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THESE

En vue de l'obtention du : **DOCTORAT**

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Structure de Recherche : Équipe de Physiologie et Biotechnologie Végétale

Discipline : Biologie

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Présentée et soutenue le 28/12/2022 par :

Soufiane FAL

Étude du potentiel des microalgues pour le traitement des eaux usées et la production du biodiesel et pour le développement des biostimulants de la tolérance des plantes aux métaux lourds

JURY

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DEDICACE

Je dédie cette thèse

A la mémoire de ma grand-mère Fadila

A l'âme de mon père

A ma mère

A mes frères Mohammed, Tijani, Rachid & Raduoane

A mes sœurs Mounia, Nabila & Zakia

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RÉSUMÉ

Face aux enjeux écologiques actuels, la recherche scientifique se tourne vers le développement des bioprocédés pour élucider de nouvelles solutions aux problèmes environnementaux. La présente thèse s'inscrit dans le cadre d'étude du potentiel des microalgues pour le développement d'un bioprocédé intégré pour le traitement des eaux usées et la valorisation de la biomasse micro-algale en bioproduits tels que le biodiesel et les biostimulants contre le stress métallique dans l'agriculture. Ces microorganismes photosynthétiques ont montré un fort potentiel d'épuration des polluants majeurs dans les eaux usées, principalement l'azote et le phosphore. Ensuite, la caractérisation de la biomasse issue du traitement des eaux usées a montré un taux élevé en lipides de bonne qualité pour la production du biodiesel. De plus, les résidus issus de l'extraction des lipides à partir de cette biomasse font l'objet d'une hydrolyse acide qui va servir en tant que biostimulant des plantes. L'application de cet extrait sur des plantes de tomate a montré une amélioration de la croissance et la tolérance des plantes au stress des deux métaux étudiés (Pb et Cd). Ces extraits ont favorisé l'accumulation de Pb et Cd dans les racines des plantes de tomate et inhibé la translocation de ces métaux vers la partie aérienne. En outre, ces extraits ont stimulé le système antioxydant des plantes de tomate face au stress métallique par réduction des enzymes et molécules antioxydantes après leurs augmentations sous ce stress. L'analyse métabolomique par GC-MS a révélé l'accumulation des AGPI, AGLC, alcanes/alcènes et stérols sous Pb et Cd stress. En effet, le traitement des plantes de tomate par les extraits des microalgues a réduit l'accumulation de ces métabolites, tout en favorisant l'accumulation des AGS principalement palmitique et stéarique. Les microalgues pourront être une base pour promouvoir une bioraffinerie efficace et durable pour le traitement des effluents liquides et gazeux et la production de la biomasse micro-algale destinée pour la production des bioproduits à haute valeur ajoutée.

Mots clés : Microalgues, Eaux Usées, Biodiesel, Métaux Lourds, *Solanum lycopersicum* L., Biostimulant.

ABSTRACT

Faced the current ecological challenges, scientific research is turning to the development of bioprocesses to provide new solutions to environmental problems. The present thesis emphasizes microalgae potential for the development of an integrated bioprocess for the treatment of wastewater and microalgal biomass valorisation in bioproducts such as biodiesel and biostimulants against metal stress in agriculture. These photosynthetic microorganisms have shown a strong potential for wastewater treatment, mainly nitrogen and phosphorus. Then, the biomass characterization from the wastewater showed a high rate of lipids with good quality to produce biodiesel. Moreover, the residues resulting from lipids extraction are subject to acid hydrolysis which will serve as a plant biostimulant. The application of this extract on tomato plants exhibited an improvement in growth and plant tolerance to heavy metals (Pb and Cd). These extracts promoted the Pb and Cd accumulation in the roots of tomato plants and inhibited the translocation of these metals to the aerial part. Furthermore, these extracts enhanced the antioxidant system of tomato plants against heavy metals stress by reducing scavenging enzymes and molecules after their increases under this stress. Metabolomic analysis provided by GC-MS revealed the accumulation of PUFA, UFA, alkanes/alkenes and sterols under Pb and Cd stress. Indeed, the treatment of tomato plