content was obtained by transferring 2 mL of microalgae samples into centrifuge tubes containing saturated mercuric chloride (0.1 mL). Mercuric chloride was used to inhibit any further substrate utilization by the microalgae cells. The mixed samples were centrifuged at 5000 rpm for 10 min. Afterward, 1 mL aliquot of the solution was extracted for the total carbohydrate determination using the Anthrone-sulphuric method. Total carbohydrate was then deduced from a standard curve using Anthrone reagent with glucose as standard at 620 nm (Sanusi et al. 2020).

The residual glucose concentration in the supernatant (sample centrifuge at 10,000 rpm for 5 min) was determined using the dinitrosalicylic acid (DNS) method (Miller 1959). Sugar utilization was then determined using Eq. (2).

$$Sugar\ utilization = \frac{Initial\ sugar\ concentration - final\ sugar\ concentration}{Initial\ sugar\ concentration} \times 100 \tag{2}$$

The microalgae lipid content was obtained by solvent extraction method (Bligh and Dyer 1959). One millilitre of aliquot was centrifuged and the pellet with 80 mL distilled water was homogenized and heated at 2450 MHz for 5 min using a 1000 W capacity Samsung microwave oven (Model: ME9114S1, Malaysia). Thereafter, 10 mL and 20 mL of chloroform and methanol were added to the disrupted cells and vortexed for 10 min. Chloroform (10 mL) was further added to the mixture with 10 min homogenization. The mixture was vortexed for an additional 10 min after adding 10 ml of distilled water. The final mixture was filtered using acrodisc syringe filters to obtain a two-layer filtrate (lipid + chloroform) and the chloroform was evaporated from the bilayer. Then the lipid content was determined gravimetrically.

Kinetic calculations

Then the specific growth rate values (μ) and the initial substrate concentration value was used to estimate the maximum specific growth rate (μ_{max}) and Monod constant (Ks).

$$Specific\ growth\ rate\,(\mu) = \frac{InX_2 - InX_1}{\left(t_2 - t_1\right)} \tag{3}$$

where μ is the specific microalgae growth rate, X_2 and X_1 were the biomass concentration at time T_2 and $T_{1,}$ respectively. The linear representation of this equation is expressed as follows:

$$\frac{1}{\mu} = \frac{1}{\mu_{max}} + \frac{K_s}{\mu_{max}}\left(\frac{1}{S}\right) \tag{4}$$

where S is the substrate concentration.

Moreover, the logistic model (Eq. 4) was employed to depict the correlation of *S. vacuolatus* biomass (X), at particular time (t) during the log and the stationary growth phase of *S. vacuolatus* to initial biomass concentration (X_0), maximum biomass concentration (X_{max}) and maximum specific growth rate (μ_{max}).

$$X = \frac{X_0 \cdot exp\left(\mu_{max} \cdot t\right)}{1 - \left(\frac{X_0}{X_{max}}\right) \cdot \left(1 - exp\left(\mu_{max} \cdot t\right)\right)} \tag{5}$$

The kinetic growth constant was estimated by fitting a linear function to exponential phase. This was calculated based on the following equation.

$$ln\left(\frac{X}{Xo}\right) = K_g t \tag{6}$$

where X is the growth of microalgae as function of time, X_o is the initial growth of microalgae, k_g is the kinetic growth constant.

Statistical analysis

The data obtained from each experiment was analyzed with a one-way analysis of variance (ANOVA), using the Statistical Package for the Social Sciences (SPSS) version 28. The computed data are means of 3 replicates (n = 3) and all results are expressed as mean ± standard error for each strain. The significant difference was performed at a 5% level of significance.

Results and discussion

Effect of UV radiation on *S. vacuolatus* structural morphology

The UV-exposed *S. vacuolatus* showed structural cellular modifications and arrangement (Figs. 1, 2, 3). Exposed cells underwent cellular fusion, elongation and cell-to-cell pairing, SM (Fig. 1b–g), BFM (Fig. 2b–g) and SEM (Fig. 3b–g) microscopy in comparison with the wild-type (Figs. 1a, 2a and 3a) respectively. Other studies have observed similar cellular morphological alterations in *Scenedesmus* sp after exposure to mutagen (Akgül et al. 2017; Beherepatil and Deore 2013; Sarwa and Verma 2017). The cell elongation, cell fusion and pairing of cells could be ascribed to the effect of the UV radiation on *S. vacuolatus* during cellular development (Choi et al. 2017; Colina et al. 2020). The elongation of the cells and cell fusion increases the cell surface area, which has the potential to improved *S. vacuolatus* photosynthetic activities and cellular metabolic performance. Microalgae with higher surface area to volume ratio and large cell size are good bioremediators (Subashchandrabose et al. 2013). Moreover, the cell's

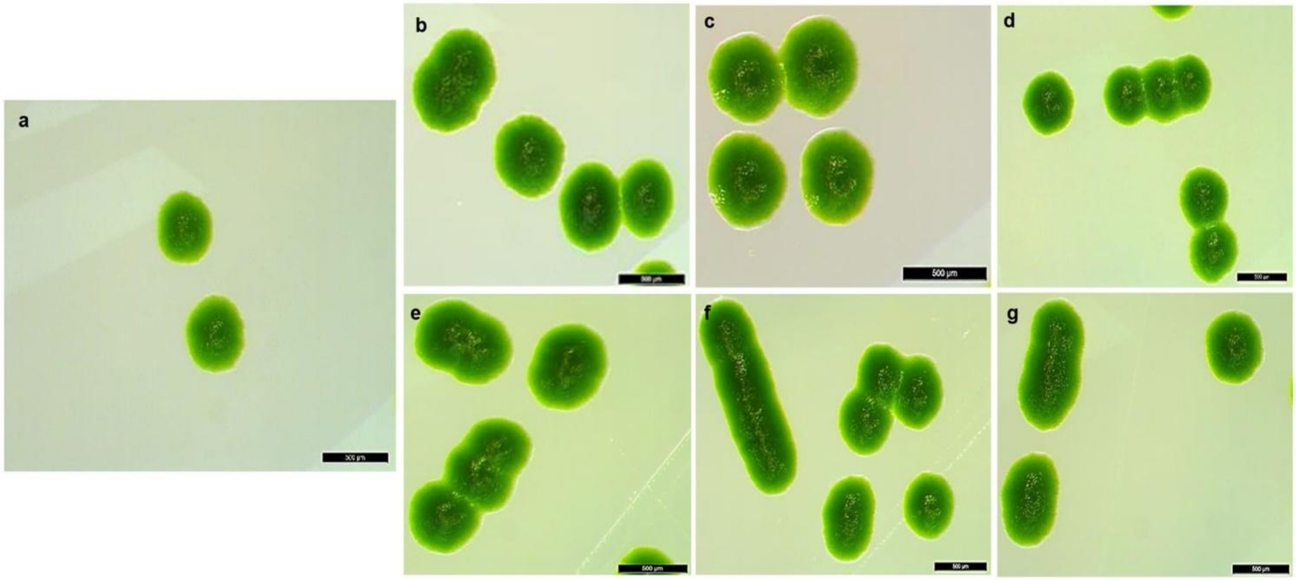


Fig. 1 Colonies of microalgae strains as observed under stereo microscope (500 µm). **a** Wild *S. vacuolatus* microalgae, and **b**, **c**, **d**, **e**, **f**, and **g** are UV exposed *S. vacuolatus* for 2, 4, 6, 12, 24 and 48 h, respectively

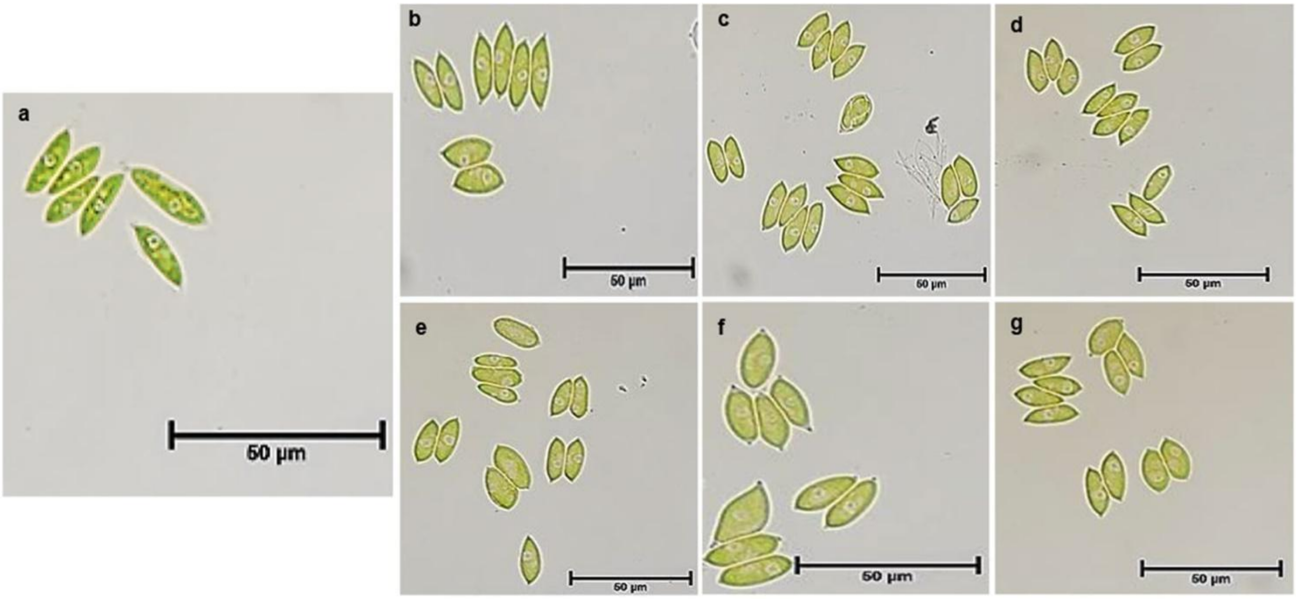


Fig. 2 Brightfield microscopy (BFM) micrograph of microalgae cells under ×40 magnification. **a** Wild-type microalgae (unexposed); **b**, **c**, **d**, **e**, **f**, and **g** *S. vacuolatus* exposed to UV light at different time intervals (2, 4, 6, 12, 24, and 48 h) respectively

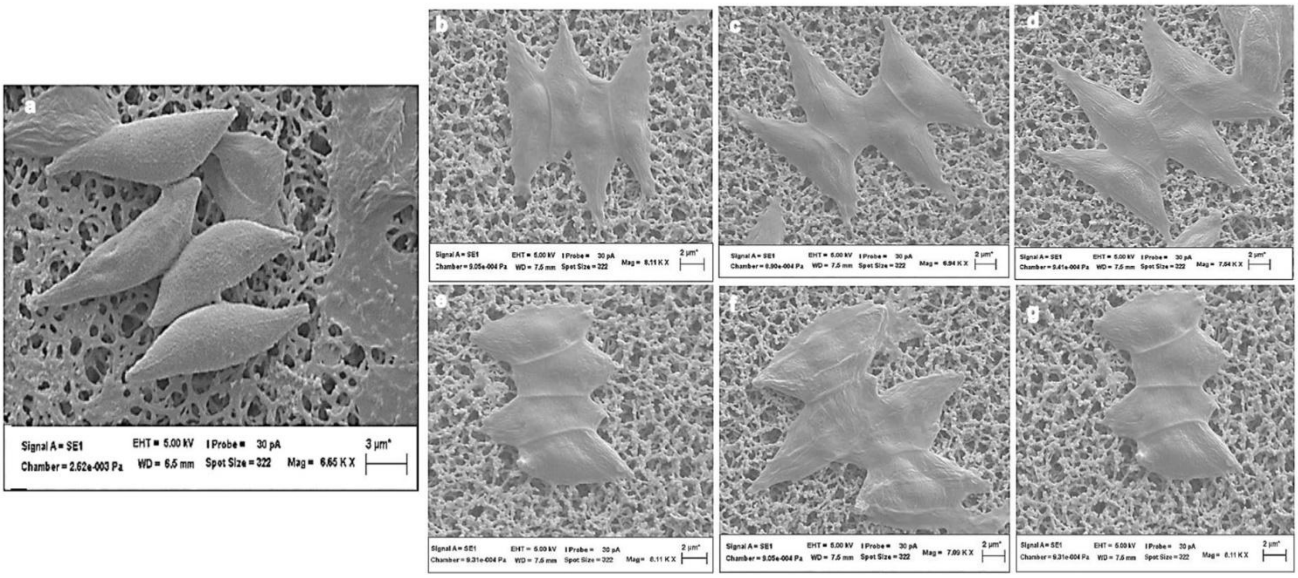


Fig. 3 Scanning electron microscopy (SEM) micrograph of microalgae cells. **a** Wild S. vacuolatus, **b**, **c**, **d**, **e**, **f**, and **g** are UV exposed S. vacuolatus for 2, 4, 6, 12, 24 and 48 h respectively

morphological modification, especially the concave shape, could also improve the photosynthetic activities through improved angulation of the light ray on the cell wall. Similarly, the non-damage to the green chlorophyll pigment is an indication of the cell integrity not been damaged, this desirable since chlorophyll is a photosynthetic apparatus (Choi et al. 2017; Sarwa and Verma 2017). The findings in this study were consistent with earlier studies on using UV light radiation for microalgae mutagenesis (Carino et al. 2022; Colina et al. 2020). The differences in cellular modifications observed in this study compared to other studies can be attributed to the differences in the choice of microalgae species, the length of UV exposure and the operational condition implemented (Carino et al. 2022; Choi et al. 2017; Colina et al. 2020).

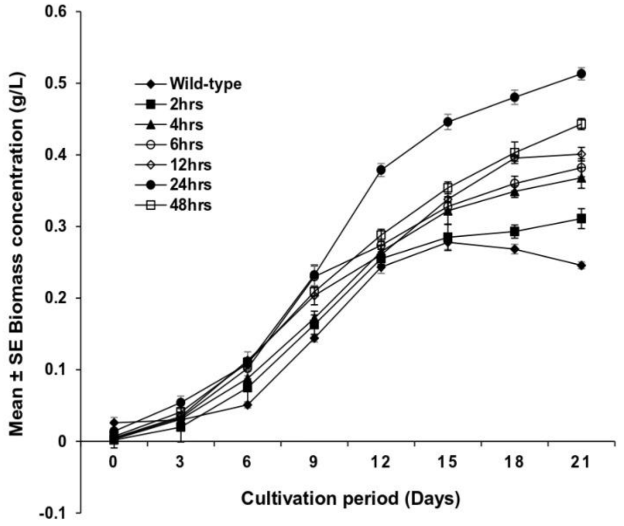


Fig. 4 The growth profile of the wild *S. vacuolatus* (unexposed *S. vacuolatus*) and UV exposed *S. vacuolatus* at different time interval (2, 4, 6, 12, 24, 48 h). Mean ± standard error of three replicates (n = 3)

Effect of UV radiation exposure on *S. vacuolatus* growth

The growth curves of the UV-exposed *S. vacuolatus* at different exposure times are shown in Fig. 4. A shortened lag period and a sharp extended exponential phase was observed for the 24 h UV exposed *S. vacuolatus* compared with the growth patterns obtained for 2, 4, 6, 12, 48 h UV exposed *S. vacuolatus* and the wild *S. vacuolatus*. Similarly, the specific growth rate (μ) of the 24 h UV exposed *S. vacuolatus* (0.024 day^{-1}) was higher compared to 0.015, 0.017, 0.018, 0.019, 0.020 and 0.012 day^{-1} obtained for 2, 4, 6, 12, 48 h UV exposed *S. vacuolatus* and the wild *S. vacuolatus*, respectively. Furthermore, the obtained biomass concentration (0.513 ± 0.008 g/L) of the 24 h UV exposed *S. vacuolatus* after 21 days of cultivation period showed significant increase ($p < 0.001$) in biomass concentration in comparison to 0.311 ± 0.009 g/L, 0.368 ± 0.015 g/L, 0.382 ± 0.013 g/L, 0.401 ± 0.009 g/L, 0.443 ± 0.008 g/L and 0.268 ± 0.005 g/L, for 2, 4, 6, 12, 48 h UV exposed *S. vacuolatus* and the wild-type respectively. The high μ and biomass concentration values obtained for the UV-exposed *S. vacuolatus* suggest the impact of UV radiation on *S. vacuolatus* growth. Also, the substantial improvement in specific growth rate observed in the study could be attributed to improved substrate affinity observed with the UV-exposed *S. vacuolatus*. Higher growth rates due to substrate availability and enhanced affinity could trigger certain cellular activities, thus increasing photosynthetic capacity and productivity. Likewise, the higher growth rates and biomass accumulation observed in the present study by the UV-exposed *S. vacuolatus* could be ascribed to the impact of UV light radiation on *S. vacuolatus* photosynthetic apparatus (chlorophyll a and b, carotenoids, large and elongated cell size) (Figs. 1 and 5), that could have resulted in enhanced photosynthetic activities (Subashchandrabose et al. 2013). The modification of the cellular structure through UV exposure has been reported to potentially improve both photosynthetic and metabolic activities of microalgae species (Liu

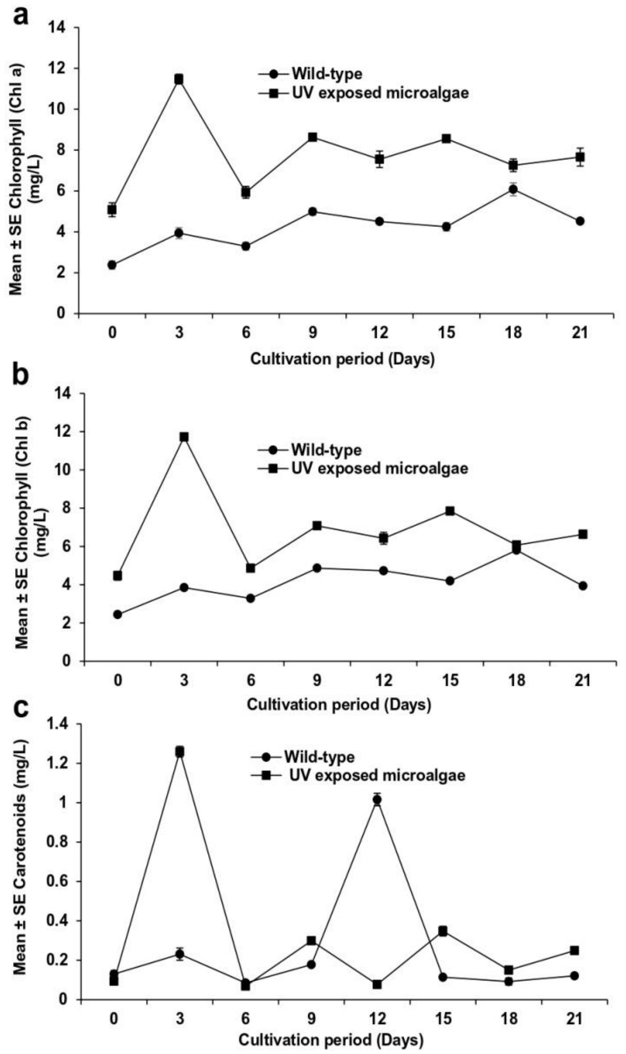


Fig. 5 Effect of UV light radiation on the total chlorophyll content of wild *S. vacuolatus* and UV exposed *S. vacuolatus*. **a** Chlorophyll a; **b** chlorophyll b; **c** carotenoids. Mean ± standard error of three replicates (n = 3)

et al. 2015). Amongst the UV-exposed *S. vacuolatus*, 24 h UV-exposed *S. vacuolatus* showed the best desirable traits such as increased chlorophyll content, enhanced substrate affinity, high sugar utilization and growth rate compared to other UV-exposed *S. vacuolatus*. Hence, the 24 h UV-exposed *S. vacuolatus* was selected for further studies.

Chlorophyll-carotenoid content of UV exposed *S. vacuolatus*

The chlorophyll and carotenoid contents of the 24 h UV exposed *S. vacuolatus* is presented in Fig. 5a–c. Significantly ($p < 0.001$) higher Chl a (11.470 ± 0.003 µg/mL), Chl b (11.725 ± 0.002 µg/mL) and carotenoid (1.259 ± 0.008 µg/mL) pigment were observed for the 24 h UV exposed *S. vacuolatus* compared to the wild-type (Chl a = 6.080 ± 0.006 µg/mL, Chl b = 5.806 ± 0.003 µg/mL and carotenoid = 1.016 ± 0.009 µg/mL). This is 1.89, 2.02 and 1.24-fold higher than the wild *S. vacuolatus* pigments, respectively. High Chl a, Chl b and carotenoid pigment obtained for the UV-exposed *S. vacuolatus* indicate a positive impact of the UV radiation on the pigment production apparatus (Gonçalves 2021). Improved pigmentation could be attributed to alterations in the genetic make-up responsible for pigment production. High pigmentation may enhance the capturing of light energy and consequently, high photosynthetic activities. It has been reported that Chl a, Chl b and carotenoid are the principal photosynthetic biomolecular apparatus in microalgae that captures light energy and CO_2. These photosynthetic apparatuses are also UV filters to protect the photosystems from the damaging effects of UV light radiation (El-Sheekh et al. 2021). A similar increase in chlorophyll contents by *Chlorella* sp and *Chlamydomonas reinhardtii* was reported by Liu et al. (2015) after their exposure to UV radiation. On the other hand, the total Chl content of 24 h UV-exposed *S. vacuolatus* in the current study differs from those of Kumar et al. (2018). They reported a decrease in the chlorophyll contents of *C. sorokiniana* (Chl a 1.51 µg/mL, Chl b 0.49 µg/mL), and carotenoid (0.81 µg/mL) after UV exposure. These variances in the Chlorophyll contents are most likely due to variations in exposure time, microalgae species and growth conditions employed.

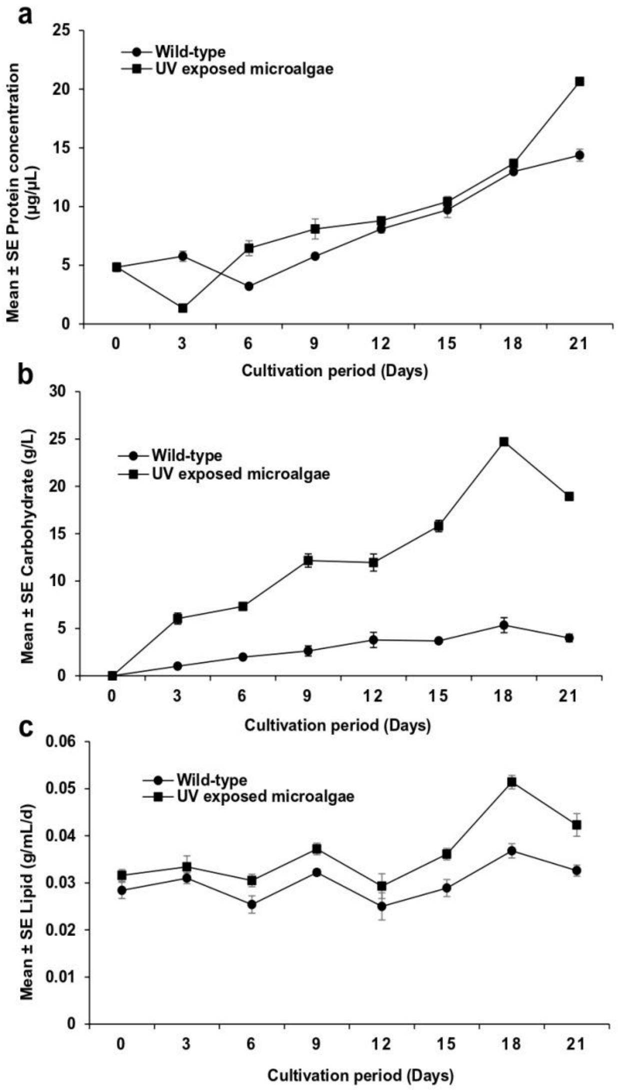


Fig. 6 Effect of UV light radiation on the total protein, carbohydrate, and lipid content of UV exposed S. vacuolatus. **a** Protein; **b** carbohydrate; **c** lipid. Mean ± standard error of three replicates (n = 3)

Protein, carbohydrate, and lipid accumulation in UV exposed *S. vacuolatus*

The protein content in the 24 h UV-exposed *S. vacuolatus* was 20.651 ± 0.003 g/L (Fig. 6a). This was 1.44 times significantly ($p < 0.001$) higher than that of the wild-type (14.372 ± 0.010 g/L). This increase in protein content can be attributed to the impact of UV radiation on the cellular protein machinery. On the hand, the ratio of protein to biomass (P:B) accumulation shows the wild-type (54:1) has higher P:B compared to the UV-exposed. *vacuolatus* (1:40). UV radiation has been used to positively bioengineer microorganisms to improved cellular biomolecules such carbohydrate and lipid (Ganapathy et al. 2017; Tenorio et al. 2022; El-Sheekh et al. 2021). The UV radiation in the present study favoured protein synthesis and accumulation and subsequently enhanced the *S. vacuolatus* enzymatic and metabolic activities. Proteins are one of the most abundant components in microalgae, accounting for up to 50% of total cell weight. Thus, alteration in the protein concentration in the cell could positively affect the overall microalgal metabolic performance (Colina et al. 2020). Moreover, the high protein accumulation suggests the activation of additional protein types such as the defense proteins and enzymes, during UV treatment. These defense proteins could be an adaptive response to protect the organism against UV radiation. The increase in the protein of the UV-exposed microalgae in this study correlates with the report of Kumar et al. (2018), who observed a 23% protein content for *C. sorokiniana* after exposure to UV radiation. Similarly, El-Sheekh et al. (2021) reported a twofold increase in the protein content of *Microcystis* after UV exposure. In addition, Colina et al. (2020) reported that the exposure of microalgae to UV radiation elevates microalgae adaptive mechanisms and the accumulation of biomolecules such as lipids, proteins, chlorophyll, and carbohydrates associated with the defensive mechanism.

The carbohydrate (CHO) accumulation by the wild *S. vacuolatus* and the 24 h UV-exposed *S. vacuolatus* is depicted in Fig. 6b. The result revealed that the total carbohydrate obtained for the wild-type increased from 1.017 ± 0.001 to 5.345 ± 0.016 g/L after 21 days of cultivation period. While the total CHO content obtained with UV-exposed *S. vacuolatus* increased significantly ($p < 0.001$) from 6.039 ± 0.008 g/L (day 3) to 24.707 ± 0.006 g/L (day 18), followed by a decline. This sharp decline in the CHO accumulation observed with the UV-exposed *S. vacuolatus*, could be an indication of rapid depletion of nutrient due to rapid growth. The 24.707 g/L CHO content observed for the UV-exposed *S. vacuolatus* is 4.62-fold higher in carbohydrate content compared to the wild *S. vacuolatus*. Moreover, the fraction of biomass to CHO is substantially higher with the UV-exposed *S. vacuolatus* (1:48) in comparison with fraction obtained with the wild-type *S. vacuolatus* (1:20). This further support the effect of UV treatment on the build-up of biomolecules such as CHO in *S. vacuolatus*. This increase in CHO is consistent with El-Sheekh et al. (2021) report, who also observed an increase in total CHO accumulation of *S. acutus* after a 24 h exposure to UV radiation.

Figure 6c depicts the significant difference ($p < 0.001$) of total lipid obtained in *S. vacuolatus* after UV exposure. The lipid build-up of the UV-exposed *S. vacuolatus* was 0.0514 ± 0.012 g/mL/day and 1.40-fold higher than the wild-type 0.0368 ± 0.015 g/mL/day. This result shows UV radiation's impact on the lipid biosynthesis metabolic pathway (Sivaramakrishnan and Incharoensakdi 2017). Carino and Vital (2022) and Choi et al. (2017) also reported a similar increase in lipid accumulation

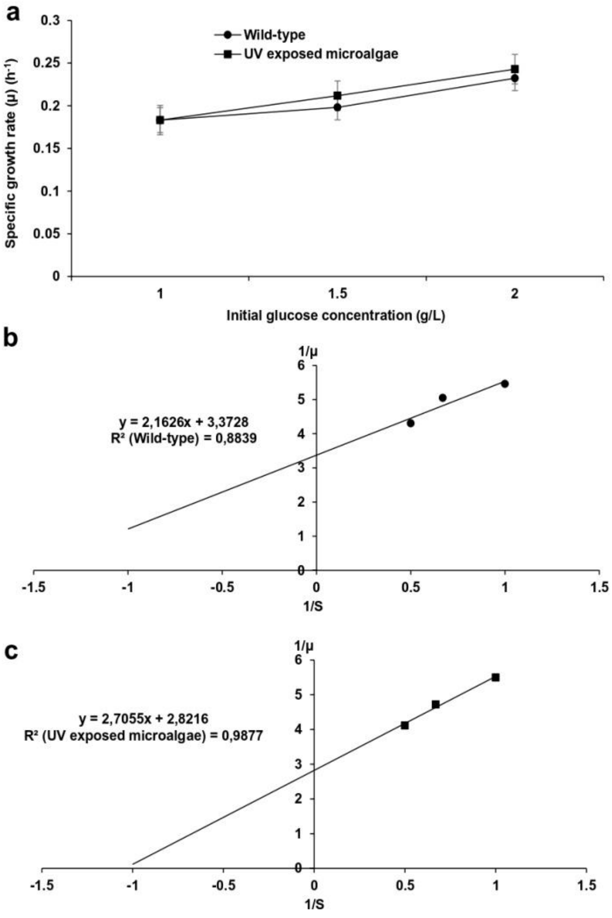


Fig. 7 Effect of UV radiation on *S. vacuolatus* growth. **a** Wild-type and UV-exposed microalgae specific growth rates (μ) at varied initial glucose concentrations increases with increase in initial glucose concentration. The specific growth rate of the UV-exposed microalgae was higher compared to the wild-type. **b**, and **c** Lineweaver–Burk plot estimating Monod constants for wild-type and UV exposed microalgae using glucose as substrate. Mean ± standard error of three replicates (n = 3)

in *C. vulgaris* after UV exposure. The increase in protein, CHO and lipid contents observed in this study show the potential of UV radiation to enhance *S. vacuolatus* cellular biomolecules to improve cellular activities.

UV exposed *S. vacuolatus* growth kinetics

The specific growth rate of 24 h UV-exposed *S. vacuolatus* was obtained from the log phase of *S. vacuolatus* growth. The obtained specific growth rate (μ) was 0.183, 0.212 and 0.243 day^{-1} at initial substrate concentrations of 1.00, 1.50 and 2.00 g/L, respectively. Alternatively, lower specific growth rate values of 0.183 day^{-1} (1.00 g/L), 0.198 day^{-1} (1.50 g/L) and 0.232 day^{-1} (2.00 g/L) were obtained with the wild *S. vacuolatus* (Fig. 7a). These outcomes suggest the observed μ increases with increasing substrate concentration and could continue until substrate saturation is reached (Okpokwasili and Nweke 2006). Moreover, the higher μ values achieved with the 24 h UV-exposed *S. vacuolatus* suggest the impact of UV radiation on substrate uptake and growth of *S. vacuolatus* (Choi et al. 2017; Ganapathy et al. 2017; Kumar et al. 2018). Microbial engineering developments such as the use of UV radiation and different cloning techniques for inducing improved genetic changes at cellular levels to simultaneously obtained enhanced traits such as shorter lag phase, improved growth rate and metabolic activities have been reported (Choi et al. 2017; Ganapathy et al. 2017; Kumar et al. 2018). Furthermore, taking into account the aforementioned impact of UV radiation on *S. vacuolatus* such as enhanced photosynthetic features and increased growth rate that could have improved *S. vacuolatus* substrate uptake, utilization and improved specific growth rate in the 24 h UV-exposed *S. vacuolatus* was expected.

The estimated Monod constant (K_S) and maximum specific growth rate ($\mu_{\max}$) from the specific growth rate values and initial substrate concentrations are shown in Fig. 7b and c. A maximum specific growth rate (μ_{max}) value of 3.30 day^{-1} and 2.85 day^{-1} were observed for the 24 h UV exposed *S. vacuolatus* and wild-type, respectively. The Ks value obtained for UV-exposed *S. vacuolatus* was 1.56 g/L, while the Ks value of 1.043 g/L was obtained for the wild-type. In addition, the UV-exposed *S. vacuolatus* had a higher affinity constant (1/Ks) for the available substrate (0.959) compared to the wild-type (0.641). The affinity for the substrate by the UV-exposed *S. vacuolatus* was 1.50 times higher than the wild *S. vacuolatus*. The higher $\mu_{\max}$ and $1/K_s$ values observed for the 24 h UV-exposed *S. vacuolatus* demonstrate the suitability of UV radiation as a physical agent to improve *S. vacuolatus* substrate uptake and metabolic activities.

The experimental data from the biomass concentration over the cultivation period were used to fit the logistic model with correlation coefficients (R^2) > 0.979. Higher maximum biomass concentration (X_{max} = 0.511 g/L) was obtained with 24 h UV-exposed *S. vacuolatus* in comparison with wild *S. vacuolatus* (X_{max} = 0.268 g/L). On the other hand, the wild-type had a higher maximum specific growth rate ($\mu_{\max}$ = 0.556 day^{-1}) than that obtained with 24 h UV-exposed *S. vacuolatus* ($\mu_{\max}$ = 0.365 day^{-1}). Though lower $\mu_{\max}$ was obtained with 24 h UV-exposed *S. vacuolatus*, higher substrate affinity and biodegradation potential were observed in contrast with the wild-type. Therefore, this suggests that the exposure of *S. vacuolatus* to UV radiation largely enhanced *S. vacuolatus* affinity for the substrate and subsequently enhanced the degradation of SCW to harmless by-products.

Furthermore, the kinetic growth constant (k_g) (0.1615 day^{-1}) was obtained for the UV exposed *S. vacuolatus* during the SLW degradation process (see supplementary document). This was 1.27-fold high compared to wild *S. vacuolatus* (0.1274 day^{-1}) under the same experimental condition. Interestingly, the high k_g obtained with UV exposed *S. vacuolatus* corroborate with the high substrate affinity, specific growth rate and high biomass accumulation also observed with the UV exposed *S. vacuolatus*. This could be attributed to the positive impact of UV radiation on *Scenedesmus vacuolatus* photosynthetic apparatus, production of enzymes and other physiological cellular behaviour to improve cellular growth and simultaneously degrade the lubricant oil waste.

Scenedesmus vacuolatus substrate usage versatility

Figure 8 illustrates the variation in the growth rate of the 24 h UV-exposed *S. vacuolatus* cultivated with sodium carbonate (Na_2CO_3), glucose and glycerol as the only carbon source. The results obtained revealed *S. vacuolatus* showed a significant difference in growth pattern with the studied substrates

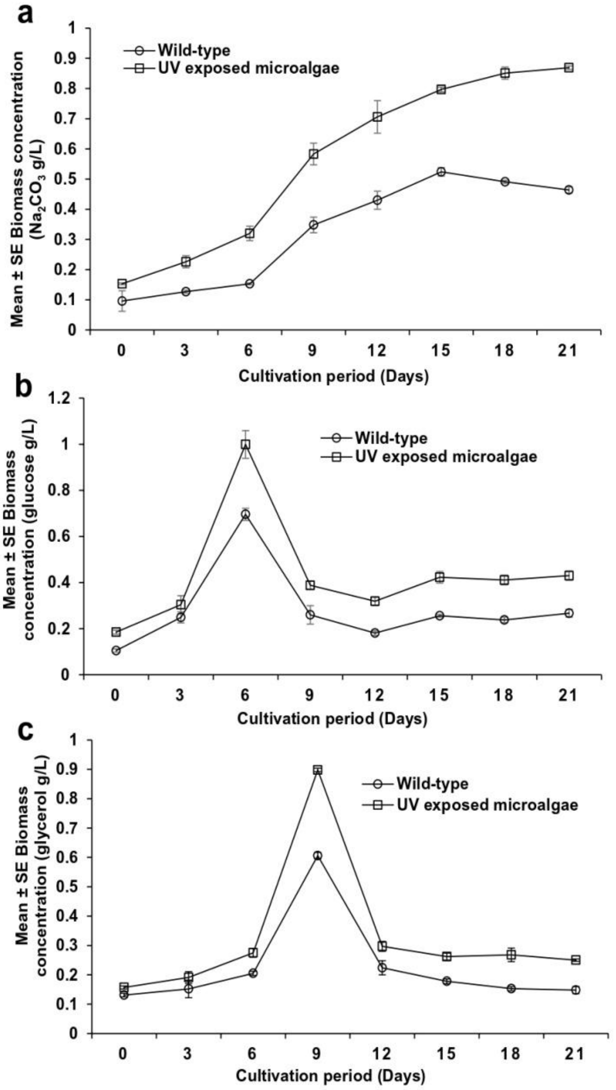


Fig. 8 The cultivation of wild-type microalgae and UV exposed *S. vacuolatus* using different substrate. **a** Sodium carbonate NaCO2; **b** glucose; **c** glycerol. Mean ± standard error of three replicates (n = 3)

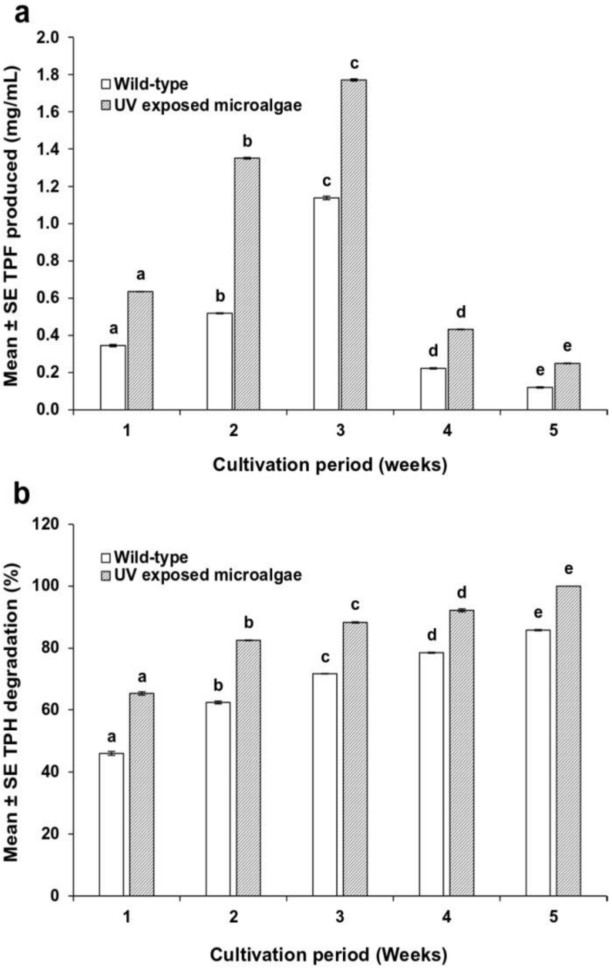


Fig. 9 The dehydrogenase activity and total petroleum hydrocarbon degradation of spent coolant waste after 5 weeks. **a** TPF production by *S. vacuolatus*, **b** TPH degradation by *S. vacuolatus*. The share letters (a, b, c, d and e) show that the TPF and TPH at different weeks are significantly different for each microalgae strain. Mean ± standard error of six replicates (n = 6)

(Na_2CO_3, glucose and glycerol) as the sole carbon source. These variations in growth patterns may be attributed to the differences in the chemical structural complexity of the compounds. Also, the variations in the specific growth rates demonstrate variations in the metabolism of the different substrates by *S. vacuolatus* (Danesh et al. 2019; Rambhiya1 et al. 2021). *S. vacuolatus* showed the highest growth rate and biomass concentration with glucose as the sole carbon source. This suggests that glucose is the most preferred carbon source by *S. vacuolatus* of the three substrates, probably due to its simple carbon–carbon bond (Danesh et al. 2019). Moreover, higher specific growth rates were observed in all the studied substrates with 24 h UV-exposed *S. vacuolatus* compared to the wild-type (Fig. 8a–c). The maximum specific growth rates of 0.054, 0.136 and 0.082 day^{-1}, were observed for the UV-exposed *S. vacuolatus* compared to that 0.028, 0.099 and 0.053 day^{-1} obtained for Na_2CO_3, glucose and glycerol of the wild *S. vacuolatus*, respectively. The UV-exposed *S. vacuolatus* also showed significantly increased biomass concentration up to 0.869 ± 0.006 g/L, 0.999 ± 0.060 g/L, 0.898 ± 0.006 g/L for Na_2CO_3, glucose and glycerol, respectively. The attained biomass concentration was 1.67, 1.44 and 1.48 times higher than those obtained with the wild-type (0.524 ± 0.014 g/L, 0.696 ± 0.026 g/L, 0.606 ± 0.011 g/L). The higher specific growth rates and biomass concentrations obtained by the UV-exposed *S. vacuolatus* can be ascribed to enhanced adaptability, metabolic capacity and substrate consumption versatility.

Effect of UV radiation on *S. vacuolatus* biodegradation potential

To determine the potential of waste lubricant of biodegradation by the 24 h UV-exposed *S. vacuolatus*, a preliminary assessment for spent coolant waste (SCW) biodegradation was carried out. The DHA production and TPH degradation of SCW after 5 weeks is presented in Fig. 9a and b (The TPF produced and gas chromatogram of the TPH degradation are shown in the supplementary document). The results showed the 24 h UV-exposed *S. vacuolatus* has significantly higher ($p < 0.001$) DHA and TPH degradation efficiency in comparison with the wild *S. vacuolatus*. The highest DHA with TPF production of 1.771 ± 0.006 mg/mL was obtained at week 3, while the highest TPH degradation (100%) was obtained at week 5. These were 36% and 14% improvements over the DHA and the TPH obtained with the wild-type. The increment in DHA and TPH degradation might also be attributed to enhanced photosynthetic and metabolic activities of UV-exposed *S. vacuolatus* resulting from improved cellular activities after the UV exposure (Choi et al. 2017; Ganapathy et al. 2017; Kumar et al. 2018). Previous studies by Megharaj et al. (2000) and Eregie and Jamal (2019, 2021) also reported an increase in DHA and TPH degradation of HCs using the *Chlorella* and *Scenedesmus* sp, respectively.

The HCs detected in the SCW sample after treatment with the 24 h UV-exposed *S. vacuolatus* are shown in the supplementary document. A relatively high biodegradation of HCs of SCW was observed compared to the control. The possible reasons for the high degradation of HCs after 5 weeks of treatment include (1) high tolerance and metabolism of SCW as substrate, (2) increased enzymatic metabolic activities that improved the metabolic activities towards SCW degradation (Ibrahim 2016; Ichor et al. 2016; Medic et al. 2020). The different alkanes (C8 to C43) present in the control (see supplementary document) were degraded by the UV-exposed *S. vacuolatus* into environmentally friendly products such as alkanols, alcohols, ketones, aldehydes, fatty acids, and carboxylic acids. Similar degradation products were also observed in the SCW treated with the wild-type (see supplementary document). Wild-type and 24 h UV-exposed *S. vacuolatus* probably exhibited a subterminal oxidation pathway for the degradation of alkanes (Patowary et al. 2017; Medic et al. 2020). Subterminal oxidation of alkane HCs have also been reported, resulting in the formation of an alcohol that is then hydroxylated to form ketone and ester by-products. The ester formed is further oxidized to produce fatty acid (Kumar et al. 2014; Patowary et al. 2017). Moreover, mono and polycyclic aromatics were degraded to their harmless degradation products by UV-exposed *S. vacuolatus* within the first week of SCW treatment compared to the control and the wild-type that occurred in subsequent weeks. A suggestive of rapid utilization of SCW as substrate resulting from high photosynthetic and metabolic activities (Patowary et al. 2016; Choi et al. 2017; Żyszka-Haberecht et al. 2019). The ability of the UV-exposed *S. vacuolatus* to degrade the aromatics to metabolic products that are non-toxic within the first week of treatment is significant and could be of biotechnological importance in the biodegradation of SCW, oil waste, or spillage. After 5 weeks of SCW treatment by 24 h UV-exposed *S. vacuolatus*, no HCs nor their degradation products (signifying complete metabolism/degradation) were detected compared to the control and degradation profiles obtained from the wild *S. vacuolatus* experiments where undegraded HCs were detected after 5 weeks. The results obtained further demonstrate the enhanced biodegradation potential of the UV-exposed *S. vacuolatus*.

Conclusion

This study demonstrates the impact of UV light radiation on the metabolic performance and biodegradation potential of *S. vacuolatus*. Higher growth rate, substrate affinity, chlorophyll, protein, carbohydrate, and lipid accumulation were obtained with the 24 h UV-exposed *S. vacuolatus*. Moreover, the exposure of *S. vacuolatus* to UV radiation enhanced the DHA by 1.55-fold and biodegradation efficiency by 100% with SCW as substrate. This study substantiates the potential of microalgae engineering to improve waste biodegradation towards eco-friendly environmental sustainability.

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Author contributions SBE conceived the experiment, SBE and IAS designed the experiments; SBE performed the experiments and analysed the data. SBE wrote the manuscript for publication with the help and guidance of IAS, GEBK and OA.

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Code availability Not Applicable.

Declarations

Conflict of interest All authors declare no conflict of interest in this study.

Ethical approval This research article does not contain any studies involving human participants or animals performed by any of the authors.

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Consent to participate I Stella Eregie gives the consent of participation.

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CHAPTER 4

Comparison of biodegradative efficiency of wild-type versus mutagenized *Scenedesmus vacuolatus* of spent coolant waste: dehydrogenase activity and total petroleum degradation studies

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of during industrial routine processes, it produces spent oil waste that is hazardous as a result of the accumulation of impurities such as carbon, water, metals, ash, dust, hydrocarbons (HCs), and other compounds [2,3]. These impurities reduce the oil emulsion efficiency and quality, which become unsuitable for use [4]. More than 28,220 tons of spent oil waste are produced yearly by oil and manufacturing industries worldwide [2]. For instance, it was reported that each year between 1.7 and 8.8 million tons of spent oil waste are deposited in water and on land, of which 90% are linked to industrial activities, including the intentional release of oil waste [5]. It was also reported that about 30% of the spent oil waste flow into groundwater [6], and nearly six million deaths are registered every year due to the pollution of groundwater [7].

Poor management and illegal disposal of spent oil wastes have been reported to contribute to environmental pollution [8,9]. Spent oil waste contains harmful pollutants such as aliphatic compounds, monoaromatics, polycyclic aromatic hydrocarbons (PAH), and chlorinated compounds that end up in water bodies and lands and result in severe pollution [10,11]. These harmful pollutants persist in the contaminated sites for a long time without being biodegraded due to their recalcitrant properties [12]. Studies have also shown that their persistence in the contaminated sites causes these pollutants' transportation into water systems via runoff and across the atmosphere via volatilisation and condensations [1,12,13]. Oil leaks resulting from the disposal of spent oil wastes are becoming a more visible issue and are regarded as one of the most prevalent environmental contaminants, much more than other petroleum-based products [13]. Scientists reported that the accumulation of spent oil waste contaminants in the environment causes pollution in several ways. For example, the incineration of the spent oil waste emits toxic greenhouse gases and other harmful compounds, including furans, dioxins, carbon monoxide, and heavy metals, contributing significantly to the ozone layer depletion [14]. Moreover, some of the compounds have negative health implications. For instance, dioxins cause damage to the endocrine hormone in humans. Additives and hydrocarbons pollutants present in the spent oil wastes cause different kinds of cancers, respiratory and nervous system disorders in humans, and endocrine, developmental, and epigenetic disorders in marine animals [1,11]. These compounds also cause severe damages and problems to residents living around the area where the oil waste has been released [5,15]. Many traditional and chemical treatment methods have been used to treat and remove organic pollutants in spent oil waste [13]. These methods are considered environmentally not safe, inefficient, incomplete decomposition of pollutants, and the operational costs make applying these treatment methods costly [8]. According to past research, spent oil waste pollution is becoming a highly sought-after field of study due to its impact on the natural ecosystem [5,16]. Due to the rise in environmental pollution of spent oil wastes, the need for a cost-effective method that can be used to treat and remove these pollutants across a wide range of industrial applications is of great concern.

The use of microalgae either as wild-type or modified, have sparked a lot of scientific interest in the past years as an innovative treatment as a result of their crucial role in the ecosystem used as biofuel, food, feeds, and pharmaceuticals [12]. Microalgal treatment has proven to be a simple, safe, viable, efficient, cost-effective, and environmentally friendly method compared to the traditional and chemical treatment [10,14]. Microalgae are photosynthetic and highly adaptive to any environment with extreme conditions. They get accustomed to the environment by resisting toxic pollutants and using them as the carbon source to release oxygen for the breakdown of the pollutants [14,17,18]. Their by-products and biomass are also used as feedstocks for animals and biofuel production [19]. These bioremediation potentials of microalgae are beneficial to the ecosystem, especially with regard to the removal of contaminants and other hazardous chemicals. In the past years, the use of microalgal treatment of lubricant oil waste has been successfully demonstrated, with the primary focus on hydrocarbon pollutant removal [12,19,20]. It has been reported that microalgae completely remove HCs present in oil waste [12,19,20]. For instance [21] reported the complete removal of HCs detected in spent lubricant oil emulsion waste by wild-type microalgae *Scenedesmus vacuolatus* [21,22]. In another study [23] reported the total oxidation of HCs found in the oil waste by the wild-type microalgae *S. obliquus* and *Chlorella vulgaris*. The catabolic abilities and the utilisation of oil wastes by these microalgal species, further demonstrates that microalgae possess extraordinary capabilities that could be used for bioremediation of oil contaminated sites. Although studies on biodegradation have been limited to wild-type microalgae, no studies yet have been reported on the use of mutagenised microalgae *Scenedesmus vacuolatus* for biodegradation of spent coolant oil. Thus, making this study the first to report for the biodegradation of oil waste using mutagenised microalgae *S. vacuolatus*.

The microalgal biodegradation treatment uses microalgae to transform toxic pollutants into compounds that are non-hazardous than the initial compounds. Microalgal biodegradation takes place through two mechanisms; the first is by metabolic degradation, in which the pollutants are used as substrates for the microalgae, and the second is by co-metabolism, in which the pollutants are broken down by enzymes that are catalysing other existing hydrocarbons [24]. Microalgal degradation can also occur intracellularly or extracellularly via various catabolic processes; these include methylation, isomerisation, subterminal oxidation, decarboxylation, hydroxylation, carboxylation, and dehydrogenation. However, these catabolic processes are yet to be fully understood [25,26]. The first phase of degradation starts in the extracellular, and the transformed products are then mineralised within the cells (intracellular) [24]. The degradation pathway for aliphatic compounds is initiated by a methyl group's terminal oxidation to form an alcohol, further transform into fatty acid. The fatty acid is then broken down via decarboxylation to release carbon dioxide and produce a secondary fatty acid through β-oxidation [27]. In some instances, the aliphatic compound is oxidised via a subterminal oxidation pathway to form an alcohol, transformed into a ketone, and further converted into fatty acid. This metabolic pathway is carried out by the class of enzymes called oxygenases [21]. The metabolic pathway for aromatic compounds involves forming a diol via cis hydroxylation of the aromatic ring. The aromatic ring is further cleaved to form a dicarboxylic acid by the enzyme oxygenases [27].

Over the years, several methods have been reported in evaluating microorganisms' capabilities and efficiencies during the biodegradation process. These include the enumeration method, dehydrogenase activity (DHA), gas chromatography, and mass spectrometry (GCMS). These methods have also been used as indicators to monitor microbial oxidative activity during degradation [28]. Among the methods mentioned above, DHA and GCMS are of much interest for this study. DHA has been successfully used to determine the oxidative activity of microorganisms with the majority of studies focusing on bacteria and fungi [29–32]. Thus far, there are limited studies on the use of DHA to quantify the biodegradation efficiency of microalgae in oil waste contaminated environment. Two approaches using triphenyl tetrazolium chloride (TTC) and iodonitrotetrazolium chloride (INT) are used to assess dehydrogenase activity, particularly TTC [30]. In this study, DHA using the TTC method is of significant interest because it is considered a simple and efficient technique for assessing oxidative activity and degradation ability of microorganisms [29–3133]. The INT method is costly and insoluble in water compare to TTC, which has led to this method not being commonly used [30]. Dehydrogenases are oxidoreductase class of enzymes, which forms an essential part of the enzyme system found in all living microorganisms [30,32]. In aerobic degradation, dehydrogenase enzymes facilitate the oxidation of HCs via the dehydrogenation mechanism by transferring electrons through a co-enzyme, which acts as an electron acceptor. After the dehydrogenation of HCs by these enzymes, the HCs become more hydrophilic, hydrophobic, and available to microorganisms by substituting the HCs with methyl groups via methylation and subterminal oxidation [26,30]. The use of GCMS to monitor the total petroleum hydrocarbon (TPH) degradation of various oil wastes, ranging from crude oil to lubricant emulsion has been reported in several biodegradation studies [17,23,34–36]. The TPH is an important parameter in oil biodegradation, it represents the total amount of oil degradation [23,34]. In this study, the TPH further elucidate the microalgae ability and potential degradation metabolic pathway to degrade SCW. This study aimed to evaluate the biodegradation capabilities of wild-type versus mutagenised *Scenedesmus vacuolatus* for the biodegradation of spent coolant wastes (SCW). The degradation efficiency of both groups of microalgae was evaluated based on the DHA and TPH biodegradation using GCMS.

2. Methodology

The SCW used in this study was supplied by a local manufacturing company in Pietermaritzburg, South Africa. The biodegradation analysis of SCW was conducted in large volumes (500 mL) studies under optima abiotic conditions established previously [21]. The indigenous *S. vacuolatus* was mutagenised using the ultraviolet light radiation in a previous study.

2.1. Microorganism

The indigenous microalgae wild-type and mutagenised *S. vacuolatus* was used in this study are of the same species previously isolated from lubricant oil waste [21].

2.2. SCW biodegradation by microalgae experimental setup

To evaluate the degradation efficiency by wild-type and mutagenised indigenous *S. vacuolatus*, biodegradation analysis was carried out. The biodegradation experiments were conducted in large volumes (500 mL). The experimental analysis was carried out in BG 11 medium. The wild-type and mutagenised *S. vacuolatus* (50 mL) each were inoculated into a 500 mL BG11 media separately, supplemented with 50 mL of SCW as substrate. The flasks were agitated and incubated at 25°C for five weeks. The control experiment was made with BG11 medium having SCW without inoculum. All experimental analysis was performed in replicates (n = 6) and monitored for five weeks. Each week SCW samples were taken from the different flask and analysed for DHA and further evaluated via GCMS to monitor the SCW biodegradation by both group of microalgae.

2.2.1. Dehydrogenase activity (DHA)

The method by [33], was used to assess the DHA of the wild-type and mutagenised *S. vacuolatus*. This method thus serves as an indicator of the oxidative metabolic activity and degradation capability of microalgae. This experiment was replicated six times and conducted over a period of five weeks. After each week of incubation, the DHA were analysed. The incubated samples of SC waste (20 mL) were placed in a sterile centrifuge tubes with the addition of 2.5 mL distilled water, 0.2 g $CaCO_3$, and 1 mL triphenyl tetrazolium chloride (TTC). The oil wastes samples were incubated at 37°C for 24 hours. To extract the triphenyl formazan (TPF) a red/pink colour, 10 mL of methanol was added to each of the centrifuge tubes and shake vigorously for 10 mins. The oil wastes samples were filtered using a cotton wool through Whatman paper in a funnel and the supernatants were collected. The obtained solution was decanted into clean centrifuge tubes, and the absorbance of the reddish/pinkish coloured solution was measured using a spectrophotometer at a wavelength of 485 nm. The concentration of the TPF produced was calculated using a standard calibration curve (see supplementary document 1, Fig. S1) for standard calibration curve.

The standard calibration curve was constructed by preparing a standard solution of TPF. 0.1 g of TPF was dissolved in 80 ml of methanol and made up to 100 mL with methanol. A set of five TPF solutions with concentrations of 0.005, 0.010, 0.015, 0.020 and 0.025 mg/mL was prepared using 100 mL volumetric flasks. The absorbance of the prepared TPF solutions were measured (in triplicates) using spectrophotometer at a wavelength of 485 nm and against the known TPF concentration for the determination of the unknown concentration of TPF produced.

2.2.2. Gas chromatography and mass spectroscopy (GCMS) analysis

To ascertain the efficacy of the two groups of microalgae (wild-type and mutagenised *S. vacuolatus*) for SCW degradation, GCMS analysis was carried out. The SCW degradation was evaluated in terms of the total petroleum hydrocarbon (TPH) accompanied with the HCs degradation which was carried out in replicates (N = 6). Prior to the GCMS analysis, the following extraction procedure including extracellular and intracellular HCs extraction analysis was performed for each sample. For extracellular HCs extraction, 50 mL of each samples (treated SCW samples with mutagenised *S. vacuolatus* and wild-type and control) was extracted with dichloromethane. The extracellular oil extracts were filtered using sodium sulphate. Then, the oil extracts were placed under the fume mood for the dichloromethane to evaporated.

The intracellular HCs in the microalgae cells (wild-type and mutagenised *S. vacuolatus*) were extracted using the beads beating methodology by [37]. Both groups of microalgae cultures (50 mL) were transferred into sterile centrifuge tubes each and centrifuge at 11,000 rpm for 5 mins to obtain the microalgae pallets. The pallets were transferred into 2 mL tubes with glass beads and nuclease free water. The microalgae solutions were agitated for 5 mins. The supernatants were obtained and decanted into sterile test tubes,

Table 3b. Extracellular compounds detected after week four and five treatment of SCW by mutagenised indigenous *S. vacuolatus*.

Week 4		Week 5
Fatty acids/other compounds	Formula	
Hexanoic acid	$C_6H_{12}O_2$	Hydrocarbons not detected
Heptanoic acid	$C_7H_{14}O_2$	
Octanoic acid	$C_8H_{16}O_2$	
Tridecanoic acid	$C_{13}H_{26}O_2$	
Pentadecanoic acid	$C_{15}H_{30}O_2$	
Acetic acid, trifluoro-, dodecyl ester	$C_{14}H_{25}F_3O_2$	
Tetradecanoic acid, 2,3-dihydroxypropyl ester	$C_{17}H_{34}O_4$	
Hexadecanoic acid,2,3-bis(acetyloxy)propyl ester	$C_{23}H_{42}O_6$	
n-Decanoic acid	$C_{10}H_{20}O_2$	
Hexanedioic acid, dioctyl ester	$C_{22}H_{42}O_4$	
alpha-Ketostearic acid	$C_{18}H_{34}O_3$	
Octadecanoic acid,2,3-bis[(1-oxotetradecyl) oxy] propyl ester	$C_{49}H_{94}O_6$	
9-Oxononanoic acid	$C_9H_{16}O_3$	
Dodecanoic acid	$C_{12}H_{24}O_2$	
Octanedioic acid	$C_8H_{14}O_4$	
Palmitoleic acid	$C_{16}H_{30}O_2$	
Tridecanoic acid, methyl ester	$C_{14}H_{28}O_2$	
Octadecanoic acid,9,10-epoxy-, isopropyl ester	$C_{19}H_{36}O_3$	
Oleic acid	$C_{18}H_{34}O_2$	
Cyclohexane carboxylic acid hydrazide	$C_7H_{14}N_2O$	
Palmitic acid	$C_{16}H_{32}O_2$	
Decanoic acid,5,5-dimethyl-9-oxo-, methyl ester	$C_{13}H_{24}O_3$	

The table shows the list of extracellular hydrocarbons present with their molecular formula after mutagenised *S. vacuolatus* treatment of SCW.

monoaromatic HCs. Furthermore, the production of these benzene alcohol, monocarboxylic and dicarboxylic acids may serve as a mechanism or a strategy by which the microalgae protect its cells from the toxicity of the parent benzene HCs [11]. Similar to the finding of [52], the authors reported the degradation of BTEX compounds (benzene, toluene, ethylbenzene, and p-xylene) into different metabolic products by the microalga *Paurachlorella kessleri* after 72 hours of treatment.

The PAHs detected were also efficiently degraded into their naphthalene derivatives after two weeks of SCW treatment compared to the control. The naphthalene derivatives including, naphthalene,1-methyl, naphthalene,1,3-dimethyl, 1,2-naphthalenediol, 1-naphthol,1,2,3,4-tetrahydro-2-methyl, 1,2,3,4-tetrahydro-1,2-naphthalenediol, and 2-naphthalenecarboxylic acid, 4,4′-methylenebis [3-methyl] were formed. The presence of these compounds shows the degradation of the PAHs which were probably produced by hydroxylation and methylation pathway [12]. After the third, fourth, and fifth weeks of SCW treatment (Tables 2a and 2b), no monoaromatic HCs and PAHs or their oxidised products were detected, only the non-toxic degradation products of alkanes were observed. This therefore clearly confirms the complete degradation of the aromatic HCs and the utilisation of the oxidised products. Naphthalene and its derivatives have been reported to be degraded by the *Ankistrodesmus* species [53]. The degradation of both monoaromatics and PAHs by microorganisms into different extracellular metabolites such as their corresponding diol and carboxylic acids have been reported to occur by hydroxylation and carboxylation mechanism [12,25,53,54,55]. It should be noted that monoaromatic (benzene) and PAHs compounds (naphthalene and chlorinated HCs) detected in the control as stated above, are highly toxic and carcinogenic. They cause different kinds

Table 4a. Intracellular metabolites extracted from the cells of the wild-type microalgae after three weeks of treatment of spent coolant waste.

Week 1		Week 2		Week 3	
Alkanes Compounds	Formula	Alkanes Compounds	Formula	Alkanes compounds	Formula
Pentane,3-ethyl-	C7H16	Hexane,3,3,4-trimethyl-	C9H20	Cyclobutane,1,1,2,3,3-pentamethyl-	C9H18
Hexane,3,3,4-trimethyl-	C9H20	Octane,2,6-dimethyl-	C10H22	Cyclohexane,1,4-dimethyl-	C8H16
Octane	C8H18	Cyclopentane,1,2,4-trimethyl-	C8H16	Hexane,2,2,3,3-tetramethyl-	C10H22
Undecane,3,7-dimethyl-	C13H28	Nonane,2,6-dimethyl-	C11H24	Nonane,3,7-dimethyl-	C11H24
Octadecane,5-methyl-	C19H40	Silane, trichlorodocosyl-	C22H45Cl3Si	2-Cyclohexylnonadecane	C25H50
Hexadecane,1-chloro-	C16H33Cl	Decane,2,5-dimethyl-	C12H26	7,7-Diethylheptadecane	C21H44
Docosane, 2,4-dimethyl	C16H32O	Undecane,2,5-dimethyl-	C13H28	2-methyltetracosane	C25H52
Dodecane,2,5-dimethyl-	C14H30	Tetradecane,2,6,10-trimethyl-	C17H36	Dodecane,2-methyl-6-propyl-	C16H34
Decane,4-ethyl-	C12H26	Heptadecane,2,6-dimethyl-	C19H40	Tetrapentacontane,1,54-dibromo-	C54H108Br
Eicosane,10-methyl	C21H44	Pentadecane,5-methyl-	C16H34	Heneicosane,5-methyl-	C22H46
Hexane,1,6-dicyclohexyl-	C18H34	Heneicosane,5-methyl-	C22H46	Cyclohexadecane	C16H32
10-Heneicosene,11-phenyl	C27H46	Tridecane,2-methyl-	C14H30	Alkene compounds	
Alkene compounds		Alkenes compounds		Squalene	C30H50
10-Heneicosene,11-phenyl	C27H46	2-Pentene,2,3-dimethyl-	C7H14	7-Heptadecene,17-chloro	C17H33Cl
cis-1-Chloro-9-octadecene	C18H35Cl	1-Tetracosene	C24H48	3-Hexene,3-ethyl-2,5-dimethyl	C10H20
7-Heptadecene,17-chloro-	C17H33Cl	4,5-Nonadiene	C9H16	2,4,4,6-Tetramethyl-6-phenyl-2-heptene	C17H26
7-Heptadecene	C17H34	17-Pentatriacontene	C35H70	Alcohols	
Squalene	C30H50	Alcohols		1-Decanol,2-methyl-	C11H24O
4,5-Nonadiene	C9H16	1-Decanol,2-ethyl-	C12H26O	Z, E-3,13-Octadecadien-1-ol	C18H34O
3-Hexene,3-ethyl-2,5-dimethyl-	C10H20	Cholesta-8,24-dien-3-ol,4-methyl, (3. beta.,4.alpha	C26H46O	n-Tridecan-1-ol	C13H28O
2-Pentene,2,3-dimethyl-	C7H14	1-Hexadecanol	C16H34O	1-Dodecanol,2-octyl	C20H42O
1-Octadecene	C18H36	2-Octanol,3-methyl	C9H20O	Fatty acid/carboxylic acids	
1-Decene,2,4-dimethyl-	C12H24	(+-)-trans-1-Isopropenyl-4-methyl-1,4-cyclohexanediol	C10H18O2	Carbonic acid, ethyl isobutyl ester	C11H24O
Fatty acid/carboxylic acids		Fatty acid/carboxylic acids		Trichloroacetic acid, 1-cyclopentylethyl ester	C9H13Cl3O2
Cyclobutane carboxylic acid,2-octyl ester	C13H24O2	Oxalic acid, propyl tridecyl ester	C18H34O4	Methoxyacetic acid,4-tridecyl ester	C16H32O3
Hydrazine carboxylic acid, phenylmethyl ester	C8H10N2O2	Palmitic acid	C16H32O2	Tetratriacontyl pentafluoropropionate	C37H69F5O2

(*Continued*)

Table 4a. (Continued).

Week 1		Week 2		Week 3	
Cyclohexane carboxylic acid	C7H12O2	Dichloroacetic acid,2-tridecyl ester	C15H28Cl2O2	Palmitoleic acid	C16H30O2
Cyclopentane carboxylic acid	C6H10O2	Dodecanoic acid, methyl ester	C13H26O2	Dodecanoic acid methyl ester	C13H26O2
Oxalic acid, isobutyl hexadecyl	C22H42O4	Pentadecanoic acid	C15H30O2	Tetradecanoic acid	C14H28O2
Sulphurous acid, dicyclohexyl ester	C12H22O3S	Eicosanoic acid,2,3-bis(acetyloxy)propyl ester	C27H50O6	9,12-Octadecadienoic acid (Z,Z)-	C18H32O
Malonic acid,4-methylpent-2-yl octadecyl ester	C27H52O4	Carbonic acid, octadecyl 2,2,2-trichloroethyl ester	C21H39Cl3O3	Cyclobutane carboxylic acid, 3-pentadecyl ester	C20H38O2
Palmitoleic acid	C16H30O2	Octanoic acid, hexadecyl ester	C24H48O2	3-Methylcyclohexane carboxylic acid	C8H14O2
Acetic acid, phenyl ester	C8H8O2	1-Cyclopentene carboxylic acid	C6H8O2		
Carbonic acid, butyl hexadecyl	C19H38O3	Pentafluoropropionic acid, tridecyl ester	C16H27F5O2		
Dodecanoic acid, methyl ester	C13H26O2	Cyclohexane carboxylic acid	C7H12O2		
Palmitic acid	C16H32O2	Cyclopentane carboxylic acid	C6H10O2		

The table shows the list of intracellular hydrocarbons with their molecular formula found in the cells wild-type microalgae.

Table 4b. Intracellular metabolites extracted from the cells of the wild-type microalgae after four and five weeks of treatments of spent coolant waste.

Week 4		Week 5	
Alkanes Compounds	Formula	Alkanes Compounds	Formula
Nonane,4-methyl-	C10H22	Hexane,3,3,4-trimethyl-	C9H20
Octane	C8H18	Cyclopentane,1,2,4-trimethyl-	C8H16
Cyclohexane, ethyl-	C8H16	Nonane,3-methyl-	C10H22
Undecane,5,7-dimethyl-	C13H28	Undecane,5,6-dimethyl-	C12H26
3,5-Dimethyldodecane	C14H30	2-Cyclohexylnonadecane	C25H50
Hexadecane,2,6,11,15-tetramethyl	C20H42	2,4,4,6-Tetramethyl-6-phenylheptane	C17H28
Dodecane,2,6,11-trimethyl-	C15H32	Tetrapentacontane,1,54-dibromo-	C54H108Br2
Heptadecane-2,6,10,15 tetramethyl	C21H44	Alkene compounds	
Pentadecane,2,6,10,14-tetramethyl	C19H40	cis-3,6,9,12,15,18-heneicosahexaene	C21H32
Alkene compounds		2,4-Dimethyl-1-heptene	C9H18
4,5-Nonadiene	C9H16	4,5-Nonadiene	C9H16
3,7,11-trimethyl-4-dodecadiene	C15H28	1-Butene,2,3,3-trimethyl-	C7H14
3-Hexadecene, (Z)-	C16H32	Isoprene	C5H8
2,4-Dimethyl-1-heptene	C9H18	17-Pentatriacontene	C35H70
Z-5-Nonadecene	C19H38	7 heptadecene	C17H34
Alcohols		1-Nonadecene	C19H38
Cholest-22-ene-21-ol,3,5-dehydro-6-methoxy-, pivalate	C33H54O3	Alcohols	
1,12-Dodecanediol	C12H26O2	cis-1,2-Cyclododecanediol	C12H24O2
1-Nonanol,4,8-dimethyl-	C11H24O	11-Methyldodecanol	C13H28O
1-Decanol,2-octyl-	C18H30O	1-Undecanol	C11H24O
Fatty acid/carboxylic acids		Fatty acid/carboxylic acids	
Cyclopentane carboxylic acid	C6H10O2	Cyclobutane carboxylic acid,2-octyl ester	C13H24O2
Cyclohexane carboxylic acid	C7H12O2	1,2-Cyclohexane dicarboxylic acid	C8H12O4
1,2-Cyclohexanedicarboxylic acid	C8H12O4	Methoxyacetic acid, 6-ethyl-3-octyl ester	C13H26O3
3-Methylcyclohexane carboxylic acid	C7H14O3	Hexadecanoic acid	C16H32O2
Cyclobutane carboxylic acid, 3-pentadecyl ester	C20H38O2	1.4-Cyclohexane dicarboxylic acid	C8H12O4
3-Methylcyclohexane carboxylic acid	C8H14O2	Oleic acid	C18H34O2
linoleic acid	C18H32O2	Octadecanoic acid	C18H36O2
Oleic acid	C18H34O2	Linoleic acid	C18H36O2
Palmitoleic acid	C16H30O2	Dodecanoic acid, methyl ester	C13H26O
Hexanedioic acid, dioctyl ester	C22H42O	Tetradecanoic acid	C14H28O2
Carbonic acid, butyl tetradecyl ester	C19H38O3	Cyclobutane carboxylic acid, 3-pentadecyl ester	C20H38O2
Trichloroacetic acid,1-cyclopentylethyl ester	C14H19Cl3O2	Palmitoleic acid	C16H30O2
Carbonic acid, ethyl isobutyl ester	C7H14O3	Isopropyl palmitate	C19H38O2
Malonic acid,2-heptyltetradecyl ester	C24H46O4	palmitic acid	C16H32O2
Cyclopentane dicarboxylic acid	C7H10O4	Methoxyacetic acid, 4-hexadecyl ester	C19H38O3
2-Thiopheneacetic acid,2-tridecyl ester	C19H32O2S	Fumaric acid, 8-chlorooctyl hexyl ester	C18H31ClO4

The table shows the list of intracellular hydrocarbons with their molecular formula found in the cells of the wild-type microalgae.

of cancers, respiratory and nervous system disorders, and genetic defects in humans [1,11,56,57]. Hence, the ability of the wild-type microalgae to completely break down these hazardous HCs and transform them into non-toxic compounds is seen as a unique ability, that may be beneficial to the health of the natural ecosystem.

Table 5a. Intracellular metabolites extracted from the mutagenised indigenous *S. vacuolatus* cells after three weeks of treatments of SCW.

Week 1		Week 2		Week 3	
Alkanes Compounds	Formula	Alkanes Compounds	Formula	Alkanes compounds	Formula
Undecane,4,7-dimethyl-	C13H28	Undecane,2,6-dimethyl-	C13H28	Hexadecane,4-methyl-	C17H36
2-Bromododecane	C12H25Br	Undecane,4-cyclohexyl-	C17H34	Undecane,2,8-dimethyl-	C13H28
Pentadecane,5-methyl-	C16H34	Dodecane,4,6-dimethyl-	C14H30	Hexane,2,2,3,3-tetramethyl-	C10H22
Dodecane,2,6,10-trimethyl	C15H32	Heptadecane,2,6-dimethyl-	C19H40	2,4-Dimethyldodecane	C14H30
Heptadecane	C17H36	Tetradecane,4-methyl-	C15H32	2-Cyclohexylnonadecane	C25H50
Heptadecane,2-methyl-	C18H38	Hexadecane,2-methyl-	C17H36	7,7-Diethylheptadecane	C21H44
4-Methyldocosane	C23H48	Pentadecane,5-methyl-	C16H34	Hexatriacontane	C36H74
Nonadecane,3-methyl-	C20H42	Heptadecane,2,6,10,15-tetramethyl-	C21H44	Pentadecane,4-methyl-	C16H34
Heneicosane,5-methyl-	C22H46	Heptadecane,3-methyl-	C18H38	9-methylheptadecane	C18H38
Alkene compounds		Alkenes compounds		Hexacontane	C60H122
cis-3,6,9,12,15,18-heneicosahexaene	C21H32	9-Tricosene, (Z)-	C23H46	Squalane	C30H62
Pentaeicosene	C25H52	3-Hexadecene, (Z)-	C16H32	Alkene compounds	
3-Hexadecene, (Z)-	C16H32	Heptadecene	C17H34	Squalene	C30H50
9-Hexacosene	C26H52	cis-1,6,9,12,15,18-heneicosahexaene	C21H32	7-Heptadecene,17-chloro	C17H33Cl
Squalene	C30H50	7-Heptadecene	C17H34	3-Hexadecene, (Z)-	C16H32
Trieicosene	C23H46	Alcohols		3-Hexene,3-ethyl-2,5-dimethyl	C10H20
17-Pentatriacontene	C35H70	1,1-Dimethyl-3-chloropropanol	C5H11ClO	Hexaene	C6H12
Fatty acid/carboxylic acids		1-Dodecanol,2-octyl-	C20H42O	Alcohols	
Hexadecanoic acid,2-(octadecyloxy)ethyl ester	C36H72O3	n-Pentadecanol	C15H32O	1-Decanol,2-methyl-	C11H24O
. alpha. -Hydroxyisocaproic acid	C6H12O3	n-Nonadecanol-1	C19H40O	1-Eicosanol,2-hexadecyl-	C36H74O
Hexanedioic acid, diisooctyl ester	C22H42O4	n-Tetracosanol-1	C24H50O	n-Pentadecanol	C15H32O
6-Hexadecenoic acid,7-methyl, methyl ester	C18H34O2	1-Decanol,2-octyl-	C18H38O	11-Methyldodecanol	C13H28O
Hexanedioic acid, dioctyl ester	C22H42O	Fatty acid/carboxylic acids		n-Nonadecanol-1	C19H40O
Dichloroacetic acid, heptadecyl ester	C19H36Cl2O2	Butanoic acid,2-methylcyclohexyl ester,	C11H20O2	Fatty acid/carboxylic acids	
Methoxyacetic acid, 4-hexadecyl ester	C19H38O3	Dodecanoic acid, cyclohexyl ester	C16H30O2	Succinic acid,3,5-dimethylcyclohexyl heptadecyl ester	C17H30O4
l-(+)-Ascorbic acid, 2,6-dihexadecanoate	C38H68O8	Hexanedioic acid, bis(2-ethylhexyl) ester	C22H42O	Alpha. -Hydroxyisocaproic acid	C6H12O3

(*Continued*)

Table 5a. (Continued).

Week 1		Week 2		Week 3	
Carbonic acid, allyl tetradecyl ester	$C_{22}H_{42}O_3$	Methoxyacetic acid,4-hexadecyl ester	$C_{19}H_{38}O_3$	2-Thiopheneacetic acid, 3-tetradecyl ester	$C_{20}H_{34}O_2S$
Cyclohexane carboxylic acid, undec-10-enyl ester	$C_{18}H_{32}O_2$	Nonahexacontanoic acid	$C_{69}H_{138}O_2$	2-Pentenoic acid,3,4,4-trimethyl-, ethyl ester	$C_{10}H_{18}O_2$
Eicosanoic acid	$C_{20}H_{40}O_2$	Palmitoleic acid	$C_{16}H_{30}O_2$	cis-4,7,10,13,16,19-Docosahexaenoic acid	$C_{22}H_{32}O_2$
Palmitoleic acid	$C_{16}H_{30}O_2$	1,2-Cyclohexanedicarboxylic acid, dinonyl ester	$C_{26}H_{48}O_4$	Carbonic acid, octadecyl propyl ester	$C_{22}H_{44}O_3$
2-Ethylbutyric acid,5-methoxy-3-methylpentyl ester	$C_{12}H_{24}O_2$	Lauroyl peroxide	$C_{24}H_{46}O_4$	Hexanedioic acid, bis(2-ethylhexyl) ester	$C_{22}H_{42}O$
3-Cyclopentylpropionic acid, 4-tetradecyl ester	$C_{22}H_{42}O_2$	8-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	Methoxyacetic acid,4-hexadecyl ester	$C_{19}H_{38}O_3$
Tetradecanoic acid, 2,3-dihydroxypropyl ester	$C_{17}H_{34}O_4$	Eicosapentaenoic acid	$C_{20}H_{30}O_2$	Nonahexacontanoic acid	$C_{69}H_{138}O_2$
cis-4,7,10,13,16,19-Docosahexaenoic acid	$C_{22}H_{32}O_2$	Hexatriacontyl trifluoroacetate	$C_{38}H_{73}F_3O_2$	Eicosanoic acid	$C_{20}H_{40}O_2$
Hexadecanoic acid,2,3-bis(acetyloxy)propyl ester	$C_{23}H_{42}O_6$	l-(+)-Ascorbic acid,2,6-dihexadecanoate	$C_{38}H_{68}O_8$	Eicosapentaenoic acid	$C_{20}H_{30}O_2$
Octadecanoic acid	$C_{18}H_{36}O_2$	Dodecanoic acid, cyclohexyl ester	$C_{16}H_{30}O_2$	Heptafluorobutyric acid, heptyl	$C_{11}H_{15}F_7O_2$
Hexadecanoic acid	$C_{16}H_{32}O_2$	Oleic acid	$C_{18}H_{34}O_2$	8-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$
1,2-Cyclohexanedicarboxylic acid, nonyl 4-octyl ester	$C_{25}H_{46}O_4$	Linoleic acid	$C_{18}H_{36}O_2$	l-(+)-Ascorbic acid, 2,6-dihexadecanoate	$C_{38}H_{68}O_8$
Palmitoleic acid	$C_{16}H_{30}O_2$				

The table shows the list of intracellular hydrocarbons with their molecular formula found in the cells of mutagenised *S. vacuolatus*

Table 5b. Intracellular hydrocarbon extracted from the mutagenised *S. vacuolatus* cells after four and five weeks of treatments of spent coolant waste.

Week 4		Week 5	
Alkanes Compounds	Formula	Fatty acid/carboxylic acids	Formula
Butane,2,3-dichloro-2-methyl-	C5H10Cl2	Hexanoic acid	C6H12O2
Eicosane,7-hexyl-	C26H54	Alpha. -Hydroxyisocaproicacid	C6H12O3
Pentadecane,8-hexyl-	C21H44	1,2-Cyclohexane dicarboxylic acid	C8H12O4
Heptadecane,3-methyl-	C18H38	Heptanoic acid	C7H14O2
Eicosane	C20H42	Octanoic acid	C8H16O2
Heptadecane,2,3-dimethyl-	C19H40	Decanoic acid	C10H20O2
Nonane,5-(2-methylpropyl)-	C13H28	Dodecanoic acid	C12H24O2
6,6-Diethylhexadecane	C16H34	Tetradecanoic acid	C14H28O2
Hexacontane	C60H122	Nonahexacontanoic acid	C69H138O2
Heneicosane,5-methyl-	C22H46	Hexadecanoic acid	C16H32O2
Alcohols		9,12,15-Octadecatrienoic acid	C18H30O2
Oleyl alcohol, trifluoroacetate	C20H35F3O2	Oleic acid	C18H34O2
Z-7-Pentadecenol	C15H30O	Cyclobutane carboxylic acid, 3-pentadecyl ester	C20H38O2
1-Dodecanol,2-hexyl-	C18H38O	Dodecanoic acid, methyl ester	C13H26O2
1-Hexadecanol,3,7,11,15-tetramethyl-	C20H42O	Heptadecanoic acid	C17H34O2
1,1-Dimethyl-3-chloropropanol	C5H11ClO	Palmitoleic acid	C16H30O2
Fatty acid/carboxylic acids		Eicosanoic acid	C20H40O2
Octanedioic acid,4-methoxy-, dimethyl ester	C11H20O5	1-Cyclohexanecarboxylic acid, 1-(4-methyl-2-oxopentyl	C10H16O3
Hexadecanoic acid	C16H32O2	9-Octadecenoic acid (Z)-, (tetrahydro-3-furanyl) methyl	C23H42O3
Malonic acid, di(2-heptyl) ester	C24H46O4	Propanedioic acid	C3H4O4
Malonic acid, 2,4-dimethylpent-3-yl tetradecyl ester	C24H46O	Oleic acid	C18H34O2
Pentadecanoic acid,13-methyl-, methyl ester	C17H34O	Linoleic acid	C18H36O2
Boric acid, ethyl-, dodecyl ester	C22H47BO2	Eicosapentaenoic acid	C20H30O2
l-(+)-Ascorbic acid,2,6-dihexadecanoate	C38H68O8	Palmitic acid	C16H32O2
Carbonic acid, neopentyl cyclohexyl methyl ester	C13H24O3		
Bromoacetic acid,2-tetradecyl ester	C16H31BrO2		
1,3-Cyclohexanediacetic acid	C10H16O4		
Fumaric acid, 8-chlorooctyl hexyl ester	C18H31ClO4		
6-Octadecenoic acid, methyl ester, (Z)-	C19H36O		
Docosahexaenoic acid	C22H32O2		
Carbonicacid, allyl2-ethylhexyl ester	C12H22O3		
Cyclobutane carboxylic acid, 3-pentadecyl ester	C20H38O2		

The table shows the list of intracellular hydrocarbons with their molecular formula found in the cells of the mutagenised *S. vacuolatus*

3.3.2. SCW extracellular biodegradation by mutagenised S. vacuolatus

In this study, the mutagenised *S. vacuolatus* treatments of SCW at various weeks caused increase in the TPH degradation after five weeks of treatment. This degrading trend was also seen in the SCW treatment with the wild-type microalgae. The TPH degradation of SCW degradation by mutagenised *S. vacuolatus* are shown in supplementary document 7, Fig. S7. Significant differences ($p < 0.001$) in TPH degradation among the treatment weeks were observed. The range of TPH degradation observed were 65.22% ± 0.52, 82.57% ± 0.11, 88.33% ± 0.21, 92.26% ± 0.46 and 100%, for week one, two, three, four and five, respectively. TPH degradation was highest in weeks five and four, and lowest in week one. The increased TPH degradation was probably due to improved metabolic activities of the mutagenised *S. vacuolatus* [58].

Tables 3a and 3b shows the HCs degradation by mutagenised *S. vacuolatus* after five weeks of treatments. The result obtained revealed higher oxidation of extracellular HCs of SCW. An increase in the oxidation activity with increasing degradation products were observed from week one to week four in comparison to the control. The alkanes ranging from C8 – C43 and their isomers were completely degraded resulting to new alkanes derivative ranging from C12 – C54 were observed in week one – week four. The synthesis of the new alkanes suggests the metabolic activity of the mutagenised *S. vacuolatus* in the course of utilising SC waste as carbon source. Alkanes compounds C8 – C15 and C16 – C36 have been reported to be easily oxidised by microorganisms as a result of the non-complex nature of these compounds [46]. Similarly, *Microcoleus chthonoplastes* and *Phormidium corium* has been reported to oxidise n-alkanes [59]. Another interesting result observed were the complete removal of all alkenes (C7 – C20) and cycloalkanes (C8 – C13) in all the weeks of treatment by mutagenised *S. vacuolatus*. The degradation of these compounds clearly proves the excellent extraordinary ability of this mutagenised *S. vacuolatus*. Our finding also shows that the mutagenised *S. vacuolatus* successfully utilised and metabolised the alkanes, alkenes, and cycloalkanes fractions of SCW.

The oxidation of the aliphatic compounds resulted in various intermediate degradation products including alcohols, ketones, carboxylic acids consisting of fatty acids and other organic compounds compared to the control (Table 1). For instance, the fatty alcohols (1-decanol,2-methyl, 11-methyl dodecanol, 1-hexadecanol, 3,7,11, 15-tetramethyl), ketones (2-pentadecanone,2,6,10,14-trimethyl, 5,8-tridecadione, 3,6-undecandione and 2-Pentadecanone,6,10,14-trimethyl) and fatty acids (tetradecanoic acid, decanoic acid, dodecanoic acid, hexadecanoic acid, tridecanoic acid, undecanoic acid, and pentadecanoic acid) were produced. These compounds were found to be the major intermediate products of alkanes degradation, which were likely synthesised by oxidation or subterminal oxidation [36,49]. The production of these compounds further confirms the active metabolic activity of the mutagenised *S. vacuolatus*. In addition, the accumulation of these degradation products with the attachments of methyl groups suggests the degradation of the alkanes HCs occurring by methylation oxidation pathway. The degradation of alkanes also resulted in the production of monocarboxylic acids such as acetic acid,7,7-dimethyl-2-oxobicyclohept-1-ylmethyl ester, acetic acid, trifluoro-, dodecyl ester, and methoxyacetic acid, 4-hexadecyl ester. These acetic compounds could have been produced via beta oxidation pathway [36].

The cycloalkanes oxidation products detected in all the treatment weeks were the cyclic alcohols, ketones, and carboxylic acids including cyclohexanol,2,4-dimethyl, (+-)-trans-1-Isopropenyl-4-methyl-1,4-cyclohexanediol, cyclohexanone, 1,3-cyclohexanedione,5,5-dimethyl-2,2-dipropyl, cyclohexanone,3-(4-hydroxybutyl)-2-methyl, cyclohexane, carboxylic acid, 3-methylcyclohexane carboxylic acid, and cyclohexane carboxylic acid, 4-pentyl-,2,3-dicyano-4-ethoxyphenyl ester. This shows that the mutagenised *S. vacuolatus* has a high multi-degradation ability for the various HCs of SCW. It was found that the cycloalkane oxidation pathway observed in this study was similar to the alkane oxidation pathway reported by [36]. Interestingly, the metabolic degradation of cyclic alkanes compounds by the mutagenised *S. vacuolatus* has never been reported, thus making this study the first to report this observation.

Apart from degrading the alkanes and cyclic alkanes, mutagenised *S. vacuolatus*, completely degraded all monoaromatics, PAHs and chlorinated compounds after week one with only the degradation products present in the treated oil sample. The early breakdown of the aromatics could be explained by their solubility [59] and utilisation these HCs as substrates, which in turn stimulated the degradation of SCW. Among the monoaromatics degradation products formed were the benzene alcohols and carboxylic acids including, benzyl alcohol,4-methoxy-6-fluoro, 2,5-di-tert-Butyl-1,4-benzoquinone, dihydroartemisininoxymethyl benzoic acid, 1,2-benzenedicarboxylic acid, butyl,2-methyl propyl ester, benzyloxy tridecanoic acid, 4-trifluoromethylbenzoic acid, tridecyl ester, etc., (Tables 3a and 3b). The PAHs degradation products detected were also the naphthalene alcohols ketone and carboxylic acids such as 1-naphthol,1,2,3,4-tetrahydro-2-methyl, 3,4-dihydro-3-methyl, naphthalen-1-one, 2-naphthalenecarboxylic acid, 4,4′-methylenebis, 2-naphthoic acid, 3-methoxy-4-methyl etc. Some of the monoaromatic and PAHs products produced by the mutagenised *S. vacuolatus* were similar to those produced by the wild-type microalgae. Again, these aromatic alcohols, ketones and carboxylic compounds were identified as the degradation products of the monoaromatics, and PAHs as stated above. Furthermore, the presence of these compounds demonstrates that the aromatic HCs degradation of SCW is influenced by the hydroxylation and methylation mechanism [25]. More so, the aromatics products detected in this present study were similar to the aromatic metabolic products reported by [53] and [12]. After week two, and three, no aromatic HCs were detected, the compounds formed were the alkane derivatives alcohols, fatty acids and other oxidised products which are considered to be non-hazardous to the ecosystem. By week four, only fatty acids and carboxylic acids were present. This proved the total degradation of HCs present in the SCW. After week five, no HCs were detected, indicating that the HCs in the SCW were efficient removed by the mutagenised *S. vacuolatus* (see supplementary document 9, Fig. S9) for the gas chromatogram. This therefore provides an opportunity for the exploitation of this mutagenised *S. vacuolatus* for bioremediation.

3.3.3. Intracellular compounds in the cells of wild-type and mutagenised microalgae in spent coolant waste

Tables 4a and 4b and Tables 5a and 5b shows the list of intracellular compounds detected in the cells of wild-type and mutagenised *S. vacuolatus*. The results showed the intracellular bioaccumulation and biosynthesis of HCs by both microalgae groups in comparison to the control and extracellular degradation compounds. The accumulated and synthesised HCs were variation of alkanes, alkenes, alcohols, fatty acids, carboxylic acids, and other carbon-based organic acids. In the wild-type microalgal cell extract after week one – week five, alkanes HCs that range from C7 – C54; alkenes C7 – C35; alcohols C11 – C33; fatty acids C13 – C27 and other carboxylic compounds C6 – C24 were detected. Whereas alkanes ranging from C5 – C60; alkenes C5 – C35; alcohols C5 – C36; fatty acid C16 – C69 and carboxylic acids C6 – C38 were detected in the mutagenised *S. vacuolatus* cells extracts. The presence of these compounds in the cell extracts of both microalgae groups indicates intracellular utilisation and biodegradation of SCW [24].

The alkanes such as dodecane, pentadecane, tetradecane, hexadecane, etc., present in the control were transformed by both microalgae groups. For instance, dodecane, pentadecane, tetradecane and hexadecane were transformed into their respective fatty acid derivatives such as dodecanoic acid methyl ester, pentadecanoic acid, tetradecanoic acid and hexadecanoic acid. The cycloalkanes cyclohexane and cyclopentane were also broken down intracellularly into their respective carboxylic acid derivatives. These alkanes and cycloalkanes compounds' transformation products further reveals intracellular degradation occurring via intracellular methylation and oxidation [13,21,24].

Alkenes compounds such as 7-heptadecene, 3-hexadecene, 3-hexene,3-ethyl-2-5-dimethyl, squalene, 17-pentatriacontene, etc., were common to both microalgae groups. These alkenes could have been produced from the intracellular microalgae metabolism of the fatty acids detected in this study [60]. Also, the alkenes produced in this study, were identified as those of the long-chain alkenes which have been detected in different microalga species and believed to have great industrial and biotechnological importance [60,61]. In addition, these alkenes have been reported to contribute majorly to the hydrocarbon cycle of aquatic environment [60].

4. Conclusion

In this study, the wild-type microalgae, and mutagenised indigenous microalgae *S. vacuolatus* exhibited efficient multi-degradation abilities of various HCs fractions of SCW efficiently. The mutagenised *S. vacuolatus* exhibited higher DHA and TPH with SCW as carbon source when compared to the wild-type microalgae. The identified intermediate degradation products showed the methylation, isomerisation, oxidation/subterminal oxidation, hydroxylation and carboxylation for alkanes, cyclic alkanes, monoaromatic and PAHs biodegradation. With the excellent degradative abilities for HCs possessed by the mutagenised *S. vacuolatus*, this microalgal strain could be potential candidate that could be employed for bioremediation of oil contaminated sites.

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Availability of data and materials

All the data analyzed during this study are included in this research article and its supplementary documents.

Consent for publication

I Stella Eregie and Dr Sumaiyah Jamal-Ally, co-author of the manuscript provides consent for publication of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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CHAPTER 5

Synergistic effect of process parameters and nanoparticles on spent lubricant oil waste biodegradation by UV-exposed *Scenedesmus vacuolatus*: Process modelling, kinetics and degradation pathways

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Synergistic effect of process parameters and nanoparticles on spent lubricant oil waste biodegradation by UV-exposed *Scenedesmus vacuolatus*: Process modelling, kinetics and degradation pathways

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Nanoparticles
Spent coolant waste

ABSTRACT

The study examines the impact of process parameters and nanoparticles (NPs) on the degradation of spent coolant waste (SCW) by UV-exposed *S. vacuolatus*. The model was experimentally optimized using response surface methodology (RSM) with high coefficient of determination (R^2) >0.99. Upon validation of the model, total petroleum hydrocarbon (TPH) degradation of 100 % was obtained after 15 days under the optimized conditions of 25 °C temperature, 10 % (v/v) substrate concentration and 10 % (v/v) inoculum concentration. Moreover, the sensitivity of the process parameters on the degradation process assessed using artificial neural network (ANN) revealed high sensitivity of the degradation process to operational temperature. Metabolites obtained provide strong evidence for terminal oxidation and subterminal oxidation metabolic pathways in SCW degradation. In addition, the incorporation of NPs in the SCW degradation significantly enhanced the biodegradation efficiency of UV-exposed *S. vacuolatus* resulting in shortened degradation period from 15 to 12 days. Likewise, the inclusion of NPs substantially improved UV-exposed *S. vacuolatus* affinity ($1/K_s$) for SCW, growth constant (K_g) and maximum specific growth rate (μ_{max}). The degradation kinetics followed the second-order reaction with degradation rate constant (K) in the range of 0.029 to 0.288 m^{-1} t^{-1} for monoaromatics and 0.972 to 4.130 m^{-1} t^{-1} for PAHs, at half-life ($T_{1/2}$) ranges of 0.75 to 1.52 day^{-1} and 0.08 to 0.23 day^{-1}, respectively. This knowledge will enhance the design of waste pollutant degradation towards sustainable green environment.

1. Introduction

The past few decades have witnessed increasing contamination of the environment due to the excessive generation of spent oil waste (SOW) globally (Abdulrasheed et al., 2020; Tomar and Jajoo, 2021). The environmental protection agencies (EPA, 2016) had classified SOW as a highly toxic waste due to their associated health complications and environmental impacts, hence, it is vital to lessen their harmful effect on humans and the ecosystem (Gu, 2021). The elimination of SOW from the environment is both challenging and expensive. Although, the present conventional treatment methods are effective in treating SOW, but still faced with the issues of high cost, high energy consumption, incomplete decomposition of SOW and secondary pollution. Also, several research efforts have been made to improve the conventional treatment methods, but secondary pollution remain a major challenge (Hamouda et al., 2016). Thus, there is a need for a low cost and eco-sustainable method in the removal of SOW pollutants.

Biological approach using microalgae such as *Scenedesmus* sp. has shown to be successful for treating industrial contaminants like SOW (Abdel-Shafy and Mansour, 2018; Baghour, 2019; Al-Hussieny et al., 2020). Globally, microalgae treatment has attracted extensive attention as a result of their degradative capabilities, less energy consumption, low-cost and eco-friendliness in converting environmental contaminants into valuable biomasses and metabolites (Al-Hussieny et al., 2020; Dell'Anno et al., 2021). Microalgae treatment for the removal of environmental pollutants can be applied on-site without causing disruption to natural activities. This approach eliminates the necessity of transporting enormous amounts of waste offsite, thus, reducing potential

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risks to human health and the ecosystem that can occur during conveyance (Al-Hussieny et al., 2020; Dell'Anno et al., 2021). Interestingly, the utilization of microalgae for environmental pollutants has the potential to significantly reduce degradation cost in the range of 50–70 % compared to conventional methods.

Over the years, *Scenedesmus* species has attracted a lot of attention in the biodegradation of lubricant oil wastes and organic hydrocarbon compounds (Baghour, 2019; Dell'Anno et al., 2021; Subashchandrabose et al., 2013). Different *Scenedesmus* species such as *S. platydiscus*, *S. quadricauda*, *S. bijugatus*, *S. acutus* and *S. obliquus*, have been employed in the cleaning up of contaminates from the environment (Ajala and Alexander, 2020; Dell'Anno et al., 2021; Kuttiyathil et al., 2021). For example, *Scenedesmus obliquus* was found to biodegrade crude oil waste by 90 % in 7 days (El-Sheekh et al. 2013). Similarly, García de Llasera et al. (2016) observed 95 % TPH removal of benzo(*a*) pyrene by *Scenedesmus acutus* in 72 h of treatment. Also, Lei et al. (2007) reported that *Scenedesmus platydiscus* significantly removed 70 % pyrene HC and *Scenedesmus quadricauda*, remove phenols by 90 % after 10 days of treatment. Moreover, the biodegradation efficiency of water pollutants by these *Scenedesmus* species ranges from 75 % to 100 % (Baghour, 2019; Dell'Anno et al., 2021; Eregie and Jamal-Ally, 2019; Subashchandrabose et al., 2013). Although, the microalgae *Scenedesmus* with an estimated species of 32 is still largely under explored particularly in the removal of pollutant from the environment (Akgül et al., 2017; Baghour, 2019). Only few species have been employed for biodegradation of hydrocarbons especially, the use of the microalgal strain *S. vacuolatus* to biodegrade HCs and cleanup oil wastes is still very limited.

Usually, microalgae degradation efficiency of hydrocarbon pollutants is dependent on process parameters such as temperature, pH, light, oxygen, substrate, and inoculum concentration (Al-Hussieny et al., 2020; Dell'Anno et al., 2021). Of these process parameters, temperature, substrate, and inoculum concentration are of particular concern due to their vital roles in pollutant biodegradation (Al-Hussieny et al., 2020; Dell'Anno et al., 2021). Studies have reported temperature to be one of the most crucial factors affecting HCs biodegradation. Temperature affects HCs biodegradation by influencing HCs emulsification, enzymatic reactions, and overall metabolism (Al-Hussieny et al., 2020; Dell'Anno et al., 2021). Substrate concentration also plays a significant role in microalgae biodegradation (Varjani and Upasani, 2017). For example, in a study conducted by Gangireddygari et al. (2017), it was observed that an increase in substrate concentration simultaneously influence both microbial lag phase and the HCs degradation performance. Similarly, biodegradation of HC pollutants is dependent on inoculum concentration. Varjani and Upasani (2017), investigated the impact of inoculum concentration on the biodegradation of HCs. Their results showed that increased inoculum concentration favoured the biodegradation of HCs. Likewise, Zhang et al. (2018), investigated the relationship between inoculum concentration and biodegradation rate, higher inoculum concentration resulted in higher monoaromatic HCs and PAHs removal. Despite the extensive literature available on the influence of process parameters on the biodegradation of HCs, little is known about their respective role and the interactive effect of these process parameters on HCs biodegradation by microalgae. Hence, study on process parameters is considered to be a priority research objective because they are important regulatory factor for the biodegradation in polluted site. Additionally, the majority of SOW are biodegradable, but the process is often quite slow and could take months (Eregie and Jamal-Ally, 2019; Eregie and Jamal-Ally, 2021). Consequently, process parameters are regarded as limiting variables impacting biodegradation process. Therefore, the determination of optimal process parameters set points is essential in obtaining maximum and faster degradation of complex organic compounds. The significance of process parameter in microalgae biodegradation is related to their impact on microalgae growth, enzymatic and metabolic activities. Moreover, extreme conditions could adversely affect the viability and metabolic activity of degrading microbes (Ezzat and Ahmed, 2022). It is imperative to optimize the process parameters for maximum biodegradation of pollutants using reliable and efficient optimization tools like response surface methodology (RSM) and artificial neural network (ANN).

The RSM and ANN are statistical experimental tools that have been successfully used for modelling and optimizing process parameters in biological processes (Chouaibi et al., 2020; Ezemagu et al., 2021; Sabour et al., 2022; Rehman et al., 2022; Abuhena et al., 2022a; Abuhena et al., 2022b). Moreover, ANN could be used to analyze patterns and relationships, even in cases where the exact nature of the relationship is unknown (Ezemagu et al., 2021). RSM has been effectively applied for the optimization of biodegradation of diesel oil waste (Abdulrasheed et al., 2020) and spent engine oil (Goveas et al., 2020). To date, no modelling study on biodegradation process of SOW using UV exposed *S. vacuolatus* has been reported. Also, there is a scarcity of report on sensitivity studies that focus on the impact of process input variations on pollutant biodegradation. Gaining insight into the sensitivity of biodegradation processes to input variations can contribute to the development of more efficient bioremediation strategies.

Additional approach to achieve high degradation is the use of catalytic materials. The incorporation of nano catalytic material has received increased attention as a means of enhancing biodegradation processes. The potential incorporation of nanoparticles in microalgae degradation of pollutants is an emerging area of interest. Exploring this aspect could offer innovative and sustainable solutions for environmental clean-up of SOW polluted sites (Borji et al., 2020; Punnoose et al., 2021). Supplementation of *S. vacuolatus* biodegradation treatment with nanoparticles could promote growth, stimulate degrading enzymes as well as increase enzyme activity of the microalgae. The applications of nanoparticles in the biodegradation of HCs have been reported (Remya et al., 2022). Likewise, Ehmedan et al. (2021) reported that Fe_2O_3 and ZnO NPs highly improved and accelerated HCs (polychlorinated biphenyls and polycyclic aromatic) and crude oil degradation. In spite of these reports, there is scarcity of studies on the impact of NPs on *S. vacuolatus* biodegradation of oil waste.

Moreover, despite the increasing interest in employing microalgae for waste lubricant oil biodegradation, limited information is available regarding the kinetic processes involved under the various process parameters. Kinetic models could be used to predict the biodegradation rates of HCs and biodegradation potential at contaminated sites (Abdulrasheed et al., 2020). Several kinetic models have been proposed with potential to describe the variation in microbial growth, substrate utilization, and different biological responses during degradation processes (Abdulrasheed et al., 2020; Das and Deka, 2019; Hammershøj et al., 2020; Raffa et al., 2020). These models include first or second-order reaction kinetics, Monod, and logistic models (Hammershøj et al., 2020; Ghorbannezhad et al., 2022). The first and second-order reaction kinetics could evaluate biodegradation rate of waste to be degraded or removed (Abonyi et al., 2022). Usually, biodegradation rate is often a major function of biomass growth rate. Moreover, employing the Monod model, biomass growth rate in relation to the limiting substrate and utilization of HCs by microorganism can be elucidated. Likewise, logistic function model could be used to depict the changes in microbial growth rate by relating it to initial and maximum biomass concentration during waste biodegradation (Mohd et al., 2021). There is still limited knowledge on HCs biodegradation process kinetics utilizing microalgae. This kinetic study could provide additional insight on efficient SOW (HCs contaminants) biodegradation towards sustainable green environmental clean-up of SOW polluted sites.

Therefore, the aim of this study is to model the biodegradation process of spent oil waste (SOW) using UV-exposed *S. vacuolatus* towards improve degradation performance. The following specific objectives were carried out to achieve the above goal: (1) investigate the interactive effect of temperature, substrate concentration, and inoculum concentration process parameters on SOW total petroleum hydrocarbon (TPH) degradation and potential degradation pathways; (2) elucidate the biodegradation kinetics of SOW under optimized conditions using the zero, first, second-order reaction kinetics, Monod and logistic function models; as well as (3) evaluate the impact of nano-biocatalysts incorporation on the SOW biodegradation process also under optimized conditions.

2. Materials and methods

2.1. *Scenedesmus vacuolatus isolation and cultivation*

S. vacuolatus was isolated from SOW and cultivated in blue-green 11 (BG11) medium (detailed composition already specified in Eregie and Jamal-Ally (2019) using growth parameters in the range of temperature (20–40 °C), pH (5–8), substrate concentration (5–20 % v/v), shaking (100–250 rpm), inoculum concentration (5–20 % v/v) under fluorescent light tube of fixed dimension (63 × 17.5 × 17.5 cm; 20 W), light intensity <4.3 K for 35 days.

2.1.1. *Identification of microalgae by rbcL gene sequencing*

The genomic DNA of microalgae was obtained using the Favorgen DNA extraction Kit, Princeton (USA), and purified using the Zymoclean™ DNA recovery kit (USA). The DNA was then amplified using the ribulose-bisphosphate carboxylase (rbcL) gene universal primers: 192 rbcL Forward 5′GGTACTTGGACAACWGTWTGGAC–3′ and 1089 rbcL Reverse 5′ATACCACGRCTACGRTCTTT–3′ (Hadi et al., 2016). The PCR reaction mixture of 20 μL included 10 μL of kapal 2G, 2 μL of each primer (10 μM), 3 μL of DNA template (50 ng/μL) and 3 μL millipore water. PCR cycling conditions involved initial denaturation at 98 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 35 s, annealing at 52 °C for 35 s, extension at 72 °C for 40 s, and a final extension at 72 °C for 8 min. The resulting amplified rbcL gene was visualized on a 1.5 % agarose gel and subsequently sequenced using the ABI V3.1 Big dye Kit at Inqaba Biotechnology in Pretoria, South Africa. The obtained rbcL gene sequence was deposited into the national centre for biotechnology information (NCBI) database for microalgae identification. The rbcL gene retrieved from NCBI was aligned using muscle and the Maximum Likelihood method consequently a phylogenetic tree was constructed.

The molecular identification (see Supplementary document) and the rbcL gene sequencing revealed a band size of 1200 kb and 94 % identity to *S. vacuolatus* (FJ968741) respectively, based on the top hit sequence similarity results. The result obtained was consistent with the findings of Hadi et al. (2016).

2.2. *UV-exposed Scenedesmus vacuolatus*

The UV-exposure was carried out as described by Eregie and Jamal-Ally (2021). Thereafter, colonies of UV-exposed *S. vacuolatus* were used to inoculate the BG11 medium and were incubated at 25 °C for 14 days. The obtained microalgae culture was used as inoculum for the biodegradation optimization studies.

2.3. *Response surface modelling (RSM) of process parameters*

The process parameter selected for the biodegradation study were temperature (25–40 °C), substrate concentration (5–25 % v/v), and inoculum concentration (5–30 % v/v) while the response parameter was total petroleum degradation (TPH %). The choice of model process parameters and their ranges were chosen from literature and previous study (Bhattacharya et al., 2015; Ibrahim, 2016; Eregie and Jamal-Ally, 2019; Eregie et al., 2023). RSM using three-factor Box-Behnken design (BBD) was used to generate seventeen (17) independent experimental runs for model development (Table 1). The experimental data obtained were used to generate a polynomial equation (Eq. (1)) which relate the model response parameter (total petroleum hydrocarbon degradation) to the process parameters.

$$Y = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \alpha_{11} {X_1}^2 + \alpha_{22} {X_2}^2 + \alpha_{33} X^2_3 + \alpha_{12} X_1 X_2 + \alpha_{13} X_1 X_3 + \alpha_{23} X_2 X_3 \quad (1)$$

where Y represents the response factor (TPH degradation), α_0 is the intercept, $\alpha_1 X_1$ to $\alpha_3 X_3$ are the linear coefficients, $\alpha_{11} X_1^2$ to $\alpha_{33} X_3^2$ are the quadratic coefficients and $\alpha_{12} X_1 X_2$ to $\alpha_{23} X_2 X_3$ represents the interaction of coefficients.

2.4. *Biodegradation experimental set up*

The biodegradation treatment was carried out according to the Box–Behnken design, shown in Table 1. UV-exposed *S. vacuolatus* was inoculated into Erlenmeyer flasks (250 mL) containing BG11 medium (100 mL) at specified temperature, varying concentration of substrate, and inoculum. Afterwards, the TPH degradation of SCW was determined after 35 days using gas chromatography and mass spectrometry (GCMS) (Irawan et al., 2018).

2.5. *RSM model validation*

The RSM model was experimentally validated using the model predicted optimum set points. The statistical significance and validity of the model was evaluated by conducting an analysis of variance (ANOVA). The TPH degradation of the SCW was then determined using Eq. (2).

To further underscore the efficiency of the validated model, control experiments were simultaneously set up: control A (less oil concentra-

Table 1
RSM design for total petroleum hydrocarbon degradation in SCW.

Run	A: Temperature (°C)	B: Substrate concentration (v/v)	C: Inoculum concentration (v/v)	Observed %TPH	RSM predicted %TPH	ANN predicted %TPH
1	32.5	25	5	51.12	51.15	56.14
2	32.5	15	17.5	55.66	54.19	56.73
3	32.5	5	30	55.07	55.04	57.33
4	25	5	17.5	91.50	91.51	84.93
5	32.5	15	17.5	53.14	54.19	56.73
6	32.5	5	5	52.41	52.31	55.63
7	40	5	17.5	33.19	33.32	28.03
8	40	15	30	32.99	32.89	29.14
9	32.5	15	17.5	53.01	54.19	56.73
10	25	15	30	90.67	90.70	86.03
11	40	25	17.5	31.49	31.49	28.54
12	25	15	5	88.92	89.02	84.33
13	25	25	17.5	90.59	90.37	85.43
14	40	15	5	29.78	29.76	27.44
15	32.5	15	17.5	55.31	54.19	56.73
16	32.5	15	17.5	53.83	54.19	56.73
17	32.5	25	30	53.12	53.23	57.84

Total petroleum hydrocarbon – TPH.

tion) with 25 °C temperature, 10 % (v/v) oil concentration, 10 % (v/v) inoculum concentration; control B (high inoculum concentration) with 25 °C temperature, 10 % (v/v) oil concentration, 25 % (v/v) inoculum concentration; control C (low temperature) with 4 °C temperature, 10 % (v/v) oil concentration, 10 % (v/v) inoculum concentration, and control D (high temperature) with 45 °C temperature, 10 % (v/v) oil concentration, 10 % (v/v) inoculum concentration were carried out and the degradation efficiencies were compared to the optimal validated experiment.

$$\text{Total petroleum hydrocabon degradation } (\%) = \frac{\text{TPHc} - \text{TPHr}}{\text{TPHc}} \times 100 \tag{2}$$

where, TPH_c is the total petroleum hydrocarbon of the control SCW (%) and TPHr is the total petroleum hydrocarbon of the residual SCW.

2.6. Artificial neuron network (ANN) modelling of process parameters

A multi-layered neural network was employed using the principle of backpropagation to model the biodegradation of SCW as a function of the input variables used during the biodegradation process. The multi-layered perceptron (MLP) consisted of 1 input layer of 3 neutrons, 2 hidden layers of 6 neurons each and 1 output layer (Aruwajoye et al., 2022). The input variables included the temperature, substrate concentration, and inoculum concentration with the corresponding output as total petroleum hydrocarbon (TPH) degradation. The training and validation of the ANN model were performed using the 64-bit Python version 2.4.1 on Google Collaboratory platform. The experimental data was divided into two, 80 % for training and 20 % for validation. To process the data, a logistic transfer function was utilized in the hidden layer serving two purposes: (a) incorporating weighted inputs and the linked bias as well as (b) transforming the input data into a non-linear form, as depicted in Eqs. (3) and (4) (Aruwajoye et al., 2022). The logistic transfer function is considered suitable for regression-based models.

$$\text{Sum} = \sum\nolimits_{1}^{n} = 1^{\text{xiwi}} + \Theta \tag{3}$$

where wi ($i = 1, n$) are the connection weights, Θ the bias and x_i the input variable.

$$f(sum) = \frac{1}{(1 + exp(-sum))} \tag{4}$$

2.6.1. ANN training and validation

To optimize the performance of the ANN network and prevent overtraining or memorization, a backpropagation algorithm was employed. The aim was to minimize the net error on the validation dataset. The accuracy of the model was assessed via regression analysis (RA) by comparing the predicted process outputs with the experimental values. The coefficient of determination (R^2) was obtained for each model output; an illustration of the model's capability to accurately predict the TPH degradation of SCW.

2.6.2. Sensitivity analysis and knowledge discovery

Sensitivity analysis (SA) was conducted to evaluate the model's sensitivity to changes in input parameter values (Aruwajoye et al., 2022). The SA involved systematically varying each input parameter from its minimum to maximum values, while keeping the other inputs at their median set point. Subsequently, mathematical equations were generated from the developed model to depict the functional relationships between the process parameters and the model's output.

2.7. Inclusion of nano-biocatalyst in SCW biodegradation

2.7.1. Nanoparticle preparations

The nanoparticles (NPs): iron (II) oxide (Fe_2O_3), zinc oxide (ZnO), iron (III) oxide (Fe_3O_4), silver oxide (Ag_2O), cobalt (II) oxide (CoO), nickel oxide (NiO), manganese dioxide (MnO_2), copper (II) oxide (CuO), zero valent iron (nZVI) and composite nanoparticle ($Fe_3O_4ZnCoCu$) used in the present study were synthesized using previously established protocol already detailed in Sanusi et al. (2019).

2.7.2. Preliminary screening of nanoparticles for SCW biodegradation

The biodegradation of SCW by UV exposed *S. vacuolatus* with nanoparticle inclusion was set up in a 250 mL flask containing 90 mL BG11 medium and SCW (10 % v/v) supplemented separately with the different NPs (Fe_2O_3, ZnO, Fe_3O_4, Ag_2O, CoO, NiO, MnO_2, CuO, nZVI and $Fe_3O_4ZnCoCu$) at different concentrations (0.01 and 0.05 g/mL) incubated at 25 °C for 15 days. Control experiments were set up accordingly. Sampling from each set up was performed at 3 days intervals for 15 days. Thereafter, the samples were analyzed using GCMS and the impact of each NPs was evaluated based on *S. vacuolatus* biomass accumulation and SCW biodegradation efficiency.

2.8. Analytical protocols

S. vacuolatus biomass concentration was determined by the optical density at 680 nm and the cell dry weight was afterwards extrapolated from a predetermined standard calibration curve which was an established relationship between the optical density and biomass dry weight (Savvidou et al., 2021).

The Scanning Electron Microscope (SEM)-EDX ZEISS-EVO/LS15 (United Kingdom) was utilized to examine the elemental composition of the NPs and the Transmission Electron Microscope (TEM) JEM-1400 (JEOL, USA) was used to capture the NP's size and structural shape. To investigate the functional group of the NPs, Fourier Transform Infrared Spectroscopy (FTIR) (Spectrum 100, PerkinElmer, USA) was employed, and these were recorded within the range of 450 to 4000 cm^{-1}.

The total petroleum hydrocarbon degradation (TPH) of the residual SCW of the treatments were extracted by liquid extraction method using dichloromethane (DCM). The DCM extract was filtered using 10 g of anhydrous sodium sulphate. The residual oil extract was then mixed with 20 mL hexane and analyzed using GCMS (Model; QP 2010 series, Shimadzu, Tokyo, Japan). The GC unit furnished with a methyl silicone HP-5 MS capillary column (μm) with a film thickness of 30 m × 0.25 mm. The temperature of the oven was programmed from 40 °C to 260 °C for 8 min. Helium was employed as the carrier gas, with a flow rate of 0.8 mL/min. The temperature was programmed at 250 °C, and the sample was injected using a split mode injection at a ratio of 1:25. The mass spectra (MS) unit's ion source temperature was fixed at 260 °C, and the MS was recorded at a voltage of 70 eV.

2.8.1. Process kinetics

The specific growth rate (μ) was determined using Eq. (5). Subsequently, the specific growth rate values (μ) and the initial substrate concentration data were used to calculate the maximum specific growth rate (μ_{max}), kinetic growth constant and Monod constant (K_s).

$$\text{Specific growth rate } (\mu) = \frac{(\text{In X2} - \text{In X1})}{(\text{t2} - \text{t1})} \tag{5}$$

where μ is the specific microalgae growth rate, X_2 and X_1 were the biomass concentrations at time T_2 and T_1 respectively. The linear representation of this Eq. (6) is expressed as follows:

$$\frac{\mathbf{1}}{\boldsymbol{\mu}} = \frac{1}{\mu_{max}} + \frac{K_s}{\mu_{max}} \left(\frac{1}{S}\right) \tag{6}$$

Furthermore, the relationship between the growth rate (X) of *S. vacuolatus* at specific times (t) during the exponential and stationary phases of growth to initial microalgal biomass concentration (X_0), maximum biomass concentration (X_{max}), and maximum specific growth rate (μ_{max}) was illustrated using the integrated logistic function (Eq. (7)).

$$X = \frac{X_o \bullet exp(\mu_{max} \bullet t)}{1 - \left(\frac{X_o}{X_{max}}\right) \bullet \left(1 - exp(\mu_{max} \bullet t)\right)} \tag{7}$$

The kinetic growth constant (K_g) was evaluated by fitting a linear function to the exponential growth phase of *S. vacuolatus*. This was determined using Eq. (8).

$$ln\left(\frac{X}{X_0}\right) = K_g t \tag{8}$$

2.8.2. Zero–order, first–order and second–order kinetics

The reaction kinetics of SCW degradation in terms of zero–order, first–order and second–order were determined after 12 days using Eqs. (9), (10) and (11) respectively.

$$\text{Zero} - \text{order model } [C]_F = -kt + [C]_0 = \frac{[C]_0}{2k_0} \tag{9}$$

$$\text{Fisrt} - \text{order model } ln[C]_F = -kt + ln[C]_0 = \frac{ln2}{k_1} \tag{10}$$

$$\text{Second} - \text{order model } \frac{1}{[C]_F} = kt + \frac{1}{[C]_0} = \frac{1}{k_2[C]_0} \tag{11}$$

where C_F is the final oil concentration, C_0 is the initial oil concentration, K_0, K_1, K_2 is the degradation rate constant, respectively for zero, first, and second kinetic model, and T is the time.

2.9. Statistical analysis

The data obtained from this study were analyzed with one-way analysis of variance (ANOVA), using a Generalized linear model (GLM) of Statistical Software Package for the Social Sciences (SPSS) version twenty-four. The data presented were means of three replicates ($n = 3$). All results were expressed as mean ± standard error for each microalgae treatment group. LSD test was performed at a 5 % level of significance.

3. Results

3.1. Model development

Table 1 depicts the experimental responses observed for the biodegradation model design. The maximum degradation rate of SCW (91.50 %) was observed in experimental run 4 with temperature 25 °C, substrate concentration 5 % (v/v), and inoculum concentration 17.5 % (v/v), while the minimum degradation rate of SCW (29.78 %) was observed in experimental run 14 with temperature 40 °C, substrate concentration 15 % (v/v) and inoculum concentration 5 % (v/v). The removal efficiency of SCW was found to be most influenced by temperature. This is consistent with the findings of previous studies where temperature as a process parameter strongly affects the degradation of lubricant oil waste (Dell'Anno et al., 2021; Touliabah et al., 2022). The ANOVA was used to assess the developed model's fitness, as shown in Table 2. The high F-value of 904.23 and the low P-value of <0.0001 shows that the quadratic model is highly significant. Reports have shown that for a better model fitness the coefficient of determination (R^2) value should be between 0.80 and 1.00 (Ezemagu et al., 2021; Umar et al., 2022). In this study, the RSM model R^2 was 0.9991 indicating that the model could explain 99.91 % variability of the experimental data (Table 2). Furthermore, for a model to be considered quality and adequate, the discrepancy between the predicted R^2 and adjusted R^2 should be <0.02 (Ezemagu et al., 2021; Mohammed and Mabrouk, 2020). From the result obtained, the difference between the predicted R^2 and adjusted R^2 is 0.0005, suggesting that only 0.0005 % of the experimental data was not represented by the model. The polynomial model relating the TPH degradation and the independent process parameters are represented in Eq. (12).

$$\begin{aligned}\text{TPH degradation\%} = {} & 54.19 - 29.27\,A - 0.7425\,B + 1.20\,C - 0.1750\,AB \\ & + 0.3650\,AC - 0.1650\,BC + 7.57\,A^2 - 0.0900\,B^2 - 1.17\,C^2\end{aligned} \tag{12}$$

where TPH: model response output, linear coefficient A: temperature, B: substrate concentration, C: inoculum concentration, AB, BC, and AC: interaction coefficient, A^2, B^2 and C^2: quadratic coefficient, negative (−) and positive (+) signs: antagonistic and synergistic effects of the process parameters.

3.2. Interactive effect of process parameters on percentage (%) TPH degradation

Fig. 1a–c shows the three-dimensional (3D) response surfaces and contour plots obtained for the differences of the TPH degradation of SCW as a function of values of two operational parameters, with the other two operational parameters simultaneously varied or fixed to their midpoints. The optimal values of the individual factors were illustrated by the co-ordinates of the centre point within the maximum contour levels in each of the generated figures. In Fig. 1a, as temperature and substrate concentration increased from 25 to 28 °C and 5 to 25 % (v/v) respectively, the TPH degradation of SCW increased from 38 to 91 %. Further increase in temperature from 28 °C to 40 °C led to a drastic reduction in TPH degradation of SCW from 91 to 30 %. The maximum TPH degradation of SCW was attained at the temperature of 25 °C. Temperatures that were very high were not favourable to *S. vacuolatus* growth and the TPH degradation of SCW. Similar response was obtained for the interaction between inoculum concentration and temperature

Table 2
Analysis of variance (ANOVA) for the quadratic RSM model.

Source	SS	DF	Mean square	F-value	P-value	
Model	**7113.53**	**9**	**790.39**	**904.23**	**<0.0001**	**Significant**
A – Temperature	6852.69	1	6852.69	7839.65	<0.0001	
B – Substrate concentration	4.41	1	4.41	5.05	0.0595	
C – Inoculum concentration	11.57	1	11.57	13.23	0.0083	
AB	0.1225	1	0.1225	0.1401	0.7192	
AC	0.5329	1	0.5329	0.6097	0.4605	
BC	0.1089	1	0.1089	0.1246	0.7345	
A^2	241.28	1	241.28	276.03	<0.0001	
B^2	0.0341	1	0.0341	0.0390	0.8490	
C^2	5.76	1	5.76	6.59	0.0371	
Residual	6.12	7	0.8741			
Lack of fit	0.0790	3	0.0263	0.0174	0.9964	**Non-significant**
Pure error	6.04	4	1.51			
Cor total	7119.65	16				
Co-efficient of determination (R^2)	**0.9991**					

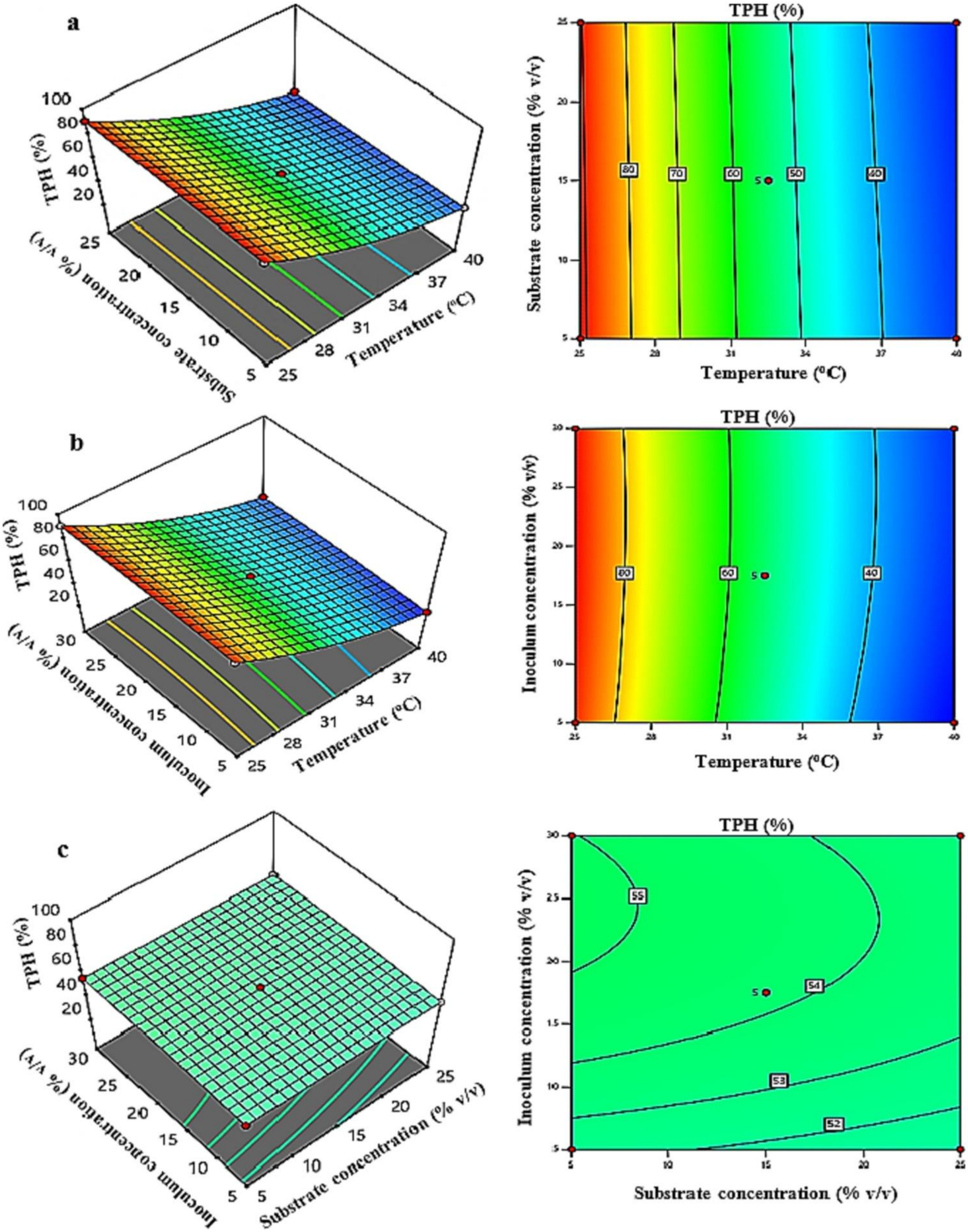

Fig. 1. The 3D and contour plot showing the interactive effect of the process parameters (a) temperature and substrate concentration, (b) temperature and inoculum concentration, and (c) inoculum concentration and substrate concentration on SCW degradation.

(Fig. 1b), as the inoculum concentration and temperature are simultaneously increased from 5 to 30 % (v/v) and 25 to 28 °C respectively, TPH degradation of SCW increased up to 91 %. A further increase in the temperature from 28 to 40 °C caused a sharp reduction in TPH degradation from 91 to 38 %. The other operational parameter, inoculum concentration, had an impact on the response as well, and the maximum TPH degradation found at around the midpoint. Moreover, the interactive effect of inoculum and substrate concentration on TPH degradation is shown in Fig. 1c. The concurrent increase in substrate concentration from 5 to 25 % (v/v) and inoculum concentration from 5 to 30 % (v/v) resulted in a linear increase in TPH degradation with a maximum degradation of 59 % and raising either substrate concentration or inoculum concentration had no noticeable influence on the TPH degradation.

3.3. Experimental validation of the developed models

Experimental validation was carried out in replicate using the predicted optimum set-points: 25 °C temperature, 10 % (v/v) substrate concentration and inoculum concentration 10 % (v/v) (see Supplementary document). Less than 100 % (Fig. 2a) and 100 % (Fig. 2b) TPH degradation was observed for the wild type *S. vacuolatus* and UV-exposed *S. vacuolatus* SCW biodegradation after 15 days respectively under optimized process conditions compared with the TPH degradation of 91.48 % predicted by the model. Furthermore, the obtained TPH degradation (100 %) of the UV-exposed *S. vacuolatus* (Fig. 2b) after 15 days of biodegradation treatment was higher compared to 83.53, 88.32, 48.64, and 30.43 % TPH degradation obtained for the control set up of less oil concentration, high inoculum concentration, low temperature,

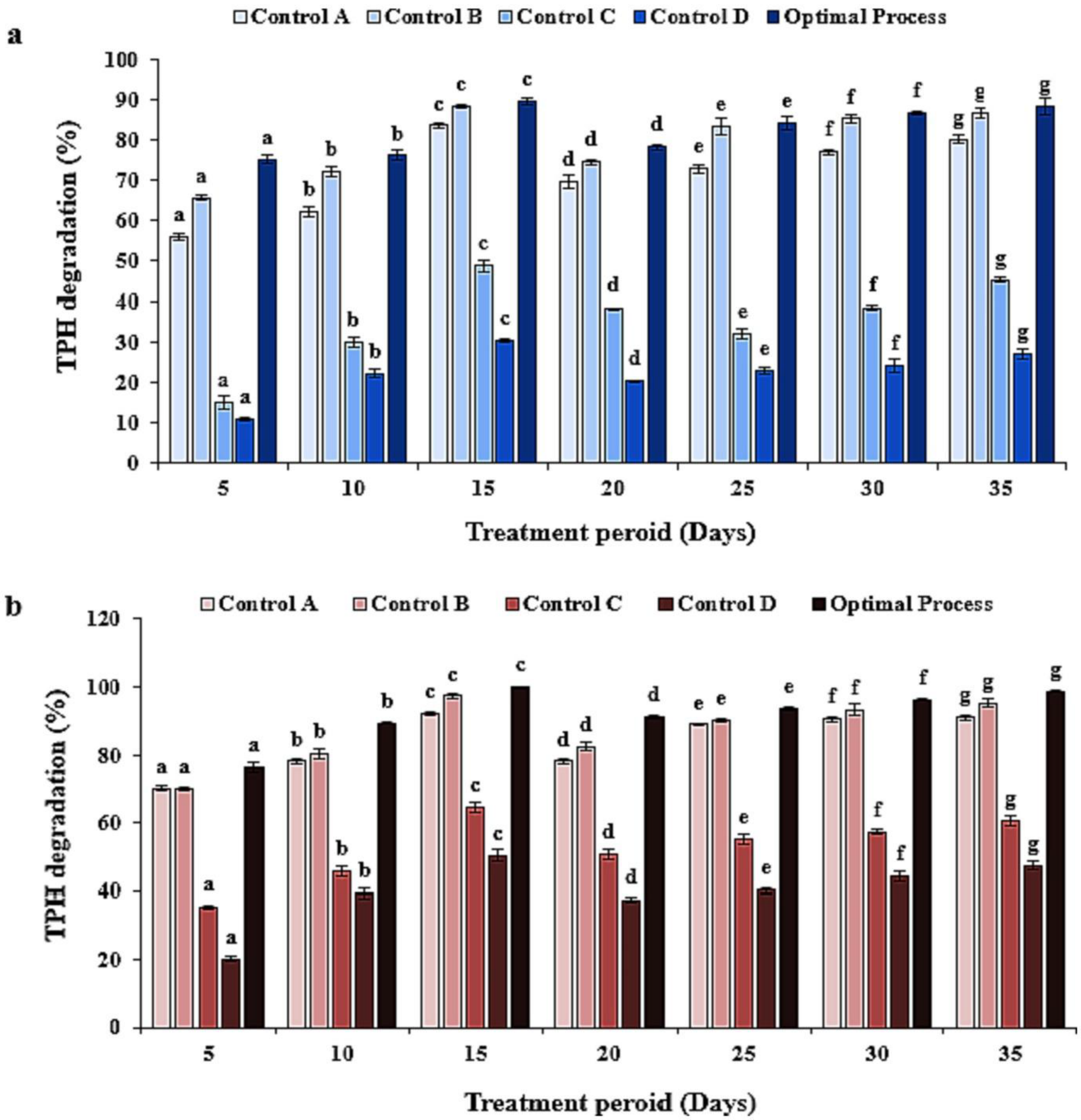

Fig. 2. Model validation for the biodegradation of SCW. (a) Experimental validation using wild *S. vacuolatus*. (b) Experimental validation using UV exposed *S. vacuolatus*. Optimal process: 25 °C temperature, 10 % (v/v) oil concentration, 10 % (v/v) inoculum concentration; Control A (Less oil): 25 °C temperature, 5 % (v/v) oil concentration, 10 % (v/v) inoculum concentration; Control B (High inoculum) 25 °C temperature, 10 % (v/v) oil concentration, 15 % (v/v) inoculum concentration; Control C (Low temperature) 4 °C temperature, 10 % (v/v) oil concentration, 10 % (v/v) inoculum concentration; Control D (High temperature) 45 °C temperature, 10 % (v/v) oil concentration, 10 % (v/v) inoculum concentration. The share letters (a, b, c, d and e) show that the TPH degradation are statistically significantly different ($P < 0.05$). Mean and standard error of 3 replicates ($n = 3$).

and high temperature respectively (Fig. 2a). Similarly, it was better in comparison to the biodegradation set up (89.49 % TPH degradation) using wild-type *S. vacuolatus* (Fig. 2a). The TPH degradation of SCW was lowest at very low and very high temperatures, while the highest TPH degradation was achieved at 25 °C. The optimum TPH degradation was achieved when the inoculum concentration and oil concentration were <15 %.

Furthermore, the HCs biodegradation of SCW by wild-type *S. vacuolatus* and UV-exposed *S. vacuolatus* under the same optimal conditions was analyzed using GCMS and compared to the control (see Supplementary document). The HCs detected in the uninoculated control were the alkanes, alkenes, PAHs, monoaromatic HCs, acid esters and alcohols (see Supplementary document). The most prevalent HCs were alkanes, followed by monoaromatics and PAHs, alcohol, alkenes, and acid esters. After the biodegradation treatment, compounds such as new alkane derivatives, fatty alcohols, ketones, fatty acids, alkane carboxylic acids, esters, and aromatic metabolites (carboxylic acid esters and diol) were detected in the treated oil sample. These compounds were identified as the metabolic by-products of alkanes, monoaromatic compounds and PAHs. For example, in the case of alkane degradation, pentadecane (C_{15}) was oxidized to n-pentadecanol (alcohol) probably by alkane hydroxylases via terminal oxidation. Then the n-pentadecanol was broken down into pentadecanal presumably by alcohol dehydrogenase, and finally metabolized into pentadecanoic acid (saturated fatty acid) by aldehyde dehydrogenase through beta (β) oxidation. Another alkane degradation metabolite identified was hexadecane (C_{16}). Hexadecane was catabolized into 2-hexadecanol by alkane hydrolases and then to 2-hexadecanone by alcohol dehydrogenase via subterminal oxidation. The 2-hexadecanone was further converted into 2-hexadecanal (ketone) and hexadecanoic acid methyl ester (fatty acid methyl ester) possibly by baeyer-villiger monooxygenase via β-oxidation. Based on the alkane metabolites identified in the SCW degradation, a potential catabolic pathway was proposed (Fig. 3a and b). In addition, monoaromatic metabolites detected include 1,2-benzenediol-4-methyl (class of catechol) and 1,2-benzenecarboxylic acid, monoethyl ester. While the PAH metabolites observed were 2-naphthol hydroxy and 3-hydroxy-2-naphthoic acid (Fig. 3c and d).

3.4. ANN-based model TPH degradation prediction

3.4.1. ANN model evaluation

Based on the ANN model, output values for the TPH degradation of SCW gave R^2 of 0.96 indicating a high replicability and accuracy in predicting the TPH degradation of SCW. The graph of predicted versus observed values showed most of the data points were clustered around the predictive diagonal line, further indicating high accuracy in the TPH degradation prediction (see Supplementary document). Moreover, the approach of employing assemblage of neural networks (assemblage averaging AA) rather than a single multilayer perceptron boosted the performance network predictions. The effect of AA on ANN model's prediction accuracy has been reported by previous studies (Piotrowski et al., 2016). The high prediction correctness of the ANN model on TPH degradation of SCW is significant as this displays the possibility for virtual analytical evaluation. This will promote the design of suitable waste biodegradation process regime with minimization of toxic side-products. This could boost the economic viability of waste degradation that are eco-friendly and environmentally sustainable.

3.4.2. Sensitivity of input parameter variations on process outputs

Fig. 4a–c demonstrates the impact of varying the different process parameter values, within the indicated ranges, on the TPH degradation of SCW. The SA indicated a drastic decrease in TPH degradation of SCW from 100 % to 9 % was observed when temperature was increased from 20 to 45 °C (Fig. 4a) illustrating a high sensitivity to temperature increase. In this case TPH degradation of SCW was largely dependent on operational temperature (see Supplementary document). On the other

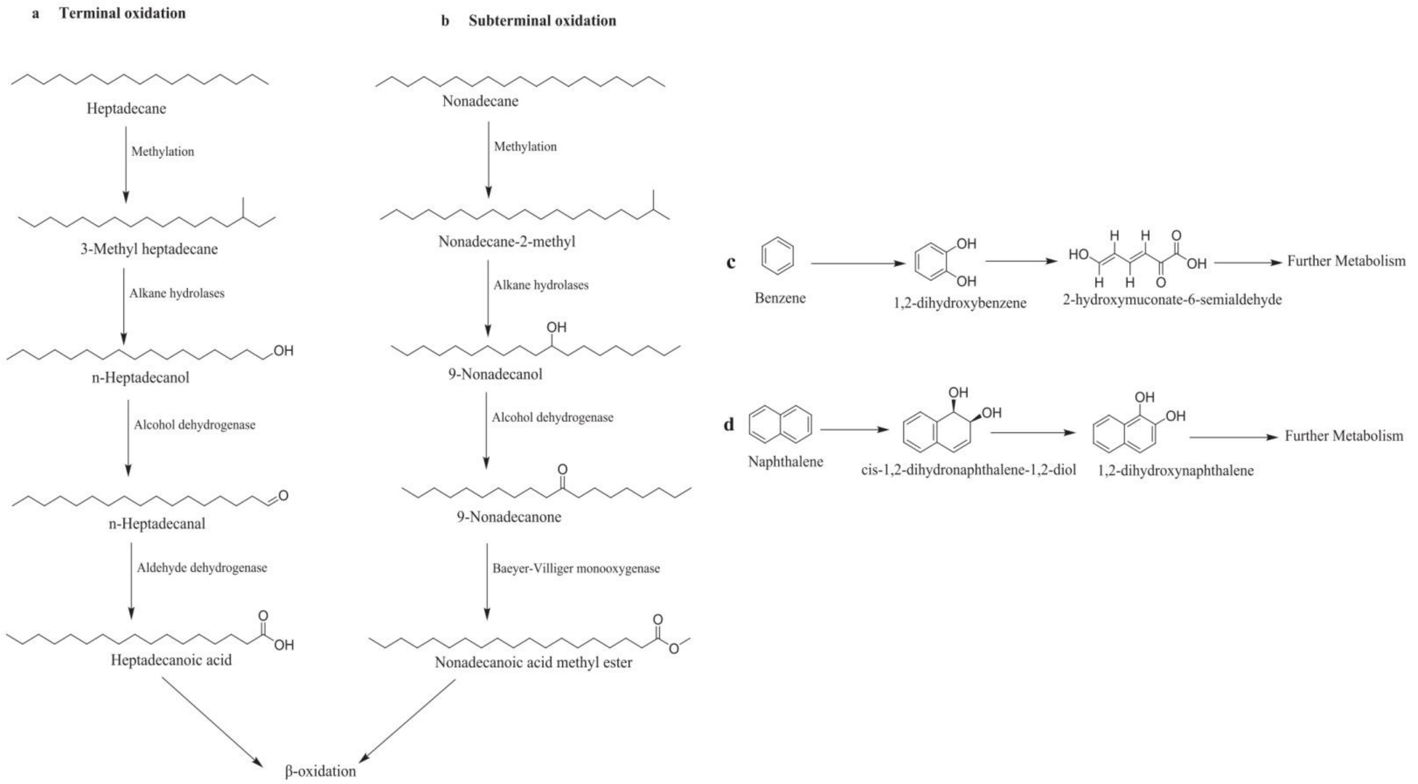

Fig. 3. Proposed pathway for the degradation of alkanes (heptadecane and nonadecane) by UV exposed *S. vacuolatus*. (a) Terminal oxidation pathway (b) subterminal oxidation pathway and the proposed degradation of aromatic (benzene and naphthalene) HCs by UV exposed *S. vacuolatus*. (c) Monoaromatic degradation (d) polycyclic aromatic degradation. The arrows represent probable enzymatic processes and pathways.

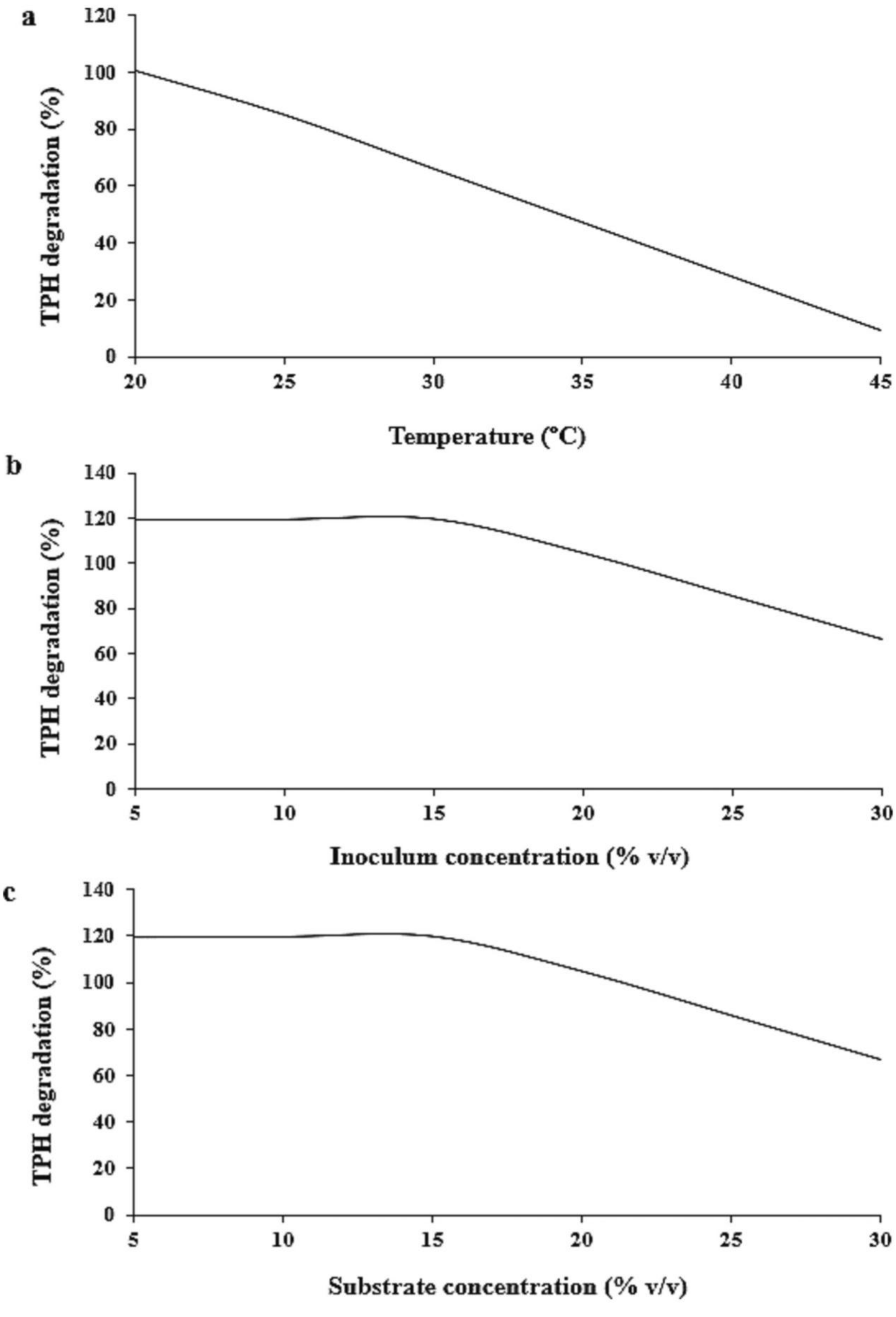

Fig. 4. The effect of fractional changes in process parameters on TPH degradation of SCW (a) temperature, (b) substrate concentration, (c) inoculum concentration.

hand, substrate and inoculum concentration parameters show similar effect on TPH degradation where an increase in both parameters from 5 and 15 % (v/v) gave a TPH degradation range of 119.11 to 119.57 % (Fig. 4b and c). This indicated that both the substrate and inoculum concentrations had no significant effect on the TPH degradation of SCW. However, an inverse relationship was observed for substrate and inoculum concentrations at value above 15 % (v/v), where a further increase in substrate and inoculum concentration from 20 to 30 % (v/v) led to a decrease in TPH degradation of SCW from 104 to approximately 67 % (Fig. 4b and c). This interaction was best illustrated by a regression type of relationship shown in Table 3.

3.5. Inclusion of nano-biocatalyst in SCW biodegradation

3.5.1. Nanomaterial characterisation

The elemental constituents and the phase purity of the synthesized NPs revealed high signal for the metallic and the oxide unit (see Supplementary document). The presence of high percentage weight of metallic unit is desirable as this determines the primary properties of the nanoparticle even the extent of their activities in biochemical reactions. The TEM image showed that most of the NPs were rough and circular in shape with particle sizes ranging from 11 to 47 nm (see Supplementary document). These must have influenced the impacts of the nanoparticles in the present degradation processes. Similar to our findings, Punnoose et al. (2021) and Karthik et al. (2022), obtained a spherical shape NPs with a particle size ranging from 11 to 40 nm. When nanoparticles with particle sizes such as obtained in the present study are employed at low concentration (<1 %), they have been reported to show desirable biocatalytic effect and high biochemical activities. This is largely in part due to their high surface area to volume ratio that provides greater number of reaction sites.

Moreover, the FTIR spectra of the NPs (see supplemented document) showed strong absorption band of Mn—O, Co—O, Cu—O, Ag—O, Zn—O and Fe_3O_4 found at 860, 659, 845, 797, 715 and 664 cm^{-1} respectively. While the absorption band below 650 cm^{-1} confirmed the presence of NiO and Fe NPs. The oxides of metallic NPs are commonly detected at absorption bands below 1000 nm wavelength resulting from interatomic pulsations (Eid, 2022). The broad absorption band peak ranging from 3628 cm^{-1} and stretching to 1031 cm^{-1} indicates the various stretching vibrations of $–CH_3$, $–CH_2$, =C–H, –C–H, C=O, –OH and NH functional groups. These peaks on each NPs spectrum may have been caused by absorbed moisture, carbon dioxide and precursor residue as well as indicating the degree of the nanoparticle purity (Kumar and Rani, 2013).

3.5.2. Preliminary screening of nanoparticles for SCW biodegradation

The biomass concentrations of *S. vacuolatus* in the presence of metallic oxide NPs are noteworthy (see Supplementary document). The results revealed significant differences ($P < 0.001$) in biomass concentration, after 15 days. The NP supplemented medium with UV-exposed *S. vacuolatus* had higher biomass concentration of 0.441 ± 0.037, 1.385 ± 0.028, 1.224 ± 0.001, 1.029 ± 0.064, 0.388 ± 0.030, 0.296 ± 0.001, 0.603 ± 0.027, 0.685 ± 0.055, 0.446 ± 0.015 and 0.323 ± 0.003 g/L for NiO, Fe_2O_3, Fe_3O_4, Ag_2O, CoO, MnO_2, CuO, nZVI, NP composite $Fe_3O_4ZnCoCu$ and ZnO in comparison to 0.25 ± 0.003, 0.603 ± 0.002, 0.537 ± 0.003, 0.489 ± 0.003, 0.199 ± 0.001, 0.193 ± 0.002, 0.438 ± 0.002, 0.301 ± 0.005, 0.285 ± 0.003 and 0.184 ± 0.036 g/L respectively obtained for the control set up. Additionally, in this study, lower concentration (0.01 g/L) of NPs resulted in higher biomass concentration compared to set up with higher NPs concentration (0.05 g/L) (see Supplementary document). Moreover, of the NPs employed to improve *S. vacuolatus* growth and degradation performances, five (5) of the nanoparticles (Fe_2O_3, Fe_3O_4, Ag_2O, nZVI, and CuO NPs) showed desirable results based on the biomass accumulation compared to other NPs. Hence, the five NPs were selected for further studies.

3.5.3. Effect of Fe_2O_3, Fe_3O_4, Ag_2O, nZVI, and CuO NPs on TPH biodegradation

The effect of Fe_2O_3, Fe_3O_4, Ag_2O, nZVI, and CuO NPs on *S. vacuolatus* TPH biodegradation of SCW showed significant difference ($P < 0.001$) in TPH degradation (Fig. 5). The maximum TPH degradation obtained for NPs supplemented UV-exposed *S. vacuolatus* biodegradation treatment was 100 ± 0.001 %, 99.44 ± 0.105 %, 99.22 ± 0.060 %, 98.43 ± 0.038 %, and 97.05 ± 0.040 % for Fe_2O_3, Fe_3O_4, Ag_2O, nZVI, and CuO NPs, respectively compared to 95.56 ± 0.102 %, 92.14 ± 0.060 %, 91.69 ± 0.013 %, 90.76 ± 0.022 % and 90.35 ± 0.962 % obtained for the control experiment, respectively. The inclusion of NPs highly enhanced the UV exposed *S. vacuolatus* growth and speed up the biodegradation of SCW from 15 days to 12 days. Analyses of metabolites formed due to the inclusion of NPs to UV-exposed *S. vacuolatus* growth medium, showed no HCs was detected in the Fe_2O_3 NP supplemented medium compared with the Fe_3O_4, Ag_2O, nZVI, CuO NPs supplemented UV-exposed *S. vacuolatus* after 12 days of degradation. The metabolites such as fatty acids (saturated and unsaturated fatty acids) were the main metabolites in the NPs supplemented UV-exposed *S. vacuolatus* degradation treatment. The presence of fatty acids metabolites strongly affirms the utilization and degradation of SCW. Among the NPs incorporated for SCW degradation, Fe_2O_3 NP inclusion was the most efficient in the biodegradation process compared to the other NPs.

3.6. Process kinetics

3.6.1. Growth kinetics

A growth constant (k_g) of 0.1549 day^{-1}, which was 1.15 times greater than the wild-type *S. vacuolatus* (0.1348 day^{-1}) (Fig. 6) was obtained with the UV-exposed *S. vacuolatus* during the SCW degradation process. Also, the maximum specific growth rate (μ_{max}) of 0.53 and 0.50 h^{-1}, Ks value of 0.070 g/L and 0.09 g/L were observed for UV-exposed *S. vacuolatus* and wild-type *S. vacuolatus*, respectively (Fig. 7). This corresponded to higher affinity constant (1/Ks) of 14.29 for the UV-exposed *S. vacuolatus*, compared to 11.11 for the wild-type *S. vacuolatus* (see Supplementary document). Similarly, higher maximum biomass concentration ($X_{max} = 0.822$ g/L) and $\mu_{max} = 0.301$ day^{-1} were obtained for UV-exposed *S. vacuolatus* compared to the wild-type (see Supplementary document).

In addition, the presence of Fe_2O_3 NP, UV-exposed *S. vacuolatus* had a k_g of 0.19 day^{-1}. This was 1.14 times higher than the control (0.1672 day^{-1}) under the same optimized condition (Fig. 6). The high k_g observed with the Fe_2O_3 NP supplemented UV-exposed *S. vacuolatus* process is also consistent with the obtained high substrate affinity, maximum specific growth rate, high biomass accumulation and enhanced TPH degradation observed with the Fe_2O_3 NP supplemented UV-exposed *S. vacuolatus* degradation process. Furthermore, in the presence of Fe_2O_3 NP, higher $X_{max} = 2.038$ g/L and $\mu_{max} = 0.487$ day^{-1} were obtained compared to the control process without Fe_2O_3 NP (Table 4). The obtained higher X_{max} and μ_{max} in the presence of Fe_2O_3 NP indicate the positive impact of the Fe_2O_3 NP on UV-exposed *S. vacuolatus* metabolic activities and growth.

3.6.2. Degradation kinetics

The kinetic degradation data obtained for the monoaromatics and PAHs degradation from the Fe_2O_3 NP supplemented UV-exposed

Table 3
Model equations illustrating the impact of fractional changes in the process parameters on the TPH degradation.

Equation	Process parameters	Model equation form	Equation type	Fitted model	R^2-value
(a)	Temperature	$y = a + b\cos(cx + d)$	Regression	$y = 4.46 + 8.66\cos(4.49x + 3.57)$	0.99
(b)	Substrate concentration	$y = \frac{a + bx}{1 + cx + dx^2}$	Regression	$y = \frac{1.10 + (-1.80x)}{1 + (-3.39x + 9.60)x^2}$	0.99
(c)	Inoculum concentration	$y = ae\frac{-(x - b)2}{x^2}$	Regression	$y = 1.22e\frac{-(x - 9.77)2}{2(1.83x^2)}$	0.99

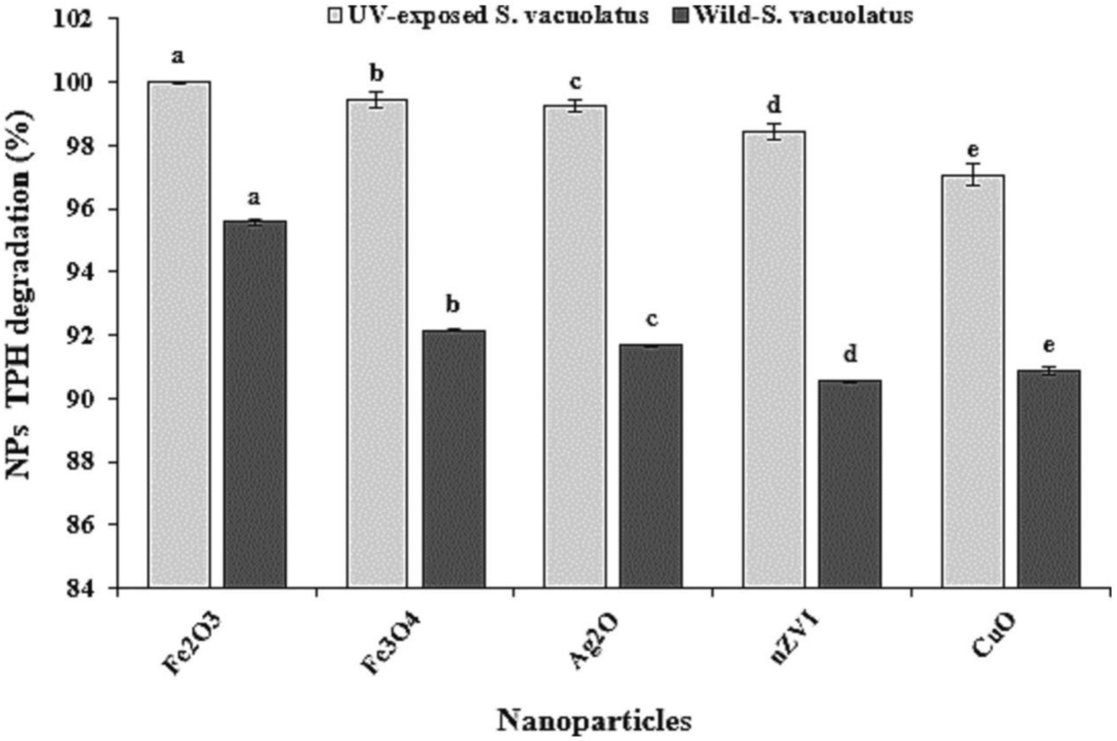

Fig. 5. The effect of 5 NPs (Fe_{2O_3}, Fe_{3O_4}, Ag_{2O}, nZVI, and CuO) on microalgae (wild *S. vacuolatus* and UV-exposed *S. vacuolatus*) after 12 days of biodegradation treatment of SCW. The share letters (a, b, c, d and e) show that the TPH degradation are statistically significantly different ($P < 0.05$). Mean and standard error of 3 replicates ($n = 3$).

S. vacuolatus degradation process was fitted to Zero, First and Second-order reaction models to further elucidate the kinetics of SCW HCs degradation in the presence of Fe_2O_3 NP (see Supplementary document). Higher K values in the range of 0.64–6.648 ($m^1\ t^{-1}$) and 0.193–0.855 ($m^1\ t^{-1}$) was obtained for the monoaromatics and PAHs, respectively in the Fe_2O_3 NP supplemented UV-exposed *S. vacuolatus* degradation process in contrast to the K values ranging from 0.153 to 3.706 ($m^1\ t^{-1}$) and 0.092–0.561 ($m^1\ t^{-1}$) obtained for the monoaromatics and PAHs in the control experiment respectively. Similarly, in the Fe_2O_3 NP supplemented degradation set up, the time ($T_{1/2}$) required to remove the PAHs and the monoaromatics by half was lower ranging from 3.027 to 3.215 day^{-1} and 3.374–3.695 day^{-1} compared to the control experiment with $T_{1/2}$ range of 4.632–6.717 day^{-1} and 5.797–6.582 day^{-1} respectively. Moreover, similar results were obtained for the first–order model (see Supplementary document). The K values obtained in the Fe_2O_3 NP incorporated degradation for monoaromatics and PAHs, ranged from 0.278 to 0.367 t^{-1} and 0.451 to 0.790 t^{-1}, respectively, at a $T_{1/2}$ of 1.88 to 2.48 day^{-1} and 0.87 to 1.53 day^{-1}, correspondingly. The higher K values and lower $T_{1/2}$ obtained in the Fe_2O_3 NP supplemented UV-exposed *S. vacuolatus* experiment suggest faster rate of degradation and lower $T_{1/2}$ for the monoaromatics and PAHs degradation.

Furthermore, in the second–order kinetic mode (see Supplementary document). The monoaromatics degradation in the Fe_2O_3 NP incorporated UV-exposed *S. vacuolatus* medium had a higher K value ranging from 0.029 to 0.288 $m^{-1}\ t^{-1}$ and lower $T_{1/2}$ of 0.75–0.94 day^{-1} in comparison to the K values (0.019–0.140 $m^{-1}\ t^{-1}$) and $T_{1/2}$ (1.2–2.38 day^{-1}) of the monoaromatics in the control experiment. Likewise, higher K values (0.972–4.130 $m^{-1}\ t^{-1}$) were obtained for the PAHs except for naphthalene, decahydro-1,2-dimethyl (1.949 $m^{-1}\ t^{-1}$) in comparison to the control experiment (0.595–3.452 $m^{-1}\ t^{-1}$) also with the exception of naphthalene, decahydro-1,2-dimethyl (3.452 $m^{-1}\ t^{-1}$). Lower half-life for the PAHs was also obtained with the inclusion of Fe_2O_3 NP in contrast with the control experiment.

4. Discussion

In the present study, the effect of process parameter on the degradation of SCW has been reported. The statistical analysis indicated that all three process conditions, viz., temperature, substrate concentration, inoculum concentration and their interaction had significant effects on the % TPH degradation of SCW. Increase in temperature and substrate concentration increased TPH degradation of SCW. At higher temperature a drastic reduction in TPH degradation of SCW was observed (Fig. 1a). Similar response was obtained for the interaction between inoculum concentration and temperature (Fig. 1b), as the inoculum concentration and temperature increases, the TPH degradation of SCW also increased and thereafter decreased with higher temperature. The significant impact of temperature on TPH degradation of SCW can be attributed to its positive effects on microalgae growth, enzymatic and metabolic activities during the degradation process (Dell'Anno et al., 2021; Touliabah et al., 2022). On the other hand, the decreased in the TPH degradation at higher temperatures (>28 °C), was most likely due to the (1) inhibition of microalgal growth, (2) oxygen limitation, (3) thermal deactivation of enzymes involved in degradation activities, and (4) cell denaturation. In addition, high temperature probably resulted in the formation of inhibitory compounds and waste products, consequently making the medium toxic and inhibiting degradation activities (Al-Hussieny et al., 2020; Khatoon and Rai, 2018; Kuttiyathil et al., 2021). Similar results have been reported by Bhattacharya et al. (2015), Ibrahim (2016) and Sonwani et al. (2019). Their studies revealed an increase in temperature from 25 to 45 °C caused a decrease in TPH degradation of waste lubricants. Moreover, the reduction in the TPH degradation of SCW at higher substrate and inoculum concentration (Fig. 1c) was most likely in part due to a lack of dissolved oxygen available to the microalgal cells. This result agrees with Ibrahim (2016), the author obtained the lowest TPH degradation at high inoculum and substrate concentration (15 % to 20 % v/v). Likewise, comparing the present study with previous studies, similar observations have been

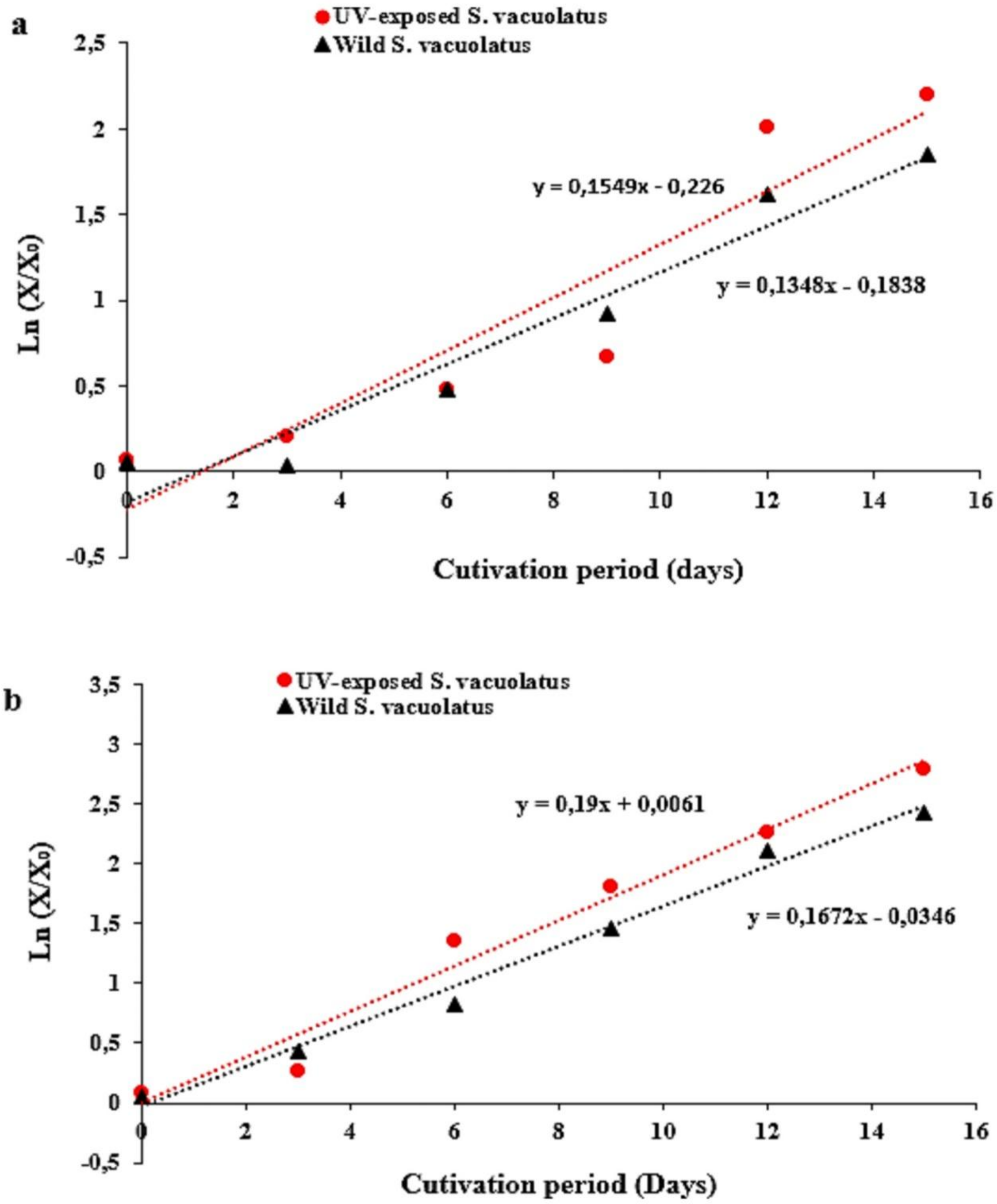

Fig. 6. The growth rate constant of microalgae. (a) The growth rate constant of microalgae (wild *S. vacuolatus* and UV-exposed *S. vacuolatus*) after 15 days of cultivation; and (b) the growth rate constant of microalgae (Fe_{2O_3} NP supplemented wild *S. vacuolatus* and UV-exposed *S. vacuolatus* growth) after 15 days of cultivation.

reported by Aldaby and Mawad (2019) and Kuttiyathil et al. (2021). These authors reported the significance of substrate concentration, inoculum concentration and temperature on HCs degradation. Higher temperature, substrate, and inoculum concentration reduced TPH degradation of HCs. Consequently, biodegradation efficiency is highly dependent on process conditions and not only could these have influenced the growth of the degrading microbe, the rates of enzyme activities, but also affect the metabolism and emulsification of HCs during the degradation process (Al-Hussieny et al., 2020; Dell'Anno et al., 2021). Due to the sensitivity of process parameters to the SCW degradation process, it is crucial to determine the optimum process conditions for maximum biodegradation rate.

After the process optimization and validation, the TPH degradation of 100 % was observed for the UV-exposed *S. vacuolatus* SCW biodegradation after 15 days under the optimized process conditions compared with unoptimized degradation process (35 days) (Fig. 2a and b). The high TPH degradation obtained after the process optimization suggests high enzymatic and metabolic activities under the optimal conditions resulting in high utilization of the SCW as carbon source (Ichor et al., 2016; Romero et al., 2018; Sutherland and Ralph, 2019). Moreover, the high TPH degradation by the microalgae could be due to increased secretion of natural emulsifiers such as biosurfactants under optimal condition. The secretion of these compounds most likely facilitates as well as improves cell-substrate interaction, thus increasing substrate degradability and consequently enhanced biodegradation (Rahman et al., 2019). The TPH degradation (100 %) obtained in the present study

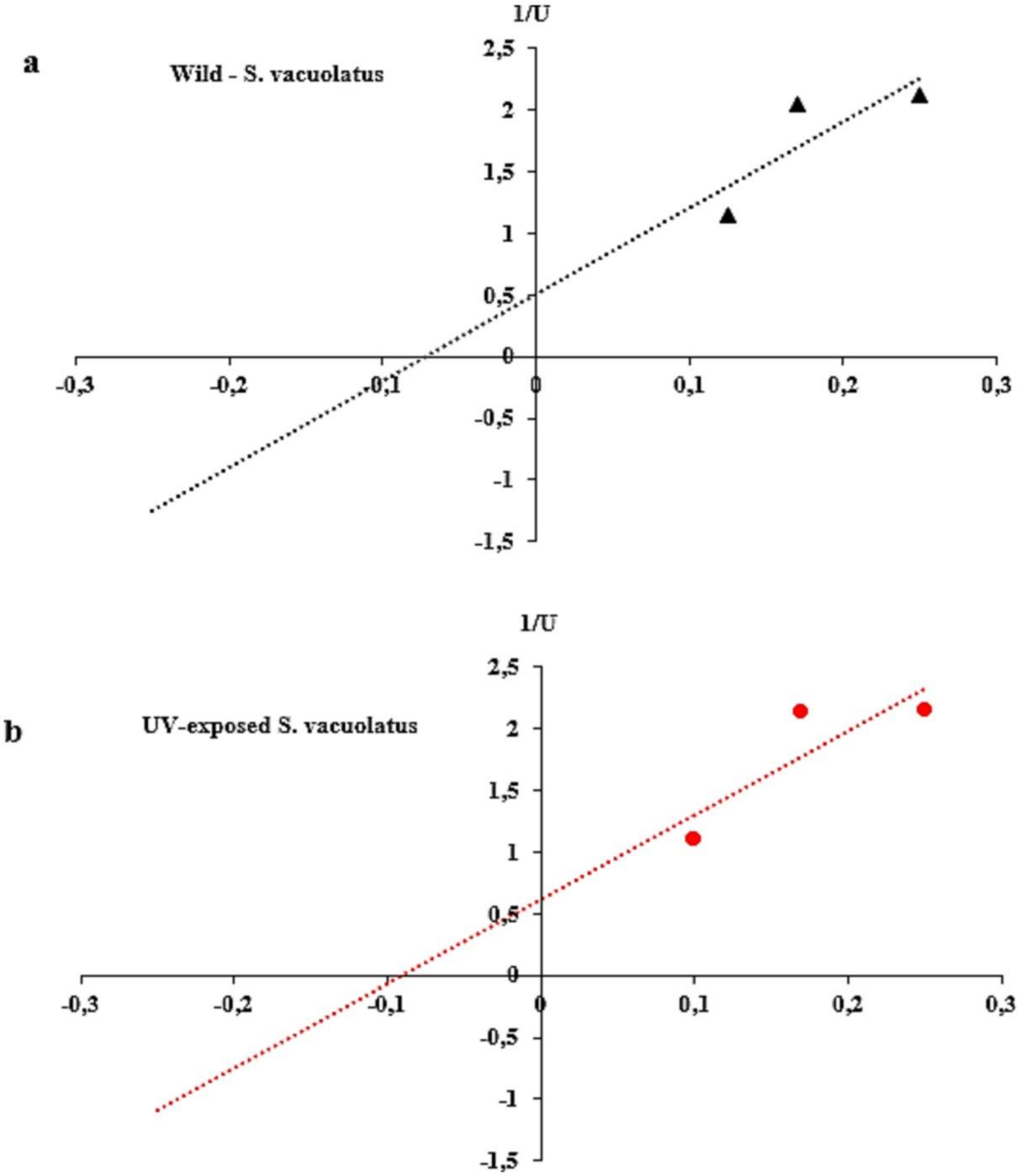

Fig. 7. Lineweaver–Burk plot estimating Monod constants for microalgae using SCW as substrate. (a) Monod constants for wild-type; and (b) Monod constant for UV-exposed microalgae *S. vacuolatus*.

Table 4
Kinetic parameters from logistic function model of *S. vacuolatus* growth without NPs.

Parameters	X_o (g/L)	X_{max} (g/L)	μ_{max} (day^{-1})	R^2
Logistic function model of S. vacuolatus growth without NPs				
Wild *S. vacuolatus*	0.027	0.742	0.208	0.965
UV exposed *S. vacuolatus*	0.033	0.822	0.301	0.977
Logistic function model of S. vacuolatus growth supplemented with Fe_2O_3 NP				
Wild *S. vacuolatus*	0.033	1.037	0.251	0.993
UV exposed *S. vacuolatus*	0.057	2.038	0.487	0.996

was also higher in comparison to those obtained in earlier reports. For example, Al-Hussieny et al. (2020) reported oil waste TPH degradation of 82 %.by cyanobacteria specie after weeks of treatment. In another related study Kalhor et al. (2017) reported soil polluted with crude oil with TPH degradation of 85 %. This was obtained after 35 days using *C. vulgaris* as the degrading microalgae.

Moreover, the result revealed that the UV-exposed *S. vacuolatus* exhibited a high HCs degradation ability and accumulation of oxidative products including n-pentadecanol, pentadecanal, and pentadecanoic acid, which were identified as the degradation products of pentadecane (C_{15}). The presence of these compounds strongly suggests that the UV-exposed *S. vacuolatus* utilized a terminal oxidization pathway to break down pentadecane (Fig. 3a) (Patowary et al., 2017). In contrast to pentadecane degradation, a different degradation pattern was observed for hexadecane. The metabolic by-products identified for hexadecane (C_{16}) degradation were 2-hexadecanol, 2-hexadecanal, and hexadecanoic acid methyl ester, signifying the utilization of a subterminal oxidation pathway by the UV-exposed *S. vacuolatus* (Fig. 3b). This variation in the degradation pathways observed between pentadecane and hexadecane shows the diverse metabolic strategies employed by the UV-exposed *S. vacuolatus* in degrading the different alkane HCs. This ability to utilize distinct pathways for different HCs is a remarkable characteristic of the UV-exposed *S. vacuolatus* metabolic capabilities.

Multi-degradative abilities exhibited by UV-expose *S. vacuolatus* enables the effective degrading of the wide range of HCs in SCW, underscoring to its potential applicability in various bioremediation processes. Additionally, the biotransformation of these alkanes was indicative of enzymatic process mediated by different enzymes such as alkane hydroxylases, dehydrogenases and baeyer-villiger monooxygenase (Wang et al., 2023). Although, various microalgae have been reported to oxidize both short and long-chain alkanes via the terminal and subterminal oxidation pathways (Ichor et al., 2016), however, the UV-exposed *S. vacuolatus* in this study accumulating the 2nd-positional-oxidation derivatives of n-hexadecane is a noteworthy observation which has not been reported. Thus, making this study the first to report these findings.

The oxidation of the monoaromatic HCs (benzene and its derivative) resulted in the formation of 1,2-benzenediol-4-methyl (a class of catechol) and benzene carboxylic acid ester strongly signifying metabolic pathway involving the monoaromatic HCs degradation (Figs. 3c and 4d). The presence of these metabolites confirms the biodegradation of the benzene and its derivative by UV-expose *S. vacuolatus*. It is also noteworthy that the observation of 1,2-benzenediol-4-methyl in our study's aligns with the report of Padhi and Gokhale (2017). According to their report, the degradation pathway of benzene starts with the production of cis-1,2-dihydrobenzene-1,2-diol, which is further transformed into catechol. Subsequently, catechol is broken down to carbon dioxide and water. Furthermore, the detection of the benzene-based metabolites in this study provides additional evidence supporting the established benzene catabolic pathway proposed by different researchers (Żyszka-Haberecht et al., 2019; Patel et al., 2020).

The oxidation of PAHs (naphthalene and its derivative) also resulted in the formation of 2-naphthol hydroxy and 3-hydroxy-2-naphthoic acid. The presence of these metabolites evidently suggests hydroxylation and carboxylation of the naphthalene and its derivatives (Żyszka-Haberecht et al., 2019; Patel et al., 2020). Also, the presence of these compounds supports an enzymatic process mediated by dioxygenase enzyme (Patel et al., 2020). Aromatic degradations by microalgae have been reported in literature (Subashchandrabose et al., 2013). For instance, cyanobacteria *Oscillatoria*, *Anabaena*, *Agmenellum*, and *Nostoc* have been reported to breakdown naphthalene into different metabolites (Subashchandrabose et al., 2013; Żyszka-Haberecht et al., 2019). In this study, the identification of the alkane and aromatic metabolites adds to the existing knowledge on microalgae HC metabolism and expands our understanding of their capabilities in degrading and utilizing different HC substrates. Additionally, these findings offer new possibilities for further research and exploration into the diverse metabolic potential of microalgae, which may have implications in various biotechnological applications.

Sensitivity analysis of the process parameters (Fig. 4a–c) indicated a drastic decrease in TPH degradation of SCW with increase in temperature suggesting a highly sensitivity and temperature-dependent process. High response to an input parameter implies that the TPH degradation of SCW will be highly influenced by slight variation on the process input parameter (Yu et al., 2023). By implication, lower temperature will rapidly increase the TPH degradation of SCW due to high enzyme activities and lower severe process condition that could result from higher degradation temperature (Al-Hussieny et al., 2020; Khatoon and Rai, 2020). On the hand, increase in substrate and inoculum concentration parameters, indicated that both the substrate and inoculum concentrations had minimal effects on the TPH degradation of SCW. However, further increase in substrate and inoculum concentration led to a decrease in TPH degradation of SCW (Fig. 4b and c). These observations align with the results of the RSM analysis (Fig. 1a–c). Thus, due to the sensitivity of temperature to the SCW degradation process, it is crucial to lower the process temperature and gradually vary the substrate and inoculum concentration. This can reduce bioremediation costs by decreasing energy consumption and minimizing the risk of contaminants migration. Additionally, this approach can aid in optimizing the biodegradation process of lubricant oil wastes and enhancing its efficacy by providing insights into the sensitivity of TPH degradation to various factors.

Likewise, remarkable outcomes were obtained with NPs inclusion indicating the positive impact of the NPs on UV-exposed *S. vacuolatus* metabolic activities and growth performance. These NPs most likely improved the growth rate of UV-exposed *S. vacuolatus* as well as serving as nutritional supplements for the microalgae growth, thus increasing biomass accumulation. Similar studies on the inclusion of NPs such as Cu, Fe, and Zn, in *C. vulgaris*, *S. obliquus*, and *Tetraselmis minutus* growth medium, revealed that NP inclusion meaningfully improve microalgal growth and biomass concentration (Sarkar et al., 2021; Wang et al., 2021). Other studies, for instance, Pádrová et al. (2015) reported the addition of Fe NP in *T. minutus* growth medium resulted in biomass concentration of 0.0317 g/L. Likewise, Rana et al. (2020) reported the effects of Fe_2O_3 NP on *C. pyrenoidosa* growth resulting in biomass concentration of 0.033 g/L. In another related study, Mykhaylenko and Zolotareva (2017) reported the biomass concentration of 0.02 g/L by *C. vulgaris* with the inclusion of Cu NP. Moreover, studies have reported the use of NPs to boost the removal of HC pollutants (Mandeep and Shukla, 2020). Recently, Ehmedan et al. (2021) observed a 95 % crude oil degradation after the addition of NPs. Likewise, Huang et al. (2019) reported 96 % TPH degradation of organic HCs from wastewater/oil after supplementation of growth medium with NPs. In another study by Borji et al. (2020), 96 % removal of PCB was observed with the addition of NPs to the waste medium. These TPH degradation values observed in the above studies were lower in comparison to the TPH degradation values obtained in the current study. The variances in TPH degradation observed were probably due to disparity in the nature of the NPs, process conditions and microalgae species. Specifically, the effect of Fe_2O_3, Fe_3O_4, Ag_2O, nZVI, and CuO NPs on *S. vacuolatus* TPH biodegradation of SCW revealed significant difference in TPH degradation. The inclusion of these NPs highly enhanced UV-exposed *S. vacuolatus* growth and accelerated the TPH biodegradation of SCW resulting in the most desirable degradation effect. The impact of NPs was elucidated by high growth constant (K_g) (0.19 day^{-1}), maximum specific growth rate (μ_{max}) (0.487 day^{-1}) and high biomass accumulation (X_{max}) (2.038 g/L). This was 1.14-fold, 1.62-fold and 2.48-fold respectively higher compared to the control experiment without NPs. These NPs possibly facilitated *S. vacuolatus* metabolic activities, enhanced degradation performance that resulted in efficient (100 %) and faster degradation of the SCW. The highly enhanced TPH degradation of SCW observed with the NPs supplemented UV exposed *S. vacuolatus* treatment medium could be attributed to improved interaction of the SCW with microalgae in the presence of the NPs. Also, this might be ascribed to their large surface area to volume ratio, oxidation-reduction potential (ORP), catalytic and buffering capacity (Sanusi et al., 2019; Sanusi et al., 2020).

Interestingly, the GCMS analysis of HCs in the NPs supplemented UV-exposed *S. vacuolatus* degradation treatment medium, showed successful and complete biodegradation of HCs in SCW after 12 days compared to 15 days before the inclusion of NPs. This was evidence by the metabolites observed after the degradation process. Fatty acids (saturated and unsaturated fatty acids) were the only metabolites in the NPs supplemented UV-exposed *S. vacuolatus* degradation treatment. The presence of fatty acids metabolites strongly affirms the utilization and degradation of SCW by bi-terminal oxidation pathway via beta-oxidation (Hu et al., 2020). This correlates with previous report on bi-terminal oxidation pathway of HCs degradation (Wang et al., 2023). During bi-terminal oxidation HCs are oxidized to produce fatty acid via ω-hydroxylation. Then the ω-hydroxy fatty acid is further transformed to a dicarboxylic acid via β-oxidation (Wang et al., 2023). Additionally, these fatty acids produced are also known to have no harmful effects on the environment and could be of biotechnological importance (Varjani and Upasani, 2017). The complete removal of the HCs in the SCW treated with UV exposed *S. vacuolatus* supplemented with Fe_2O_3 NP further underscores the catalytic potentials of nanoparticles in this case Fe_2O_3

NP in HCs degradation. In addition to aforementioned impacts of NPs, Fe_2O_3 NP have been reported to have high reactivity, increased the formation of bioemulsifiers and significantly reduce interfacial tensions (Khatoon and Rai 2018; Mandeep and Shukla, 2020; Ehmedan et al., 2021; Thangadurai et al., 2021; Salama et al., 2023). Moreover, iron (Fe) is a vital nutrient for microbial metabolic activities because it promotes cell growth, function as a cofactor for numerous enzymes. It is also needed for various biochemical processes, including photosynthetic activities and degradation process (El-Hoshoudy et al., 2019; Ehmedan et al., 2021). Although, NPs at high concentrations can impede microbial activities and growth through different mechanisms but at lower concentration (<1 %) they have improved both enzymatic and metabolic activities of microorganisms such as *S. vacuolatus* in HCs degradation (Sanusi et al., 2019). Hence, the enigma effect of NPs on biodegradation makes it an appealing area of research.

Other intriguing observations were the high k_g (Fig. 6a and b), maximum specific growth rate and the high substrate affinity (1/Ks) (Fig. 7a and b). The higher μ_{max} (0.53 day^{-1}) and 1/Ks (14.29) observed for the UV-exposed *S. vacuolatus* demonstrates improvement in the utilization of SCW as a source of carbon for growth (Hu et al., 2020; Pete et al., 2021; Varjani and Upasani, 2021). Moreover, the logistic model analysis revealed that the UV-exposed *S. vacuolatus* had a higher maximum biomass concentration (X_{max}) and maximum specific growth rate (μ_{max}). The obtained higher X_{max} and μ_{max} with UV-exposed *S. vacuolatus*, thus suggest that this microalgae strain is not only able to acclimatize to the SCW but also efficiently utilized the oil waste as a source of nutrient and energy. The μ_{max} obtained in the present study is consistent with the reports of other researchers (Hanief et al., 2020; Mohd et al., 2021). For instance, Ajala and Alexander (2020) observed μ_{max} of 0.16 and 0.18 day^{-1} for *S. obliquus* and *C. vulgaris*, respectively which were lower compared to the μ_{max} (0.53 day^{-1}) obtained with Fe_2O_3 NP supplemented UV-exposed *S. vacuolatus* degradation process.

The degradation kinetics (zero, first and second-order kinetic model), rate constants (K) and half-life ($T_{1/2}$) for the monoaromatics and PAHs degradation in the presence of Fe_2O_3 NP shows that the data fitted well into the second–order kinetic model. The relatively high $R^2 > 70$ value obtained indicated that the models successfully described the kinetics of the SCW degradation process. Higher K values and lower $T_{1/2}$ was obtained in the Fe_2O_3 NP supplemented UV-exposed *S. vacuolatus* experiment compared with the K values obtained for monoaromatics and PAHs in the zero–order, first–order and the control experiment, suggesting faster rate of degradation and lower $T_{1/2}$ for the monoaromatics and PAHs degradation. Similar results have been reported by Hammershøj et al. (2020) and Ghorbannezhad et al. (2022). The authors reported increase in K values and lower half-life as biodegradation rate improves. Although, the three (3) models successfully described the SCW degradation kinetics, the second-order model was the best model to describe the degradation of monoaromatics and PAHs in SCW. Interestingly, benzene and naphthalene derivatives were aromatics that were degraded effectively at shorter time in presence of Fe_2O_3 NP. This is desirable as aromatic HCs have been known to induce toxic effects in plants, animals, and human when inhaled or ingested (Ichor et al., 2016). Toxic effects include inflammation of the eyes and skin, nausea, kidney as well as liver and immue system impairment (Abdel-Shafy and Mansour, 2018).

5. Conclusion

The study demonstrated the impact of process parameters and nanoparticles (NPs) on the degradation of spent coolant waste (SCW) by UV-exposed *S. vacuolatus*. The optimization process resulted in 100 % TPH degradation of SCW using RSM ($R^2 = 0.99$) and ANN model ($R^2 = 0.96$), with significant nonlinearities. Moreover, biodegradation kinetics showed high biodegradation rate constants (K) and shorten half-life ($T_{1/2}$) of 6.65 $m^1 t^{-1}$, and 0.08 days respectively. Also, the degradation kinetics revealed the degradation of monoaromatics, and PAHs are best elucidated by the second-order reaction model. In addition, NPs inclusion significantly improved biodegradation efficiency, shortening the degradation period from 15 to 12 days. This study demonstrated the potential of an efficient, eco-friendly, cost-effective and sustainable biodegradation of environmental pollutants.

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CRediT authorship contribution statement

Stella B. Eregie: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Isaac A. Sanusi:** Conceptualization, Methodology, Formal analysis, Data curation, Writing – review & editing, Supervision. **Gueguim E.B. Kana:** Conceptualization, Writing – review & editing, Resources, Supervision, Funding acquisition, Project administration. **Olaniran O. Ademola:** Writing – review & editing, Resources, Supervision, Funding acquisition, Project administration.

Declaration of competing interest

The authors: Stella B. Eregie, Isaac A. Sanusi, Gueguim E.B. Kana, Olaniran O. Ademola declare there are no conflict of interest in this work.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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CHAPTER 6

Transcriptomic removal and mass balance of polycyclic aromatic hydrocarbons in waste spent coolant oil: Gene discovery, enzyme identification and metabolic pathway

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Abstract

The study identified the catabolic genes, enzymes and metabolic pathways involved in spent coolant oil waste (SCW) degradation by mutant S. vacuolatus (MSv) and wild S. vacuolatus cultivated without SCW and with SCW using RNA transcriptomic analysis. Moreover, total petroleum hydrocarbon (TPH) degradation and the metabolites released were determined using GC-MS. Major hydrocarbon (HC) degraded were naphthalene, decane, and benzene,1,3-dimethyl with highly enriched gene ontology obtained that provided strong evidence of enhanced cellular metabolic activities that enabled the oxidation of various hydrocarbons (HCs) present in SCW. Significant residual mass balances (<70% degradation) were obtained for the polycyclic aromatics (PAHs) and their derivatives. Also, distinct transcripts involved in SCW degradation include 6-CoA-linked acetaldehyde dehydrogenase, 3-coatomer subunit alpha-3 and 1-arginine deiminase. Additionally, a key transcript CoA-linked acetaldehyde dehydrogenase encoding alcohol dehydrogenase for the degradation of naphthalene HCs via a naphthalene degradation pathway was identified. These findings provide a major insight into HC degradation genes and enzymes that can further be exploited for the bioremediation of HC polluted water environments.

Keywords: *Scenedesmus vacuolatus*; biodegradation; spent coolant waste; transcriptomics.

6. Introduction

Excessive amounts of organic pollutants are improperly disposed in the environment (Hoang et al., 2021). Recalcitrant hydrocarbon compounds (HCs) such as aliphatic and aromatic HCs are major constituents of these organic pollutants, which have attracted global concern (Hoang et al., 2021; Lawniczak et al., 2020). Their deleterious medical and environmental effects have already been reported in different ecosystems (Ohanmu et al., 2019; Romero et al., 2018).

Numerous chemical and physical treatment methods have been applied for the removal of these organic pollutants such spent oil waste (SOW) in the environments. However, these physicochemical treatment approaches have been plagued with high costs, low pollutant removal efficiency, time consuming process and generation of high mass of toxic by-products, making them non-eco-friendly. Moreover, the by-products generated from the chemical and physical treatment can be more hazardous and require additional effort for decomposition (Al-Hussieny et al., 2020; Dell' Anno et al., 2021). In contrast, biological approach such as microalgae-based method has gained increasing interest as a viable alternative to physicochemical pollutant treatments in the field of environmental studies and sustainability (Bhatt et al., 2021).

The use of microalgae to degrade SOW is not only due to its efficient degradation capabilities, but also its low cost, environmental friendliness, and ability to grow autotrophically, heterotrophically, or mixotrophically in polluted sites. Additionally, their ability to use SOW as a source of carbon to produce new biomass and generate highly eco-friendly compounds (Eregie and Jamal-Ally, 2021; Eregie et al., 2023). Although there have been studies on the potential of microalgae to biodegrade SOW, but literature on microalgae to biodegrade HCs is still very limited compared to the use bacteria and fungi. Furthermore, detailed information on the specific strains of microalgae and their degradation pathways for SOW is also limited in literature. Several species of microalgae Scenedesmus such as S. obliquus, S. acutus, S. quadricauda, and S. platydiscus have been reported to developed nutritional strategies to obtain energy from aliphatic and several aromatic HCs available to them (Aldaby and Mawad, 2018; Baghour, 2019; Eregie and Jamal-Ally, 2019). These Scenedesmus spp. were also found to use HCs as carbon and energy sources via various intracellular, extracellular metabolic processes as well as aerobic or anaerobic pathways (Sutherland and Ralph, 2019). Furthermore, their unique genetic diversity accounts for their great photosynthetic and metabolic versatility (Gonçalves, 2021; Ismail et al., 2020; Baghour, 2019; El-Sheekh et al., 2013). For example, S. obliquus has been reported to effectively metabolize and degrade alkanes and alkyl cycloalkanes (Denaro et al., 2021). Similarly, S. obliquus demonstrated high utilization and degradation of both long and short-chain alkanes (El-Sheekh et al., 2013). In the same vein, S. acutus was found to degrade polycyclic aromatic hydrocarbon (PAHs) such as benzo(a)pyrene and the transformation resulted in the formation of cis-4,5-, 7,8-, 9,10- and 11,12-BaP-dihydrodiols which are less toxic to the environment (Chattopadhyay et al., 2023). Likewise, Ahmadi et al. (2022) and Ghodrati et al. (2022) reported the removal of benzo(b)fluoranthene by S. dimorphus from crude oil waste. Moreover, Jing et al. (2023) reported the complete degradation of phenol and nonylphenol by S. obliquus and S. quadriauda. Also, the removal of fluoranthene and pyrene were demonstrated using S. platydiscus (Ghosal et al., 2016).

While the genus Scenedesmus have been reported to biodegrade oil waste pollutants, the details of the gene-based metabolic pathways and cellular mechanisms involved are yet to be understood. Moreover, the genome-scale transcriptional responses of Scenedesmus to various HC pollutants are rarely reported. Additionally, there is a dearth of knowledge on molecular evidence to support the transcriptional responses of Scenedesmus and the involvement of enzymes such as hydrolases, dehydrogenases, and monooxygenase during HC degradation process. These probing using techniques such as RNA Sequencing (RNA-Seq) will provide additional insight into molecular-based HC degradation that can further be exploited for the biodegradation of environmental pollutants.

In recent times, the use of advanced technologies such as transcriptomics have been utilized to study microorganisms involved in HCs biodegradation (Tripathi et al., 2021; Xi et al., 2021). The transcriptomic sequencing has been studied in different species of microalgae (Wang et al., 2023). Transcriptomes analyses involve high-throughput methods, including de novo assembled-transcriptome, total transcript amplification, quantitative reverse transcription polymerase chain reaction (qRT-PCR), and transcriptomes sequencing (RNA-Seq) (Tripathi et al., 2021; Xi et al., 2021; Wang et al., 2022). Of these methods employed to analyze microalgae transcriptomes, RNA-Seq has been reported to show more desirable results in the assessment of microalgae diversity in the characterization of genes (Tripathi et al., 2021; Xi et al., 2021; Wang et al., 2022). Transcriptomic approach using RNA-Seq serves as a valuable tool for gaining deeper insights into microbial gene expression and regulation in response to different HC degradation.

This is because of its efficiency, enhanced precision, high-sensitivity, high resolution, and low cost.

Despite the abundance of literature on the RNA-Seq of microalgae, no research on the RNA-Seq of Scenedesmus vacuolatus genome has been reported. The RNA-Seq of S. vacuolatus in relation to HCs biodegradation could provide insight into the catabolic genes, enzymes and metabolic pathways used by this microorganism. This knowledge can be used for the development of an effective cleanup bioremediation strategy.

Therefore, the aim of this study is to identify the catabolic genes, enzymes and metabolic pathways involved in the metabolism of hydrocarbons in spent coolant oil waste using transcriptomics RNA-Seq. Subsequently, the differentially expressed genes and responsive genes during the spent oil waste HCs degradation were elucidated.

6.1 Materials and method

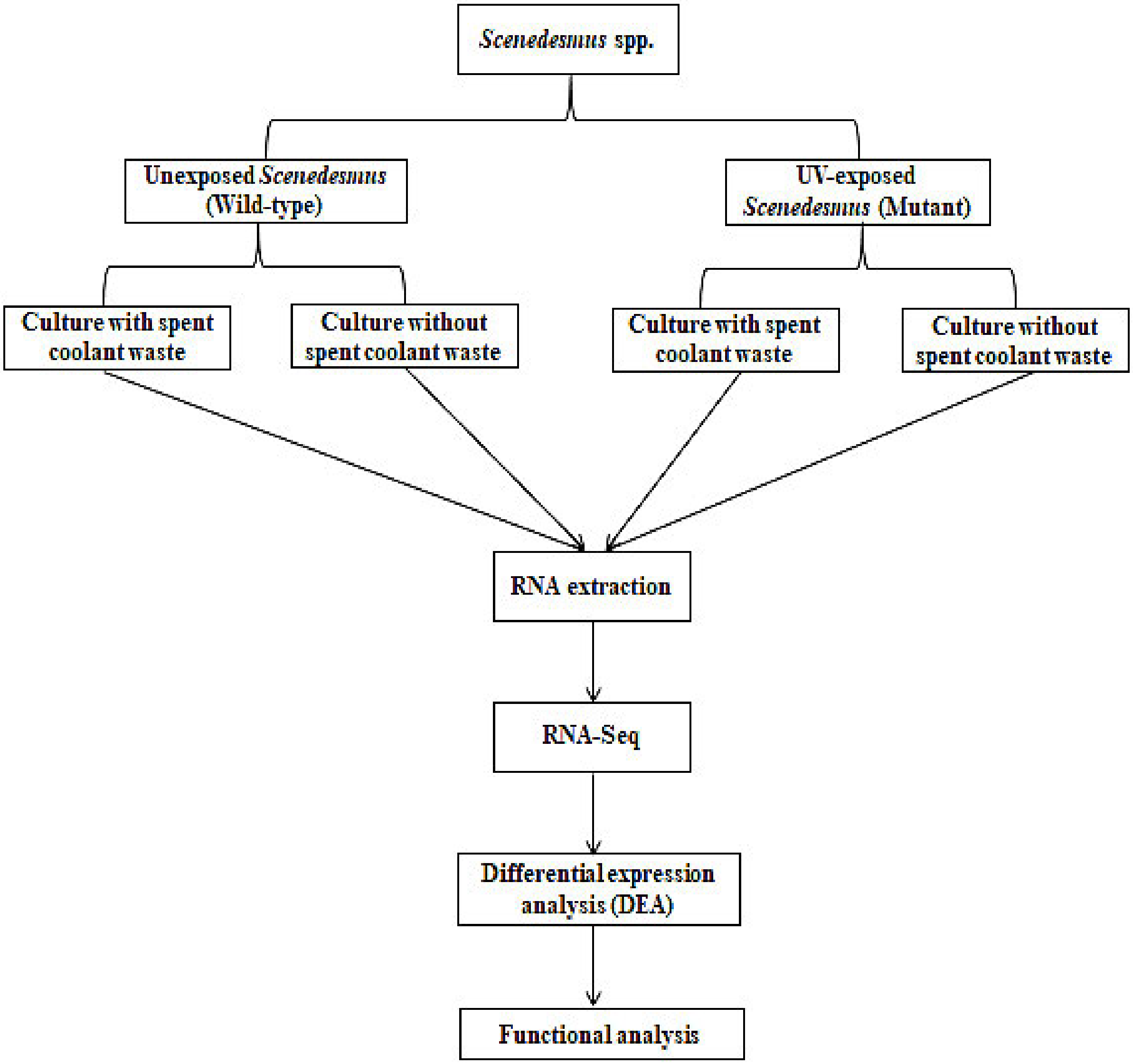


Fig. 6.1: Flow diagram of the general methodology of the study. The diagram illustrates the experimental and the bioinformatics analysis.

6.2 Microalgae strains cultivation

The wild-type and mutant *Scenedesmus vacuolatus* isolated from SOW and obtained from UV mutagenesis (exposed to UV radiation) respectively, were used in this study (Eregie and Jamal-Ally, 2019; Eregie et al., 2023). The microalgae were maintained separately on standard blue-green 11 (BG11 agar). The cultivated microalgae on BG11 agar was allowed to grow for fourteen days under standard conditions (Eregie et al., 2023). The BG11 medium used for the microalgae cultivation comprised (in litre: 2.86g H3BO3, 1.81g MnCl2.4H2O, 0.22g ZnSO4.7H2O, 0.39g Na2MoO4.2H2O, 0.08g CuSO4.5H2O, 0.05g CO(NO3)2.6H2O, 2.0g K2HPO4, 3.75g MgSO4.7H2O, 1.80g CaCl2.2H2O, 0.30g Citric acid, 0.30g Ammonium Ferric Citrate green, 0.05g EDTANa2, 1.0g NaCO2 and 5.0g NaNO3). The pH was adjusted to 7.5 using 2 M NaOH prior to autoclaving at 121oC for 15 minutes. The strains were preserved in BG11 medium supplemented with 80% (v/v) glycerol and stored in a biofreezer at - 20°C. A schematic representation of the experimental and the bioinformatics analyses are shown in Fig. 6.1.

6.3 Biodegradation studies

6.3.1 Biodegradation of spent coolant waste by microalgae

The study utilized spent coolant waste (SCW) and four (4) independent microalgae cultures BD11 growth medium: (1) culture consisting of mutant *S. vacuolatus* (10%) and growth medium (80%) supplement with 10% (v/v) of SCW, (2) culture consisting mutant *S. vacuolatus* (10%) and growth medium (90%) without SCW (3) culture containing wild-type S. vacuolatus (10%) and growth medium (80%) supplement with 10% (v/v) of SCW, and (4) culture containing wild-type *S. vacuolatus* (10%) and growth medium (90%) without SCW. Prior to the RNA extraction, the four independent cultures were incubated under optimized conditions for 12 days. Samples were taken after 3 days for total RNA extraction (used in the transcriptomic studies) and gas chromatography analysis (for the determination of release metabolites).

6.3.2 Metabolites determination

50 mL of each of the samples were extracted with dichloromethane and dried with sodium sulphate (Na2SO4). Subsequently, 3 µL of the extract were analyzed using GC-MS (QP2010 GC) equipped with a flame ionization detector and a HP-5 MS capillary column (30 mm, and 0.25 mm). Sample injection was carried out with 1:25 split ratio following previously described

conditions (Eregie et al., 2023). The extracts' constituents were identified based on the mass spectra and compared to the NIST library.

6.3.3 Mass balance

The TPH, alkanes, monoaromatics and PAHs of the initial and final masses of individual hydrocarbon was quantified using gas chromatography and mass spectrometry (GC-MS) output, with the relative amounts of individual hydrocarbon concentration calculated based on GC peak areas. The computed data were means of three replicates (n = 3). These were determined during the degradation of SCW using the mutant and the wild-type *S. vacuolatus*. The total mass/concentration (g day−1) of the hydrocarbon in the SCW and the residue (g day−1) was computed as follows (Mohammed et al., 2021):

$$\text{Starting TPH (Total concentration)} = C_{\text{start}} \times DR \text{ x } 1000$$

$$\text{Final TPH (Residue)} = C_{\text{final}} \times DR \text{ x } 1000$$

Where (C_{start}) is the initial concentration of TPH in the SCW, (DR) is the TPH degradation rate, (C_{final}) is the residual concentration of TPH after the degradation period, and the factor 1000 represents the conversion factor from mL to L.

6.4 Transcriptomics studies

6.4.3 Total RNA extraction

Important factors such as high biomass accumulation, RNA extraction under sterile condition, yield, purity, and integrity testing, were considered to obtain quality RNA extraction. Briefly, the microalgae samples were harvested by centrifugation at 11000 rpm at 4°C for 15 minutes. Then the cells were washed with 30 ml of 50 mM sodium phosphate buffer (pH 7.5) and centrifuged twice, followed by resuspension in 30 ml of the same buffer. The pellets were placed on ice to prevent denaturation of RNA and stored in a biofreezer at - 20°C. Total RNA was extracted using NucleoSpin RNA/Protein kit (Macherey-Nagel GmbH, Duren, Germany) following the manufacturer's protocol. Any contamination of DNA was removed by treating the samples with RNase free DNase. The RNA concentration was determined using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Waltham, MA, USA) and run on the bioanalyzer

(Agilent Technologies, Palo Alto, CA) to test the integrity of the RNA obtained (9.0 on averages for all the RNA samples).

6.4.4 RNA sequencing (RNA-Seq) and transcriptome assembly

The RNA sequencing was performed by Inqaba Biotechnology (Pty) Ltd, Pretoria, South Africa. The next generation sequencing (NGS) libraries were prepared using NEBNext® Ultra™ II RNA Library Prep Kit for Illumina®. The raw RNA-sequenced reads were trimmed using trimmomatic flexible read trimming tool (usegalaxy.org) and the quality of the raw reads were assessed using FastQC (version 0.12.1).

Trinity tool (usegalaxy.org) was used for de-novo assembly of the resulting clean reads. The blasting, mapping (Gotz et al., 2008), functional annotation (Gotz et al., 2008) and gene ontology annotation of the assembled cleaned RNA-Seq reads was performed using the OmicsBox software (version 3.1.1).

To identify the differentially expressed genes (DEGs) and non-differentially expressed genes (nDEGs) in each of the samples, a differential expression analysis was performed and measured using false discovery rate (FDR) and fold change (FC) (FDR p-value < 0.05; $\log FC > 1$ or $\log FC < -1$). Moreover, the gene ontology (GO) terms of the DEGs were carried out using the Fisher's exact test in Omicsbox platform to quantitatively compare the distribution of enriched GO terms between the different samples (Eltoukhy et al., 2022). In addition, to identify the possible metabolic pathways in the SCW degradation process by microalgae S. vacuolatus, annotated transcripts were mapped to the Kyoto Encyclopedia of Genes and Genomes (KEGG) using the Omicsbox platform (Ogata et al., 1999).

6.5 Statistical analysis

The data obtained from each Total petroleum hydrocarbon (TPH) degradation experiment was analyzed with a one-way analysis of variance (ANOVA), using IBM-SPSS v.28. The computed data are means of $n = 3$ and all results were expressed as mean ± standard error for each strain. To test the significant difference between the conditions, least significant difference (LSD) test was conducted at a 5 % level of significance.

6.6 Result and discussion

6.7 SCW Biodegradation by *S. vacuolatus*

The TPH degradation of SCW after 3, 6, 9 and 12 days are shown in Fig. 6.2. Mutant *S. vacuolatus* in media supplemented with SCW (MSv+SCW) has significantly higher ($p<0.001$) TPH degradation in comparison with the wild *S. vacuolatus* supplemented with SCW (WTSv+SCW), Mutant *S. vacuolatus* without SCW (MSv) and wild *S. vacuolatus* without SCW (WTSv) after day 3. The SCW TPH degradation obtained for MSv+SCW and WTSv+SCW was 70.65±0.004% and 61.74±0.003%, after 3 days, respectively. While the TPH observed for MSv and WTSv was 68.47±0.003% and 59.56±0.003%, after 3 days respectively. Further increase in the TPH degradation in the MSv+SCW from 83.45±0.001%, 91.78±0.001% and 100±0.001% were observed for day 6, day 9 and 12 day. These were higher compared to the TPH degradation in the WTSv+SCW, MSv and WTSv experiments (Fig. 6.2). The significant increase in TPH degradation with MSv+SCW was attributed to the high utilization of SCW from enhanced metabolic activities of mutant *S. vacuolatus* during the SCW degradation (Hussieny et al., 2020; Touliabah et al., 2022). Additionally, the rapid biodegradation of SCW observed just after 3 days was attributed to the microalgae's ease of adaptation to the oil medium and secretion of surfactants, which enhanced the emulsification of SCW hydrocarbons. Surfactants (lipids, fatty acids, lipopeptides, and lipoproteins) play an important role in enhancing hydrocarbon (HC) biodegradation by emulsifying the HCs into smaller molecules, thereby, increasing the bioavailability of the HCs (Patel et al., 2019). This emulsification increases the surface area of the HCs, making them more easily accessible to microorganisms. Also, they reduce the surface tension and interfacial tension between microorganisms and HCs (Fardami et al., 2022). Additionally, surfactants modify cell surfaces, enhancing microorganism's adhesion to HCs facilitating the uptake of HCs (Fardami et al., 2022). By improving the interaction between microorganisms and HCs, surfactants significantly boost the efficiency of biodegradation process. In this present study, different types of fatty acids were produced as by-products of the HCs degradation. This observation shows that the bioavailability of SCW HCs to microalgae was enhanced by the fatty acids production that acts as emulsifiers and surfactants, boosting HCs biodegradation. Similarly, surfactants have been reported to increase the uptake and assimilation of HCs (Wang et al., 2023). Previous studies by Ichor et al. (2016), Kalhor et al. (2017), Romero et al. (2018), and Touliabah et al. (2022) also reported an increase in TPH degradation of oil waste HCs using *Chlorella*. Interestingly, the rapid utilization of SCW by mutant *S. vacuolatus* as substrate and achieving more than 50% TPH degradation of SCW after 3 days is highly desirable approach for environmental pollutant remediation.

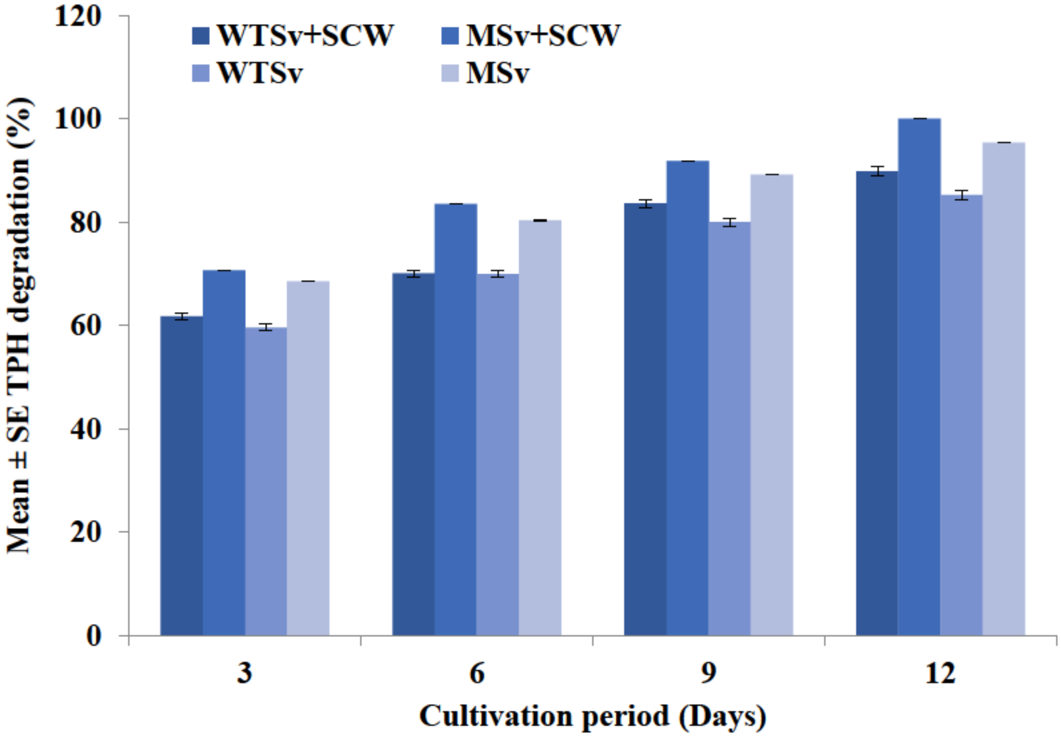


Fig. 6.2: The TPH degradation of spent coolant waste (SCW) after 12 days of cultivation period. WTSv+SCW (wild-type S. vacuolatus supplemented with spent coolant waste); WTSv (wild-type S. vacuolatus supplemented without spent coolant waste); MSv+SCW (mutant S. vacuolatus supplemented with SCW) and MSv (mutant S. vacuolatus supplemented without SCW).

6.8 *S. vacuolatus* SCW degradation metabolites

The GC-MS analysis of SCW (Table 6.1) revealed the presence of aliphatic (alkanes), alkenes, aromatic HCs, and acids metabolites. Of these HCs, alkanes, monoaromatics, polycyclic aromatics (PAHs) and their derivatives were found to be the most predominant HCs. The predominant aromatics (benzene and naphthalene derivatives) obtained from the SCW degradation has been identified as priority HCs due to their toxicity and environmentally unfriendliness (Patel et al., 2020). They have been known to induce toxic effects in plants, animals, and human beings (Ichor et al., 2016). These toxic effects include inflammation of the eyes, skin, nausea, kidney damage, liver impairment and breakdown of the immune system (Abdel-Shafy and Mansour, 2018).

Moreover, the metabolites detected in the SCW sample after treatment with mutant S. vacuolatus are shown in Table 6.2. Comparing the metabolites from the treated experiment to the control (Table 6.1), a relatively high HCs biotransformation and biodegradation of the SCW HCs was observed after 3 days. The alkanes, monoaromatics, PAHs and their derivatives were biotransformed in the treated SCW compared to the control. These observations are in line with the study of Ichor et al. (2016), the authors reported that the microalgae (Cyanobacteria, *Phormidium foveolarium, Nostoc punctiforme* and *Spirulina platensis*) completely degraded alkanes and aromatic HCs from crude oil contaminated brackish water after 7 days.

Specifically, in the present study, decane (long-chain alkane) was broken down into decanol (alcohol) and subsequently transformed into decanal (aldehyde). The decanal was further oxidized into decanone (ketone) and finally catabolized into decanoic acid (saturated fatty acid) (Table 6.2). Another specific degradation pathway is the undecane oxidation, where undecane at 2nd positions was first metabolized into 2-undecanol. The 2-undecanol underwent further oxidation, converting into 2-undecanone (methyl nonyl ketone). Thereafter, the 2-undecanone was converted into 2-methylundecanal and finally transformed into undecanoic acid methyl ester (Table 6.2). Other alkanes including dodecane, nonane, tetradecane, and pentadecane were completely biotransformed into their corresponding environmentally friendly forms such as alcohols, ketones, aldehydes, and fatty acids. The complete oxidation of the alkanes in the treated SCW to ketones, fatty alcohols, fatty aldehydes, and fatty acids revealed biotransformation as the primary removal mechanism. Also, the identification of these alkane metabolites during SCW degradation suggests that mutant *S. vacuolatus* might have degraded alkane HCs through the hydrolases, dehydrogenases, and monooxygenase enzyme systems (Pathak et al., 2018). Further study will be required to confirm the precise involvement of these enzymes during the SCW degradation. Studies have shown that the metabolic pathways mediated by these enzymes could be via terminal oxidation or subterminal oxidation (Ammar et al., 2018; Medic et al., 2020; Patowary et al., 2017). In most terminal oxidation pathway, the n-alkane is broken down to the corresponding alcohol either by hydrolases or monooxygenase. The alcohol is then oxidized to the corresponding aldehyde by alcohol dehydrogenase and finally converted into a fatty acid (Ammar et al., 2018; Patowary et al., 2017). Subterminal oxidation which has also been described for alkanes involves the formation of an alcohol that undergoes hydroxylation to form a ketone and an ester. The formed ester is then oxidized to produce alcohol and fatty acids (Pathak et al., 2015). Nonetheless, in a few cases, the alkane HC is oxidized to hydroxy fatty acids. Afterwards, the hydroxy fatty acids are transformed to carboxylic acids via the process of beta (β)-oxidation (Patowary et al., 2017). In addition, it has been reported that microalgae employ diverse enzymes for the degradation of HCs (Sutherland and Ralph, 2019). Three common categories of enzymes that have been proposed for alkane-degrading enzyme systems include: (1) short chain alkanes (C1-C4) degraded by methane-monooxygenase-like enzymes, (2) medium to long chain alkanes (C5-C17) oxidized by membrane nonheme iron or cytochrome P450 enzymes, and (3) longer chain alkanes (C17+) broken down by yet to be identified enzyme systems). This study can strongly affirm that terminal oxidation and subterminal oxidation were most likely the pathways mediated by these enzymes for alkane HC biodegradation (Patowary et al., 2017; Medic et al., 2020).

Furthermore, the mutant *S. vacuolatus* significantly degraded the monoaromatics, PAHs and their derivatives in the SCW indicating the versatility of mutant S. vacuolatus in using SCW monoaromatic as substrate. The oxidation of PAHs (naphthalene and its derivative) resulted in the formation of 2-hydroxymethyl naphthalene,1-(hydroxymethyl) naphthalene, 1-naphthoate, 2-naphthoate, 1,2-dihydroxy naphthalene, 1-naphthaldehyde and 2-naphthaldehyde (Table 6.2). The presence of these metabolites most likely indicates dihydroxylation and dehydrogenation of the naphthalene and its derivatives occurring via dehydrogenase reactions (Abdel-Shafy and Mansour, 2018; Patel et al., 2020; Żyszka-Haberecht et al., 2019). Similar naphthalene related metabolites have been reported in previous study (Ghasemi et al., 2011). Additionally, the naphthalene metabolites observed in this present study strongly supports the involvement of dehydrogenase as the key enzymes in the biodegradation of the aromatics and their derivatives (Patel et al., 2020). Dehydrogenases are well-known for their vital role in the breakdown of aromatic compounds which are further mineralized into non-toxic compounds (Padhi and Gokhale, 2017). Several enzymes from microalgae have been reported to play a role in aromatic degradation. These include cytochrome P450 monooxygenase, dehydrogenase, mono(di)oxygenase and epoxide hydrolases (García et al., 2021; Sutherland and Ralph, 2019). Due to the remarkable degradation of alkane and aromatic HCs by mutant S. vacuolatus observed in this present study, it is crucial to identify the key metabolic pathways and molecular bases involved in the transformation and degradation of these compounds.

Table 6. 1: Hydrocarbon compounds identified in SCW by GCMS

Alkanes compounds	Formula	Isomers	Alkenes compounds	Formula	Monoaromatic compounds	Formula
Heptane, 2,4-dimethyl	C9H20	4	17-Pentatriacontene	C35H70	Benzene	C6H6
n-octane	C8H18	2	7-Tetradecene	C14H28	Ethylbenzene	C8H10
2,3-Dimethyldodecane	C14H30	3	1-Tetradecene	C14H28	p-Xylene	C8H10
n-decane	C10H22	5	**Acid esters**		o-Xylene	C8H10
Undecane	C11H24	2	Oxalic acid	C2H2O4	Benzene, 1,3-dimethyl	C8H10
Dodecane	C12H26	2	Carbonic acid	H2CO3	Benzene, 1,4-dimethyl	C8H10
Tridecane	C13H28	3	cis-Vaccenic acid	C18H34O2	**PAHs**	
Tetradecane	C14H30	3			Naphthalene	C10H8
Tetradecane, 5-methyl	C15H32				Naphthalene,2,6-dimethyl	C12H12
Pentadecane	C15H32	1			Naphthalene,1-methyl	C11H10
Hexadecane	C16H34				Naphthalene,2-methyl	C11H10
Heptadecane	C17H36	2			Naphthalene,2,3-dimethyl	C12H12
Nonadecane	C19H40				Naphthalene,1,2,3,4-tetrahydro-6-methyl	C11H14
Tetracontane	C40H82				Naphthalene, decahydro-1,2-dimethyl	C12H22
n-hexatetracontane	C46H94				Naphthalene, decahydro-2,6-dimethyl	C12H22
n-octadecane	C18H38	1				
Heneicosane	C21H44					

Table 6. 2: Metabolites formed due to biodegradation of SCW hydrocarbon

Alcohol derivative	Formula	Fatty acids	Formula	PAHs compounds	Formula
Nonanol	C9H20O	Decanoic acid	C10H20O2	2-hydroxymethyl naphthalene	C10H8O
1-Dodecanol	C12H26O	Undecanoic acid	C11H22O2	1-(Hydroxymethyl) naphthalene	C11H10O
1-Tetradecanol	C14H30O	Undecanoic acid methyl ester	C12H24O2	1-naphthoate	C11H7O2
1-Undecanol	C11H24O	Dodecanoic acid, methyl ester	C13H26O2	2-naphthoate	C11H7O2
Decanol	C10H22O	Decanoic acid methyl ester	C11H22O3	1,2-dihydroxy naphthalene	C10H8O2
2-Undecanol	C11H24O	16-Hydroxyhexadecanoic acid	C16H32O3	1-Naphthaldehyde	C11H8O
1-hexadecanol	C16H34O	3-Hydroxytetradecanoic acid	C14H28O3	2-Naphthaldehyde	C11H8O
n-Pentadecanol	C15H32O	Palmitic acid	C16H32O3	**Carboxylic acids**	
Aldehydes		Pentadecanoic acid	C15H30O2	Acetic acid	CH3COOH
2-Methyl-decanal	C11H22O	Palmitoleic acid	C16H30O2	Methoxyacetic acid	C3H6O3
Nonanal	C9H18O	Tridecanoic acid	C13H26O2		
Undecanal	C11H22O	Tridecanoic acid, methyl ester	C14H28O2		
2-Methylundecanal	C12H24O	Dodecanoic acid	C12H24O2		
Decanal	C10H20O	15-hydroxypentadecanoic acid	C15H30O3		
Tetradecanal	C14H28O	Hexadecanoic acid, methyl ester	C17H34O2		
Dodecanal	C12H24O	Tetradecanoic acid	C14H28O2		
Ketone derivatives					

2-Undecanone,6,10-dimethyl	$C_{13}H_{26}O$
8-Pentadecanone	$C_{15}H_{30}O$
2-Undecanone	$C_{11}H_{22}O$
4-Hexadecanone	$C_{16}H_{32}O$
6-Undecanone	$C_{11}H_{22}O$
Decanone	$C_{10}H_{20}O$

6.9 Mass balance for different hydrocarbons in SCW

Earlier research showed that SCW was better degraded with mutant *S. vacuolatus* compared to using wild-type S. vacuolatus due to mutant *S. vacuolatus* enhanced degradative capability after UV exposure. The mass balances of alkanes and aromatics HCs in the SCW are presented in Table 6.3 and 6.4. As shown in Table 6.3, total mass balances indicate that the various alkanes (straight chain and long chain alkanes), were effectively removed after 3 days. This is typically more easily degradable than branched, cyclic or aromatic hydrocarbons. Likewise, it can be attributed to the susceptibility, bioavailability, and biodegradability of alkanes. The straight chain alkanes such as n-octane and 2,3-dimethyldodecane, and the long chain alkanes including heptadecane, nonadecane, tetracontane, n-hexatetracontane, n-octadecane and heneicosane were principally degraded. This result clearly indicated that the degradation of alkanes were fasters than that of the aromatics in line with their reported easy susceptibility, and bioavailability.

The monoaromatics and PAHs in the untreated SCW ranged from 49–1g/L (Table 6.4), with naphthalene, decahydro-2,6-dimethyl detected to a lesser extent (1%). Monoaromatics like benzene,1,3-dimethyl and benzene,1,4-dimethyl were the most degraded monoaromatic. Similarly, this can be ascribed to the high degradative potentials of mutant *S. vacuolatus* compared to the wild-type and the low molecular complexity of monoaromatics in relation to the PAHs (Venkatraman et al., 2024). PAHs are lipophilic and hydrophobic that is easily adsorbed onto different particulates, hence; effortlessly evade degradation (Venkatraman et al., 2024; Yao et al., 2024). This is beside their high molecular complexity that makes them recalcitrant. Interestingly, in this study, mass balances of significance were obtained for most PAHs after day 3. However, PAHs were degraded at different rate, for instance, naphthalene, naphthalene, 2,6-dimethyl and naphthalene,1-methyl were less easily degraded of the observed PAHs compared to naphthalene,1,2,3,4-tetrahydro-6-methyl, naphthalene, decahydro-1,2-dimethyl and naphthalene, decahydro-2,6-dimethyl which were the most degraded after day 3. The overall degradation efficiencies of the aromatics were high enough to achieve the global acceptable level for the investigated HCs. The evaluation of mass flow of target HCs (monoaromatic and PAHs) in the SCW degradation has allowed for important information on HCs in SCW, such as (i) biodegradability, (ii) recalcitrant nature of the HCs under the prevailing conditions. Evidently, PAH biomineralization with mutant *S. vacuolatus* was very effective towards a desirable approach for PAH removal. The mass balance approach for HCs gave insight into the degradability or recalcitrant nature of different HCs in SCW, as well as the overall performance of the degradation process. The overall removal efficiencies of the degradation process were satisfactory to achieve the desired removal level for the determined HCs.

Table 6. 3: Mass balance of alkane hydrocarbon compounds

Spent coolant waste treated with mutant *S. vacuolatus*			Spent coolant waste treated with wild-type *S. vacuolatus*		
Alkanes compounds	**Initial**	**Final**	**Alkanes compounds**	**Initial**	**Final**
Heptane, 2,4-dimethyl	1.48 ± 0.001	0.002 ± 0.001	Heptane, 2,4-dimethyl	1.48 ± 0.001	0.69 ± 0.001
n-octane	3.22 ± 0.001	0.00	n-octane	3.22 ± 0.001	0.53 ± 0.001
2,3-Dimethyldodecane	4.81 ± 0.02	0.00	2,3-Dimethyldodecane	4.81 ± 0.02	0.70 ± 0.001
n-decane	1.53 ± 0.005	0.02 ± 0.001	n-decane	1.53 ± 0.005	0.80 ± 0.003
Undecane	0.56 ± 0.001	0.003 ± 0.001	Undecane	0.56 ± 0.001	0.20 ± 0.002
Dodecane	0.36 ± 0.002	0.02 ± 0.001	Dodecane	0.36 ± 0.002	0.10 ± 0.001
Tridecane	0.41 ± 0.002	0.002 ± 0.001	Tridecane	0.41 ± 0.002	0.10 ± 0.001
Tetradecane	0.64 ± 0.01	0.003 ± 0.002	Tetradecane	0.64 ± 0.01	0.25 ± 0.001
Tetradecane, 5-methyl	0.57 ± 0.001	0.00	Tetradecane, 5-methyl	0.57 ± 0.001	0.11 ± 0.001
Pentadecane	0.36 ± 0.021	0.01 ± 0.003	Pentadecane	0.36 ± 0.021	0.08 ± 0.002
Hexadecane	3.11 ± 0.04	1.02 ± 0.001	Hexadecane	3.11 ± 0.04	1.20 ± 0.003
Heptadecane	0.36 ± 0.003	0.00	Heptadecane	0.36 ± 0.003	0.09 ± 0.001
Nonadecane	2.06 ± 0.001	0.00	Nonadecane	2.06 ± 0.001	0.01 ± 0.001
Tetracontane	0.41 ± 0.001	0.00	Tetracontane	0.41 ± 0.001	0.00
n-hexatetracontane	0.34 ± 0.001	0.00	n-hexatetracontane	0.34 ± 0.001	0.00
n-octadecane	0.39 ± 0.001	0.00	n-octadecane	0.39 ± 0.001	0.00
Heneicosane	0.69 ± 0.003	0.00	Heneicosane	0.69 ± 0.003	0.00

Table 6. 4: Mass balance of aromatic hydrocarbon compounds

Spent coolant waste treated with mutant *S. vacuolatus*			**Spent coolant waste treated with wild-type *S. vacuolatus***		
	Mass balances			**Mass balances**	
Monoaromatic compounds	**Initial**	**Final**	**Monoaromatic compounds**	**Initial**	**Final**
Benzene	8.23 ± 0.05	1.12 ± 0.04	Benzene	8.23 ± 0.05	4.63 ± 0.01
Ethylbenzene	5.39 ± 0.03	1.10 ± 0.02	Ethylbenzene	5.39 ± 0.03	3.01 ± 0.08
p-Xylene	3.24 ± 0.01	0.15 ± 0.01	p-Xylene	3.24 ± 0.01	1.39 ± 0.02
o-Xylene	4.59 ± 0.03	0.21 ± 0.05	o-Xylene	4.59 ± 0.03	0.85 ± 0.03
Benzene, 1,3-dimethyl	2.56 ± 0.01	0.03 ± 0.01	Benzene, 1,3-dimethyl	2.56 ± 0.01	0.07 ± 0.01
Benzene, 1,4-dimethyl	2.23 ± 0.02	0.02 ± 0.01	Benzene, 1,4-dimethyl	2.23 ± 0.02	0.05 ± 0.02
Polycyclic aromatic compounds	**Initial**	**Final**	**Polycyclic aromatic compounds**	**Initial**	**Final**
Naphthalene	49.57 ± 0.08	9.32 ± 0.98	Naphthalene	49.57 ± 0.08	15.54 ± 0.04
Naphthalene,2,6-dimethyl	25.14 ± 0.21	2.25 ± 0.25	Naphthalene,2,6-dimethyl	25.14 ± 0.21	9.23 ± 0.01
Naphthalene,1-methyl	10.69 ± 0.03	1.27 ± 0.06	Naphthalene,1-methyl	10.69 ± 0.03	5.82 ± 0.03
Naphthalene,2-methyl	6.73 ± 0.001	1.14 ± 0.03	Naphthalene,2-methyl	6.73 ± 0.001	3.99 ± 0.001
Naphthalene,2,3-dimethyl	3.61 ± 0.002	1.07 ± 0.03	Naphthalene,2,3-dimethyl	3.61 ± 0.002	1.75 ± 0.001
Naphthalene,1,2,3,4-tetrahydro-6-methyl	2.12 ± 0.003	0.03 ± 0.01	Naphthalene,1,2,3,4-tetrahydro-6-methyl	2.12 ± 0.003	0.82 ± 0.002
Naphthalene, decahydro-1,2-dimethyl	2.14 ± 0.001	0.02 ± 0.01	Naphthalene, decahydro-1,2-dimethyl	2.14 ± 0.001	0.49 ± 0.001

Naphthalene, decahydro-2,6-dimethyl	1.16 ± 0.001	0.02 ± 0.01	Naphthalene, decahydro-2,6-dimethyl	1.16 ± 0.001	0.52 ± 0.001

6.10 Transcriptomic study

6.10.1 RNA-Sequencing

The QC of all samples was >30% and GC content was approximately 57%, indicating the high quality (stability and reliability) of the RNA raw sequences. The quality of the raw RNA sequence assessed using FastQC, is an essential step to accurately interpret the biological significance of the obtained results (Gondane and Itkonen, 2023). Similarly, the quality of an RNA sequence data plays an important role in sequence assembly and gene expression analysis (Wu et al., 2024). In line with the current study, Gondane and Itkonen (2023), also reported high quality read of RNA sequence data using FastQC tool.

Moreover, the preprocessing cleaning and trimming step resulted in 4,960,051 and 4,651,562 high quality clean reads corresponding to 96.19% and 96.63% of the original raw sequences for mutant *S. vacuolatus* with SCW (MSv+SCW) and mutant *S. vacuolatus* without SCW (MSv), respectively. While a total of 4,094,114 and 3,858,688 high quality clean reads corresponding to 96.34% and 96.67% of the original raw sequences were obtained for S. vacuolatus with SCW (WTSv+SCW) and wild *S. vacuolatus* without SCW (WTSv), respectively (see supplementary document Table S1). The de novo assembly of all the clean reads resulted in 24,892 transcripts with an average length of 769.36 bp and a contig length (N50) of 1,229 bp. Hence, the throughput and desirability of the results obtained in the present study.

6.10.2 Differential expressed transcripts analysis

The transcriptomic analysis of the samples with SCW and without SCW resulted in 24,892 transcripts and low count transcripts were filtered out in all the samples to obtain a new total of 21,852 transcripts (see supplementary document Table S4). Using the false discovery rate (FDR) and fold change (FC) (see supplementary document Table S8), the obtained commonly expressed gene and differentially expressed genes (DEGs) of *S. vacuolatus* is presented in Fig. 6.3a and b. The number of induced genes (15,677) observed for MSv+SCW were significantly (FDR $p\text{-value} < 0.05$) higher compared to the MSv (14451 genes), WTSv+SCW (9132 genes) and WTSv (9292 genes) (Fig. 6.3a). This is 1.08, 1.72 and 1.69-fold higher than the MSv, WTSv+SCW and WT_Sv-SCW, respectively. Several studies on transcriptomics have shown that false discovery rate (FDR) and fold change (FC) are significant indicator for DEGs and non DGEs (Panahi and Hejazi, 2020; Peidro-Guzmán et al., 2021; Zhang and Chen, 2022). Additionally, the number of identical and unique genes obtained for the different samples are illustrated in Fig. 6.3a. Furthermore, a total of DEGs

1117 up and 131 down-regulated, 1105 up and 118 down-regulated, 255 up and 302 down-regulated and 268 up and 311 down-regulated genes were obtained for MSv+SCW, MSv, WTSv+SCW and WTSv, respectively (Fig. 6.3b). The heatmap plotted of the differential gene expression indicated similar pattern (Fig. 6.4). Most of up-regulated genes were highly expressed in the UV-exposed samples conditions compared to those of the wild-types. The high up-regulated DEGs obtained for the MSv+SCW and MSv indicates the positive impact of UV radiation on the MSv which resulted in the expression of varieties of important genes. Also, this significant increase in the expression of the up-regulated DEGs observed with the mutant microalgae could be attributed to the mutational changes in the microalgae gene pool (Eregie et al., 2023; Ibrahim, 2016; Poong et al., 2018). Similarly, Poong et al. (2018) reported 77 differentially expressed genes by Chlorella species after UV radiation exposure. The exposure of microorganisms to UV light radiation has been reported to induce the expression of a wide variety of genes (McKay et al., 2004; Kolackova et al., 2020).

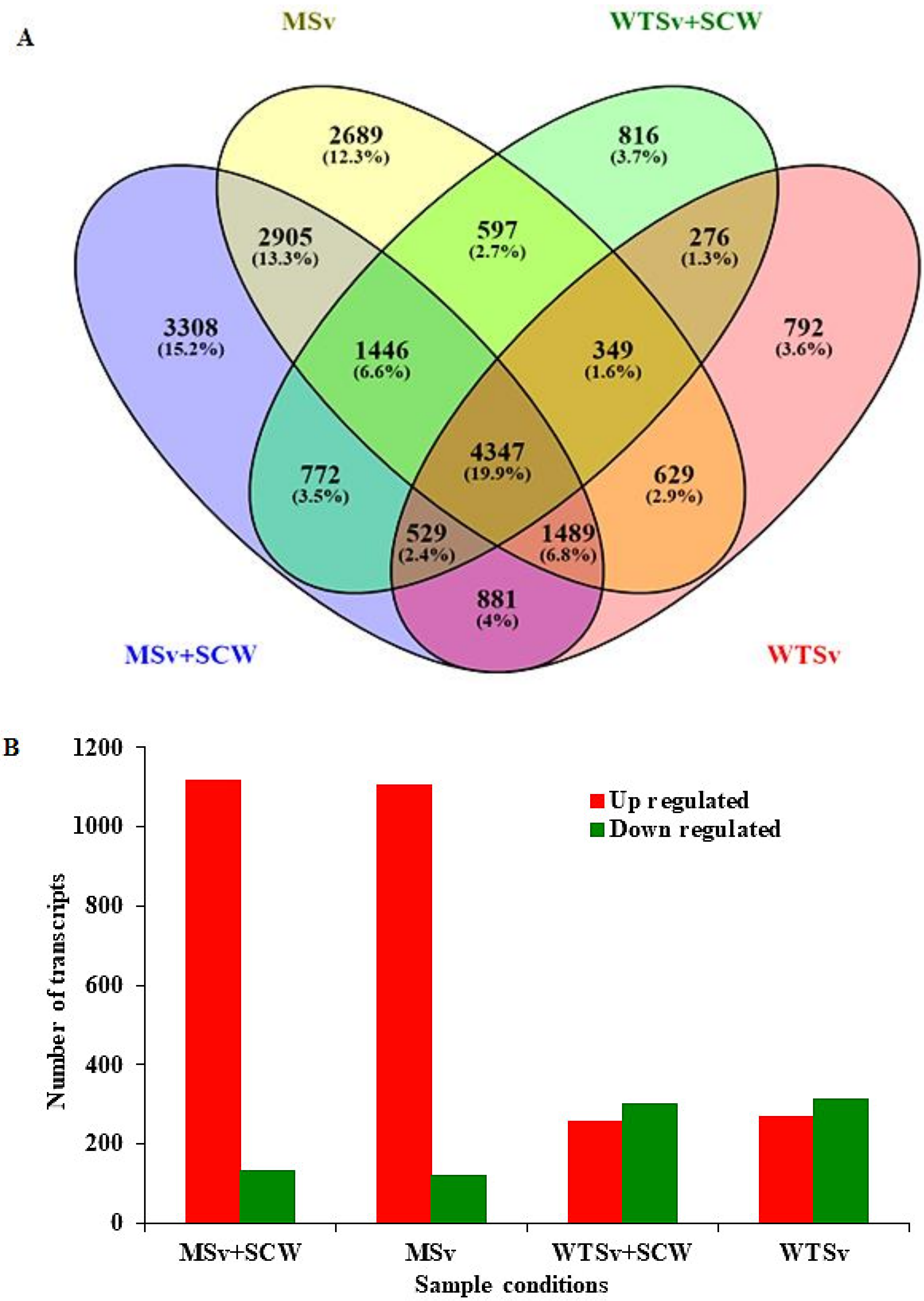


Fig. 6.3: Differential expression analysis. (a) Venn diagram of non-differentially expressed genes of *S. vacuolatus* under different conditions; (b) Differentially expressed genes (up regulated and down regulated genes). MSv+SCW (Mutant *S. vacuolatus* with SCW), MSv (Mutant *S. vacuolatus* without SCW), WTSv+SCW (Wild-type *S. vacuolatus* with SCW) and WTSv (Wild *S. vacuolatus* without SCW).

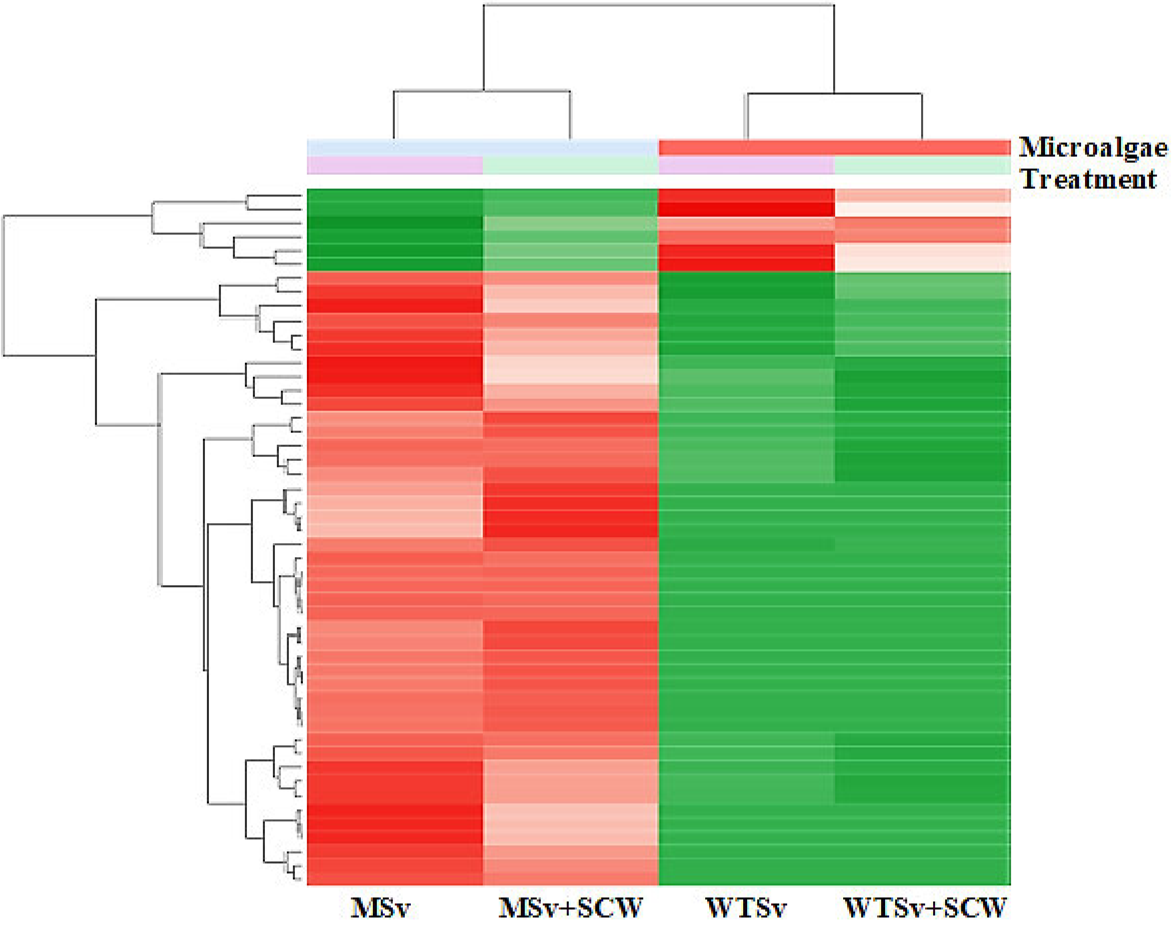


Fig. 6.4: Heat map of the differentially expressed genes (DEGs) during SCW degradation. The horizontal axis represents the samples conditions for SCW degradation and the vertical axis represents significant DEGs. The color represents the expression level and variation in gene expression. MSv+SCW (Mutant *S. vacuolatus* with SCW), MSv (Mutant *S. vacuolatus* without SCW), WTSv+SCW (Wild-type *S. vacuolatus* with SCW) and WTSv (Wild *S. vacuolatus* without SCW).

6.10.3 Functional differential and non-differential expression

The fisher functional test analysis of all DEGs under various samples conditions are presented in the supplementary document. The gene ontology (GO) shows a broader descriptions or functions of genes and genes product across microbial species (Eltoukhy et al., 2022). The total number of GO terms overrepresented of up-regulated DEGs was 102 and 98 GO terms for down regulated DEGs. The overrepresented GO terms observed under the MSv+SCW were significantly higher compared to (48 up- and 98 down), (19 up- and 46 down), (13 up- and 46 down) regulated GO terms obtain for MSv, WTSv+SCW and WTSv, respectively (see supplementary document S13 and S14). The significant increase in the GO terms of the up and down regulated DEGs observed with MSv+SCW, revealed that the exposure of *Scenedesmus* to UV-radiation and the SCW as the only carbon source resulted in highly enriched GO terms.

In addition, all DEGs detected with the MSv+SCW were categorized into three subgroups: biological process (BP), molecular function (MF) and cellular component (CC). Among the overrepresented up regulated GO terms; cellular aromatic compound metabolic process (14 DEGs), aromatic compound biosynthetic process (14 DEGs) and cellular metabolic process (46 DEGs) were the most significant biological process related to biodegradation of HC in MSv+SCW (Fig. 6.5a). While the most significant down-regulated GO term is organic cyclic compound metabolic process (14 DEGs) (Fig 6.5b). Other important biological process GO terms of up and down regulated DEGs associated with the biodegradation of SCW HCs were organic substance metabolic process (10 DEGs), primary metabolic process (56 DEGs), heterocycle metabolic process (48 DEGs) and cellular biosynthetic process (47 DEGs). The GO terms results obtained are in line with the previous report by Eltoukhy et al. (2022) which stated that the up-regulated GO terms associated with bisphenol (a) degradation by YC-AE1 were mainly cellular aromatic compound metabolic process and cellular metabolic process. Moreover, the molecular function category, monooxygenase activity (8 DEGs) and oxidoreductase activity (2 DEGs), were the most significantly up and down regulated GO terms overrepresented. While ATP synthase complex (5 DEGs) is the most up-regulated GO term significantly overrepresented in the cellular component in the MSv+SCW. The observed GO terms in the BP, MF and CC, directly reflects the cellular metabolic process enabling the oxidation of various HCs present in the SCW. These also correlate with the observed enhanced SCW HCs degradation in the MSv set up compared WTSv. Likewise, the obtained high GO terms with the MSv correlate with higher growth rate, higher biomass accumulation, increased chlorophyll, protein, and lipid content also observed with the MSv in recent study resulting to high degradative activities (Eregie et al., 2023).

In the WTSv+SCW (Table 6.5), it was observed that cellular biosynthetic process (up regulated GO term) with 12 DEGs and ATP metabolic process (down regulated GO term) with 3 DEGs were the most overrepresented BP. The most overrepresented up and down regulated GO term under MF was oxidoreductase activity acting on diphenols and related substance (3 DEGs) and oxidoreductase activity acting on CH-CH group of donors (2 DEGs), respectively. While chloroplast (12 DEGs) up regulated GO term and ATPase complex (2 DEGs) down regulated GO terms were the most significant over represented CC (see supplement document). For WTSv (Table 6.5), protein folding (7 DEGs) and carboxylic acid metabolic process (3 DEGs) was the significant up and down regulated GO terms for BP. On the other hand, response to oxidative stress (2 DEGs) and carbon-carbon lyase activity (3 DEGs) were the most significant up and down regulated GO terms for MP. While cytochrome b6f complex (1 DEGs) is the most significant down regulated GO term under CC. No significant up regulated GO terms were observed under CC. These GO terms outcomes (over representation of GO terms and gene activities) are strong evidences in the present study to support the enhanced metabolic activities of S. vacuolatus upon exposure to UV light radiation (Eregie et al., 2023).

The GO terms for the non DEGs relating to SCW HCs degradation in the MSv+SCW is depicted in Fig. 6.6. Number of total terms observed was 4553 with 1037 over represented. The overrepresented GO terms of the non DEGs observed in the MSv+SCW were significantly higher compared to 975, 562 and 536 observed for MSv, WTSv+SCW and WTSv, respectively (see supplementary document). The significant increase in the GO terms observed under MSv+SCW, revealed that positive impact of UV exposure on *S. vacuolatus* (Eregie et al., 2023). Similarly, the non DEGs transcripts detected in MSv+SCW were categorized into three subgroups (1) biological process (BP) (GO: 0008150) with 2598 (100%), (2) molecular function (MF) (GO: 0003674) 3244 (100%), and (3) cellular component (CC) 2133 (100%) (see supplementary document S25-S27). Among the over represented GO terms, cellular process (2179 genes, 83.87%), metabolic process (2169 genes, 83.49%) and cellular metabolic process (1775 genes, 68.32%) were the most significant biological process related to biodegradation of HC in MSv+SCW compared to the WTSv samples. While other important biological processes associated with biodegradation of HCs were organic substance metabolic process (1747 gene, 67.24%), cellular aromatic compounds metabolic process (465 genes, 17.9%), primary metabolic process (1645 genes, 63.32%) methylation (36 genes, 23.12%), aromatic compound biosynthetic process (273 genes, 10.51%), heterocycle metabolic process (40 genes, 30.09%), and cellular biosynthetic process (1137 genes, 43.76%).

From the molecular function category GO: 0003674, catalytic activity (2165 genes, 74.74%) and NADH dehydrogenase (ubiquinone) activity (16 genes, 20.51 %) were the most significantly overrepresented molecular function. While cellular anatomical entity (2116 genes, 99.2%) was the most significantly over represented cellular function in MSv+SCW. The observed enriched GO terms in the BP, MF and CC, directly reflects the metabolic process and enzyme systems enabling the oxidation of various HCs present in the SCW (Eltoukhy et al., 2022; Wu et al., 2024). These also correlate with high SCW HCs degradation activities in the MSv compared to WTSv set up. Also, the correlation of these GO term with high growth rate, biomass accumulation, enhanced chlorophyll, protein and lipid biomolecules have been previously reported with the MSv (Eregie et al., 2023).

Other enriched GO terms identified as significantly overrepresented in MSv+SCW that must have enhanced *S. vacuolatus* degradation efficiencies after UV exposure included fatty acid biosynthetic process (40 genes, 19.21%), fatty acid metabolic process (54 genes, 26.50%), long chain fatty acid metabolic process (8 genes, 10.89%) and fatty acid beta-oxidation (20 genes, 15.4%) (See supplementary document). These can specifically be linked to increased fatty acid accumulation and production in MSv cells. More so, this observation shows that the bioavailability of SCW HCs was enhanced by the cellular fatty acids production that acts as emulsifiers and surfactants, promoting HC biodegradation (Kumar and Shukla, 2023). For the WTSv+SCW (Fig. 6.7), it was observed that the GO term metabolic process was the most overrepresented BP (960 genes, 67.56%) and binding (936 genes, 55.34%) was the most overrepresented GO term under MF. While oxidoreductase complex (17 genes, 15.24%) is the most significant overrepresented CC (Fig. 6.7). See supplement document for WTSv fisher test analysis.

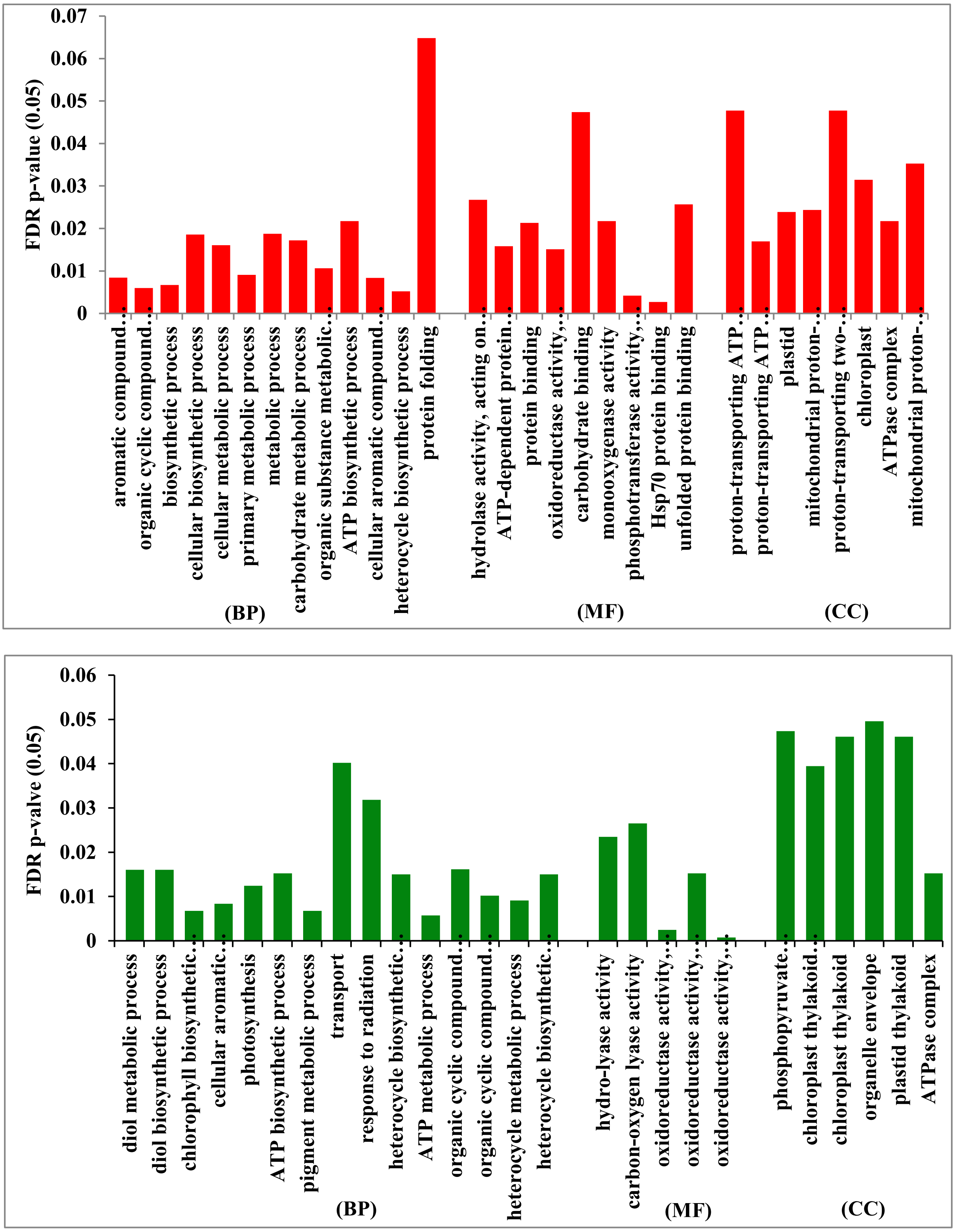


Fig. 6.5: Classification of the first thirty GO terms over represented in MSv+SCW (UV-exposed *S. vacuolatus* supplemented with oil). (a) GO terms of the up-regulated; (b) GO terms of the down regulated.

Table 6. 5: Most significant gene ontology terms under WTSv+SCW and WTSv

Most significant GO term under WTSv+SCW					
GO term	GO names	GO category	FDR p-value	Regulation	DEGs
GO:0044249	Cellular biosynthetic process	Biological process	0.0242	up	12
GO:0046034	ATP metabolic process	Biological process	0.00571	down	3
GO:0016679	Oxidoreductase activity acting on diphenols and related substance	Molecular function	0.001299	up	3
GO:0016636	oxidoreductase activity acting on CH-CH group of donors	Molecular function	0.000761	down	2
GO:0009507	Chloroplast	Cellular component	0.002635	up	12
GO:1904949	ATPase complex	Cellular component	0.00522	down	2
Most significant GO term under WTSv					
GO:0006457	Protein folding	Biological process	0.00310	up	7
GO:0019752	carboxylic acid metabolic process	Biological process	0.043825	down	3
GO:0005515	Protein binding	Molecular function	0.04073	up	6
GO:0016830	carbon-carbon lyase activity	Molecular function	0.001961	down	3
GO:0009512	cytochrome b6f complex	Cellular component	0.047345	down	1

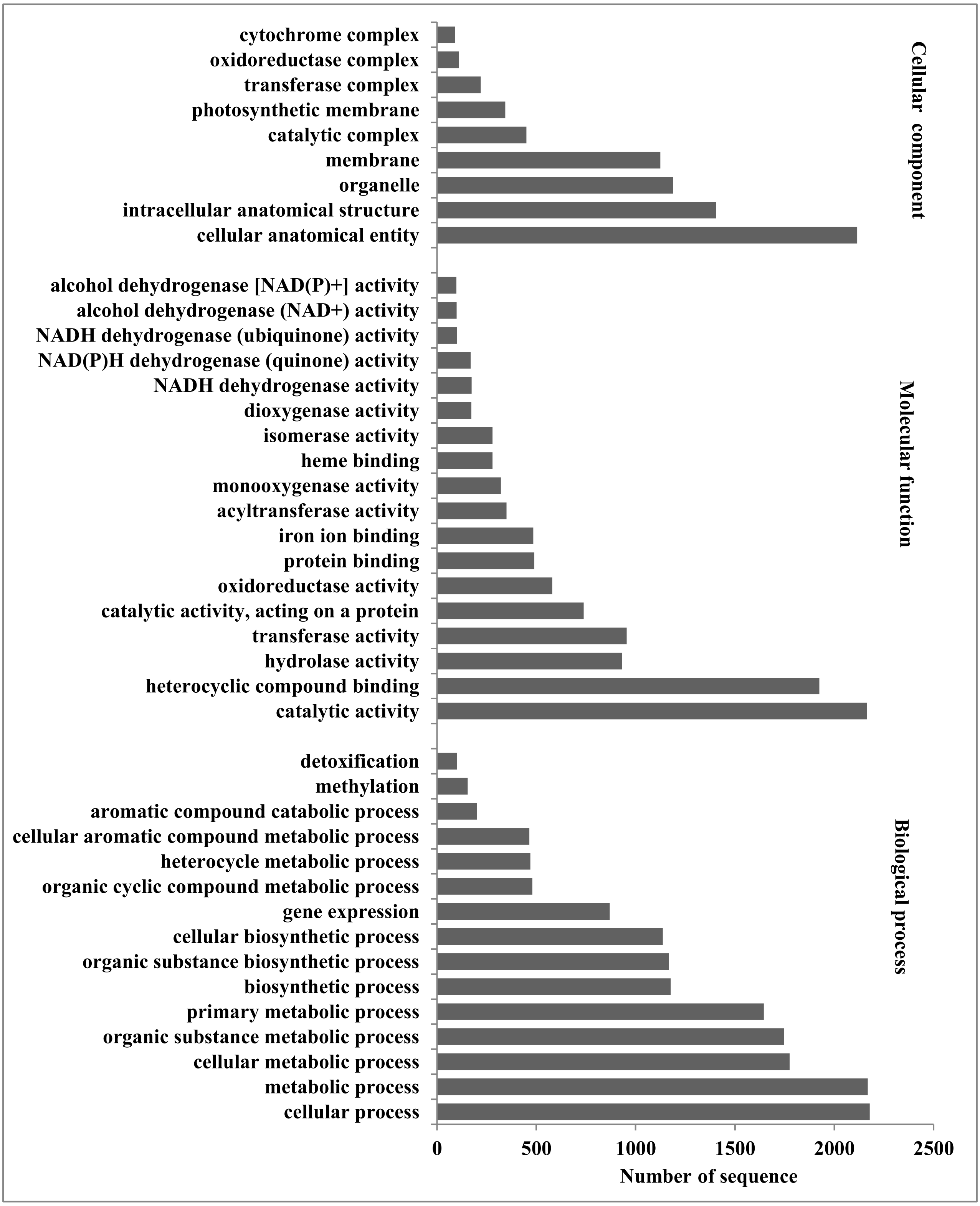


Fig.6.6: Classification of the first forty GO terms of non-differentially expressed transcripts under MSv+SCW (mutant *S. vacuolatus* with SCW) sample condition.

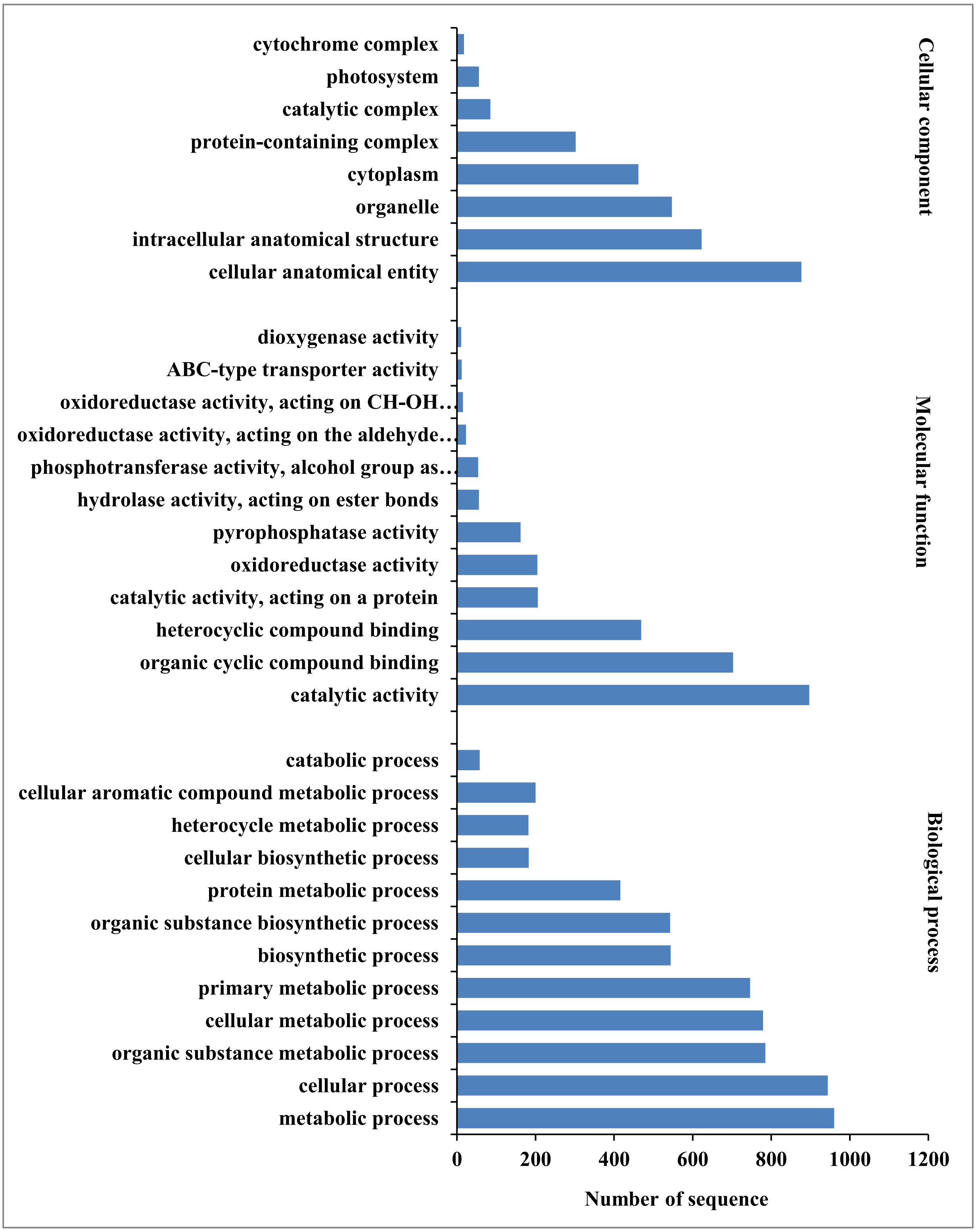


Fig. 6.7: Classification of the first thirty GO terms of the non-differentially expressed transcripts under WTSv+SCW (Un-exposed *S. vacuolatus* with SCW) sample condition.

6.10.4 KEGG metabolic pathway in SCW degradation

The functional annotation of the transcriptome, transcript encoding enzymes and DEGs involved in the degradation of SCW HCs in *Scenedesmus* was identified (see supplementary document Table S15-S24). The results revealed that the transcriptomes were mapped to different pathways, which comprised of metabolism, human diseases and genetic information processing. In the MSv+SCW sample, increase in the expression of transcripts were observed resulting 116 different metabolic pathways compared to 108, 99, 95 metabolic pathways observed in MSv, WTSv+SCW and WTSv, respectively. Similarly, increase in transcripts (1078) linking to different metabolic pathways observed in MSv+SCW was higher in comparison to 1003, 469 and 450 transcripts linked to metabolic pathways in MSv-SCW, WTSv+SCW and WTSv, respectively. The relative increase in the metabolic pathways and linked transcript in the MSv+SCW suggest the impact of UV radiation and modification in the cellular metabolic processes (Eregie et al., 2023; Ibrahim, 2016).

Of the 116 metabolic pathways linked to SCW HCs degradation, biosynthesis of ansamycine, naphthalene degradation, lysine biosynthesis, monobactam biosynthesis and retinol metabolism were the most significant and overrepresented pathway (Table 6). Moreover, other important metabolic pathways, including photosynthesis, oxidative phosphorylation, fatty acid biosynthesis, metabolism of xenobiotics by cytochrome P450, chloroalkane and chloroalkene degradation, were also identified. Metabolic pathway identification is vital to the determination of the biological functions and potential role of genes during the HCs degradation. Although, microalgae as a degradation tool have been reported to break down oil waste pollutants (Hammed et al., 2016; He et al., 2016), the metabolic pathways and the enzyme activities involved in the degradation process have not been fully understood and known. The present study has presented additional evidence and clarifications on microalgae enzymatic activity as well as metabolic pathways in HCs pollutant degradation. Interestingly, in this study, the degradation pathway that best described the oxidation of the aromatic compounds such as naphthalene in the SCW was identified and elucidated.

6.10.4.1 Naphthalene degradation pathway

Of the 1117 up and 131 down-regulated expressed transcripts in MSv+SCW, ten (10) responsive transcripts potentially involved in naphthalene biodegradation were identified compared to the rest of the samples (Table 6.6). The expression of these transcripts showed that most of them were commonly overexpressed regardless of naphthalene compound type. These responsive transcripts include 6-CoA-linked acetaldehyde dehydrogenase, 3-coatomer subunit alpha-3 and 1-arginine deiminase. The identification of these transcripts in MSv+SCW suggests a drastic modification of the cellular metabolic activity of S. vacuolatus after UV exposure. Remarkably, these genes have not been reported to be regulated by UV treatment, and many of these genes have no assigned function. Therefore, making this current study the first to report this observation. Furthermore, CoA-linked acetaldehyde dehydrogenase was the only transcript that was specifically expressed for naphthalene degradation and the other nine (9) transcripts were observed to be a support system for the main transcript in the naphthalene degradation process. The presence of these commonly expressed transcripts showed that upon UV exposure, S. vacuolatus expressed multiple transcripts to bind with the main transcript to facilitate the degradation process.

The high expression of transcripts indicates that the MSv is using the transcripts as intermediate carrier for either donating or accepting of HCs during the degradation process. This study finding corroborated with the reports of Sutherland and Ralph (2019) and Xiong et al. (2018). According to these authors, microalgae express various unique genes for the degradation of HCs. Likewise, Dell'Anno et al. (2021) and Ghodrati et al. (2021) reported the degradation of HCs by multi-gene pool. Additionally, the transcript CoA-linked acetaldehyde dehydrogenase was completely absent in MSv, WTSv+SCW and WTSv samples. The presence of CoA-linked acetaldehyde dehydrogenase only in the MSv+SCW is plausible and affirmed that one of the genes associated with the degradation of naphthalene in Scenedesmus was induced upon UV exposure. Thus, making UV mutagenesis (UV light radiation) a desirable tool for strain improvement and gene discovery. The transcript CoA-linked acetaldehyde dehydrogenase codes for a coatmer subunit alpha-3 protein-like (gene name COO60DRAFT_1702088) was located at WD40 domain: the WD40 domain is one of the most abundant domains found in eukaryotic microorganisms which have been reported to be the most common site for protein-protein interactions (Hu et al., 2023).

Moreover, WD40 domain proteins have been demonstrated to play numerous essential roles in cellular and biological processes (Hu et al., 2023).

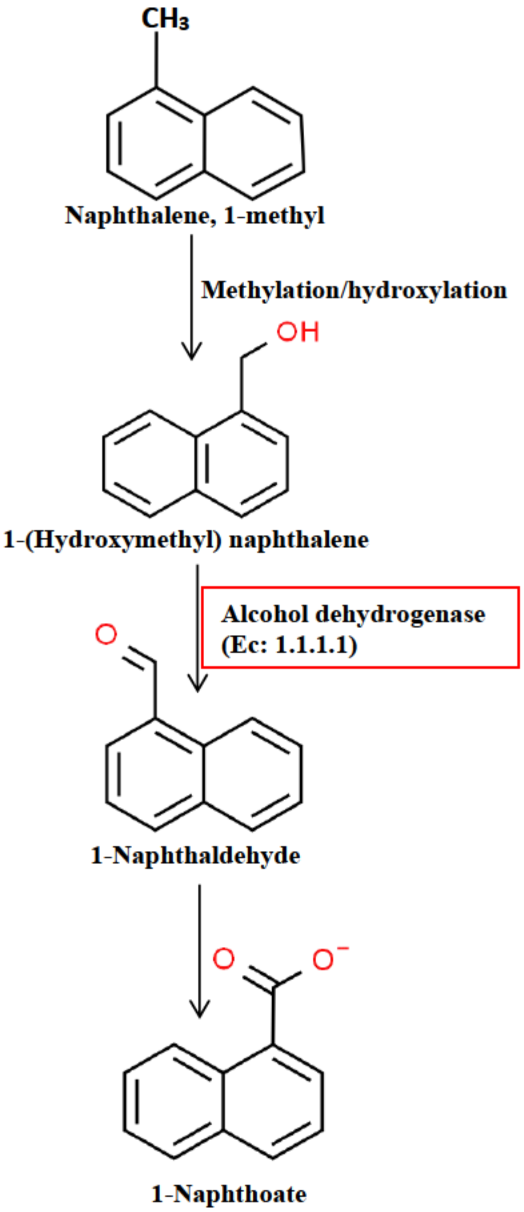
Furthermore, for the oxidation of the naphthalene compounds in SCW, CoA-linked acetaldehyde dehydrogenase was found to encode alcohol dehydrogenase (ADH) (Ec: 1.1.1.1) belonging to the oxidoreductase group of enzymes. ADHs have been reported to play a major role in PAHs degradation by catalyzing the oxidation of alcohols to aldehydes and ketones (Sui et al., 2023; Gyaneshwari et al., 2023). Additionally, looking at the naphthalene metabolites obtained in this study, naphthalene-1-methyl was oxidized to 1-(hydroxymethyl) naphthalene via hydroxylation and methylation (addition of a hydroxyl and methyl group). The 1-(hydroxymethyl) naphthalene was then converted to a central metabolite 1-naphthaldehyde by alcohol dehydrogenase (Table 6.2 and Fig. 6.8) which was further converted to 1-naphthoate. Similarly, naphthalene-2-methyl was transformed to 2-hydroxymethyl naphthalene via hydroxylation and methylation, which then underwent subsequent oxidation to 2-naphthaldehyde by alcohol dehydrogenase. The 2-naphthaldehyde therefore was converted to 2-naphthoate (Table 6.2 and Fig. 6.8) that might enter the TCA cycle to produce carbon dioxide. This suggests that CoA-linked acetaldehyde dehydrogenase used hydroxylation and methylation mechanism as the activation reaction mechanism for naphthalene,1-methyl and naphthalene,2-methyl catabolic process. While the alcohol dehydrogenase enzymes were used for the oxidation of the naphthalene compounds via both activation mechanisms. Also, as highlighted above, these results suggest that the bioavailability of the naphthalene compounds as carbon source was enhanced by the cellular production of fatty acids to emulsify the naphthalene compounds and promote solubility (Table 6.2). This observation is consistent with previous studies in which similar naphthalene metabolites and their activation mechanisms were observed (Abdel-Shafy and Mansour 2018; Patel et al., 2020; Żyszka-Haberecht et al., 2019).

Importantly, the over expression of alcohol dehydrogenases was observed in the MSv+SCW compared to the rest of the samples, indicating that the UV radiation led to the over expression of this enzyme. To the best of our knowledge, this is the first transcriptomic evidence of the involvement of CoA-linked acetaldehyde dehydrogenase and alcohol dehydrogenase during naphthalene degradation by mutant *Scenedemus*.

Table 6. 6: Most significant KEGG pathway for SCW HCs degradation

Pathway ID	Pathway	Pathway category	Linked transcripts	DEGs	Fisher FDR	Fisher p-value
MSv+SCW sample condition						
Ko01051	Biosynthesis of ansamycine	Metabolism	1	1	0.633333	0.033333
Ko00626	Naphthalene degradation	Metabolism	9	1	0.633333	0.033333
Ko00300	Lysine biosynthesis	Metabolism	6	1	0.633333	0.033333
Ko00261	Monobactam biosynthesis	Metabolism	4	1	0.633333	0.033333
Ko00830	Retinol metabolism	Metabolism	11	1	0.633333	0.033333
MSv sample condition						
ko00600	Sphingolipid metabolism	Metabolism	8	2	1	0.014079
ko00740	Riboflavin metabolism	Metabolism	1	1	1	0.023923
Ko00626	Naphthalene degradation	Metabolism	4	0	1	0.025344
WTSv+SCW sample condition						
Ko01051	Biosynthesis of ansamycine	Metabolism	1	1	0.433333	0.022223
Ko00626	Naphthalene degradation	Metabolism	1	0	0.433333	0.022223
WTSv sample condition						
ko00270	Cysteine and methionine metabolism	Metabolism	11	2	1	0.03905

(a) Naphthalene, 1-methyl



(b) Naphthalene, 2-methyl

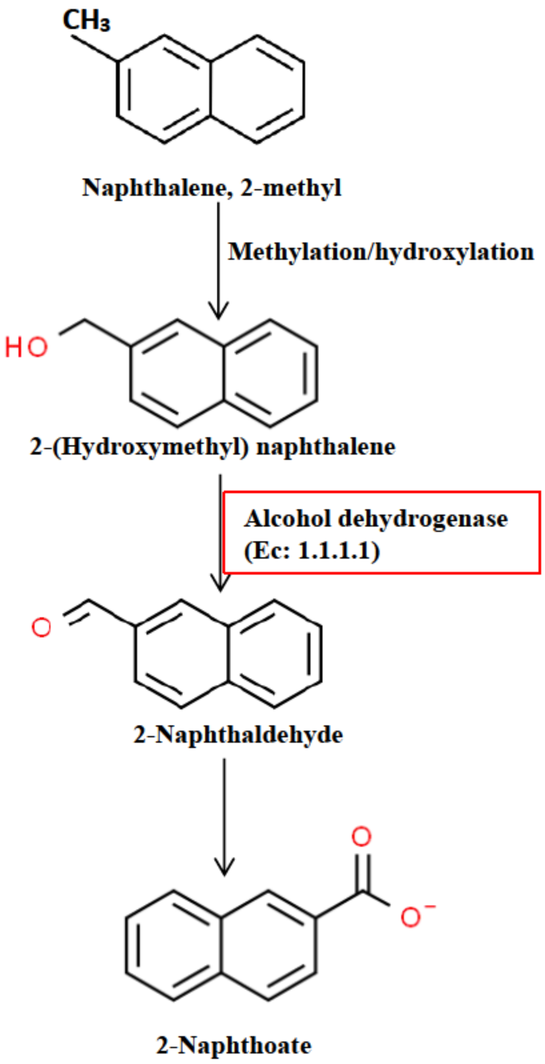
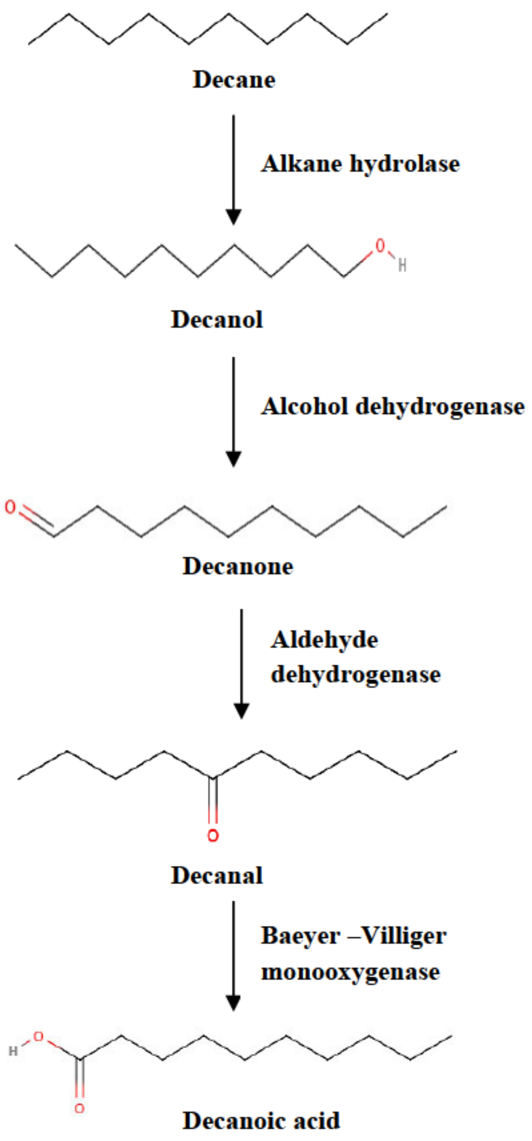


Fig.6.8: Scheme of pathways of naphthalene degradation in mutant *Scenedesmus*. (a) Pathway 1 (naphthalene, 1-methyl degradation pathway); (b) pathway 2 (naphthalene, 2-methyl degradation). Enzyme detected by transcriptomics analysis is framed in red.

6.10.4.2 Alkane degradation pathways

Alkanes such as decane, undecane, dodecane, nonane, tetradecane, and pentadecane were completely broken down into their corresponding by-products such as alcohols, ketones, aldehydes, and fatty acids. For example, decane (long-chain alkane) was broken down into decanol (alcohol) and further converted into decanal (aldehyde). The decanal was then biotransformed into decanone (ketone) and decanoic acid (saturated fatty acid) (Fig. 6.9a). Similarly, undecane was oxidized into 2-undecanol. The 2-undecanol was further converted into 2-undecanone (dialkyl ketone MNK). Subsequently, the 2-undecanone was oxidized into 2-methylundecanal and undecanoic acid methyl ester (Fig. 6.9b). The identification of these alkane metabolic products in the MSv+SCW indicates that MSv might have used hydrolases, dehydrogenases and monooxygenase enzyme systems via terminal oxidation or subterminal oxidation pathway for the alkane degradation (Ammar et al., 2018; Medic et al., 2020; Patowary et al., 2017). Despite the MSv capability to degrade the alkane fraction of SCW, the metabolic pathways and enzyme activities involved in the alkane degradation process is yet to be fully understood and known. Furthermore, it is possible that MSv employs a distinctive pathway for alkane degradation.

(a) Terminal oxidation



(b) Subterminal oxidation

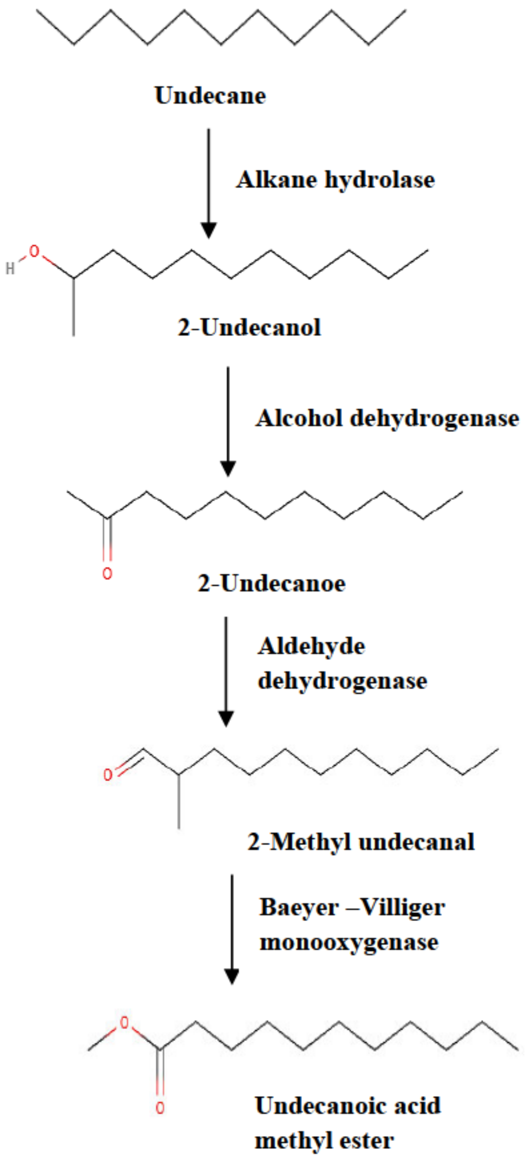


Fig. 6. 9: Proposed pathways of alkanes (decane and undecane) degradation in mutant *S. vacuolatus*. (a) Pathway 1 (terminal oxidation degradation); (b) pathway 2 (subterminal oxidation degradation).

6.11 Conclusion

This study presents the first RNA sequencing and transcriptome analysis of mutant *S. vacuolatus* in the degradation of SCW HCs. The HCs mass balance gave insight into the degradability of HCs in SCW. Key transcripts encoding enzymes and metabolic pathways used by MSv in degradation of SCW HCs were identified. These transcripts include 6-CoA-linked acetaldehyde dehydrogenases, 3-coatomer subunit alpha-3 and 1-arginine deiminase that were involved in the degradation. Moreover, strong evidence of MSv using CoA-linked acetaldehyde dehydrogenase encoding the enzyme alcohol dehydrogenase for the oxidation of naphthalene HCs in the SCW via the naphthalene degradation pathway has been presented. These findings have identified unique genes and enzymes in *S. vacuolatus* that are involved in the degradation of SCW which could be an effective tool in environmental remediation.

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CHAPTER 7

General Discussion and Conclusion

7.1 The Research in Perspectives

Environmental pollution with spent oil waste (SOW) has attracted public and scientific attention globally, due to the increased usage of lubricant oil (Hoang et al., 2021). This oil waste often contains pollutants such as long chain and cyclic alkanes, halogenated hydrocarbons, benzene derivatives, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and other hydrocarbon contaminants (Al-Hussieny et al., 2020; Dell' Anno et al., 2021). The uncontrolled dumping of SOW in soils, drains, surface and ground water pollute the environment, posing serious health risks to humans and other living organisms. Moreover, these contaminants are known to be recalcitrant, toxic, carcinogenic and mutagenic (Ohanmu et al., 2019; Romero et al., 2018). To date, techniques such as physical and chemical have been used for the removal of SOW pollutants. However, these techniques have several drawbacks, such as high processing costs, high energy consumption, and the production of secondary pollutants (Al-Hussieny et al., 2020; Dell' Anno et al., 2021). Therefore, a cost-effective and environmentally sustainable approach is required for the removal of SOW pollutants. Currently, microalgae-based biodegradation treatment has gained increased global attention as it showed desirable outcome in pollutant degradation compared to physical and chemical treatment methods. This innovative approach not only presents a cost-effective solution but also offers an eco-friendly alternative for SOW cleanup, potentially alleviating the financial burden associated with traditional waste management methods (Eregie et al., 2023). Even though microalgae have been efficient in the biodegradation of several SOWs, challenges such as slow degradation of recalcitrant pollutants and ineffective degradation process have plagued the commercialization and field application of microalgae-based SOW pollutant treatment. Thus, this study was undertaken to develop an efficient and effective microalgae-based degradation approach to enhance its metabolic performance and degradative abilities on recalcitrant pollutants.

The study first evaluated the potential of UV (ultraviolet) light radiation to enhance the metabolic and photosynthetic capabilities of *S. vacuolatus*. This was evaluated by observable physical and microscopic morphological alterations, change in chlorophyll, carotenoid, carbohydrate, protein, and lipid accumulation, as well as growth rate, substrate affinity and substrate usage versatility improvement as reported in chapter 3. Also, the degradation efficiencies of UV-exposed *S. vacuolatus* in comparison to wild-type were evaluated. Thereafter, the modelling and optimization of the key operational process parameters to enhance SOW biodegradation was assessed. Finally, the transcriptomics of the catabolic genes and enzymatic pathways were explored in biodegradation of SOW.

The effects of UV radiation (wavelength 254 nm and intensity 1.4 mW/cm^2) on *S. vacuolatus* at different time intervals (2, 4, 6, 12, 24 and 48 hours) were established. Upon analysis, the 24 hrs UV-exposure induced the most desirable hydrocarbon (HC) degradative traits on *S. vacuolatus.* These desirable traits include improved chlorophyll (chl a = 1.89–fold, chl b = 2.02–fold), carotenoid (1.24–fold), carbohydrates (4.62–fold), proteins (1.44–fold) and lipid accumulations (1.40–fold). Additionally, the 24 hrs UV-exposed *S. vacuolatus* exhibited an enhanced substrate affinity (1/Ks) (0.959) and specific growth rate (μ) (0.024 h-1) (1.50-fold, 2-fold, and 1.9-fold respectively). The obtained data proved that UV-radiation indeed improved the metabolic and photosynthetic performances of *S. vacuolatus.* The increased desirable HC degradative traits observed demonstrated that the UV-exposure of *S. vacuolatus* not only enhances its HC degradative traits but also boosts its overall cellular activity. Additionally, the substantial improvement in the photosynthetic and metabolic activities likely served as an adaptive cellular response to protect the organism against UV radiation (Choi et al., 2017; Ganapathy et al., 2017), which in turn was advantageous for improve cellular activities as observed in the study.

Considering the positive effects of UV radiation on *S. vacuolatus* growth, photosynthetic apparatus, and cellular metabolic performance, it is necessary to evaluate the potential HC biodegradation capabilities of the 24hrs UV-exposed microalgae. Hence, in chapter 4, a preliminary assessment of spent coolant waste (SCW) biodegradation was conducted based on the dehydrogenase activity (DHA) and total petroleum hydrocarbon (TPH). Remarkably, the 24 hrs UV-exposed *S. vacuolatus* had significantly higher ($p < 0.001$) DHA (55%) and TPH degradation efficiency (100% after 5 weeks) in comparison with the wild *S. vacuolatus*. The enhancement in DHA and TPH degradation efficiencies are strongly attributed to the following: Enhanced photosynthetic and metabolic activities of UV-exposed *S. vacuolatus* resulting from improved cellular activities after the UV exposure; High tolerance and metabolism of SCW as substrate; and increased enzyme activities that improved the metabolic activities towards SCW degradation (Ganapathy et al., 2017; Kumar et al., 2018). The exposure of *S. vacuolatus* to UV radiation largely enhanced *S. vacuolatus* affinity for substrate and the overall efficiency of the degradation treatment. High TPH biodegradation efficiency of SCW coupled with the release of environmentally friendly metabolites (such as alcohols, ketones, aldehydes, fatty acids, and carboxylic acids) as end products underscore the UV mutagenesis a suitable and promising approach for industrial application. However, to further improve the process performance for industrial application, process optimization for the SCW degradation using the 24hrs UV-exposed *S. vacuolatus* was implemented. Due to the sensitivity of *S. vacuolatus* to process conditions, it is vital to ensure that optimum process conditions are determined for maximum SCW biodegradation.

The interactive effects of temperature, substrate concentration, and inoculum concentration on TPH degradation of SCW was modelled and optimized using the Response Surface Methodology (RSM) and findings presented in chapter 5. This approach provides an in-depth understanding of the interaction between the process parameters being investigated and the degradation response (Chouaibi et al., 2020; Ezemagu et al., 2021). The model suggested optimal process set points of 25°C, 10% (v/v) oil concentration and 10% (v/v) inoculum concentration respectively that promoted *S. vacuolatus* metabolic activities and maximum biodegradation of SCW. Upon validation, the model gave 100% TPH degradation of SCW within 15 days of treatment (lower than the period before optimization) demonstrating the efficacy of these conditions in enhancing bioremediation performance and suggesting potential applications in environmental management and oil waste treatment processes (Touliabah et al., 2022). Moreover, the sensitivity assessment of the process parameters on the degradation process using Artificial Neural Network (ANN) revealed high sensitivity of the degradation process to operational temperature (Chouaibi et al., 2020; Ezemagu et al., 2021). The degradation process indicated potential for further improvement of SCW degradation following, provided that appropriate degradation conditions are employed. To this end, nanomaterials with catalytic potentials were incorporated to the optimized degradation process. Ten nanoparticles (NPs) were synthesized, characterized and evaluated for their potential to improve UV-exposed *S. vacuolatus* degradation performances. Upon analysis, five of the NPs positively and significantly impacted SCW degradation. The obtained result revealed that the inclusion of NPs in the biodegradation medium improved UV-exposed *S. vacuolatus* growth and thus the SCW degradation performances, through their high surface area, providing a greater site for interactions with the microalgae as well as the SCW pollutant (Borji et al., 2020). Specifically, the inclusion of Fe_2O_3, Fe_3O_4, Ag_2O, nZVI, and CuO NPs highly enhanced the UV exposed *S. vacuolatus* growth and shorten the degradation period of SCW from 15 days to 12 days.

Furthermore, the degradation process was evaluation using different degradation kinetics. The degradation kinetics showed high biodegradation rate constant (K) and shortened half-life ($T_{1/2}$) of 6.65 m1 t^{-1} and 0.08 days respectively especially in the degradation of monoaromatics, and PAHs which are important environmental pollutants. The degradation kinetics was best represented by the second-order degradation reaction, highlighting the importance of the SCW concentration to the degradation process.

In addition, from the obtained metabolites (alcohols, aldehyde, ketones, fatty acids and carboxylic acids of alkanes and aromatic compounds), enzymatic and transcriptomic assessment was performed. The result obtained, as reported in chapter 6, revealed a total of 116 metabolic pathways were found in UV_Sv+SCW. Notably, of the 116 metabolic pathways linked to SCW HCs degradation, the UV-exposed *S. vacuolatus* utilized the naphthalene degradation pathway for the oxidation of the PAHs fraction of SCW. Furthermore, transcripts of biotechnological importance were implicated in SCW degradation. These include six CoA-linked acetaldehyde dehydrogenase, three coatomer subunit alpha-3 and one arginine deiminase. Of these transcripts, the CoA-linked acetaldehyde dehydrogenase encoding alcohol dehydrogenase enzyme was used by UV-exposed *S. vacuolatus* in the breaking down of the aromatic compounds in SCW. This indicates that specific metabolic pathways are activated in response to UV exposure, enhancing the organism's ability to break down complex pollutants (Patel et al., 2020; Żyszka-Haberecht et al., 2019).

7.2 Concluding Remarks

Overall, this study provides an innovative approach to improving the biodegradation of spent oil waste using UV-mutagenized microalgae. The optimization of the degradation process and the incorporation of nanomaterials provide a scalable process, cost-effective, and environmentally sustainable solution to SOW pollution. This can pave the way for industrial and field application of microalgae-based treatments with potential to contribute significantly to the economy of environmental waste remediation. This study not only addresses the potential solution to environmental and health challenge posed by SOW pollutants but also offers a cost-effective, efficient, and environmentally friendly alternative to traditional remediation methods. The significant improvements in biodegradation performance and the potential for industrial application underscore the study's importance in advancing sustainable environmental practices.

7.3 Recommendations for future studies

This research illustrated that UV radiation remarkably enhanced the metabolic performance and biodegradation efficiency of *S. vacuolatus*. Exposing the microalgae to different UV radiation conditions (e.g., different wavelengths and intensities) may assist to further identify other effective parameters for enhancing the metabolic performance and biodegradation capabilities this organism. In addition, applying UV mutagenesis approach to other microalgae species could be investigated to enhance their degradation capabilities to efficiently eliminate various recalcitrant and persistent pollutants from contaminated sites.

Identifying the optimal conditions for SCW degradation and the optimized conditions resulting in 100% TPH degradation within 15 days is a significant achievement in this study. Further improvements and scalability of the biodegradation process require the optimization of other key parameters such as light intensity, agitation and pH, among others. This approach will provide a detailed understanding of the complex interactions of these factors and ultimately maximizing the treatment efficiency and effectiveness of spent coolant waste

The incorporation of nanoparticles further enhances the SCW degradation performance of *S. vacuolatus,* leading to a shorter degradation period from 15 days to 12 days. Further, investigation into microalgae-based degradation in the presence of nanoparticles would contribute valuable insights into the interaction between nanoparticles and essential biomolecules, including cellular carbohydrates, proteins, lipids, and chlorophyll. This is pivotal for advancing industrial desirability by understanding the intricate relationships and potential effects on microalgae-based degradation processes.

Key transcripts such as CoA-linked acetaldehyde dehydrogenase, coatomer subunit alpha-3 and arginine deiminase and 116 unique metabolic pathways were identified in this study. The identification of the key transcripts and 116 metabolic pathways involved in SCW degradation provides a foundation for deeper molecular studies. Further studies could be carried out using the quantitative real-time polymerase chain reaction (qRT-PCR) to confirm the transcripts functional roles and changes in the gene expression observed in the RNA-Seq results. This step will provide an independent verification of the expression levels of selected genes as well as provide additional insight into the complex gene regulation mechanisms following the UV exposure for further understanding of the metabolic pathways

In addition, cloning, purification and functional characterization of the identified transcripts particularly CoA-linked acetaldehyde dehydrogenase and alcohol dehydrogenase could further be investigated, to understand better the specific roles, activities, metabolic conditions and catalytic mechanisms of these enzymes in SCW biodegradation. This will aid in elucidating their substrate specificities and kinetic parameters, and optimal operational conditions. Moreover, these studies could reveal potential for enzyme engineering to enhance biodegradation efficiency, contributing to more effective bioremediation strategies.

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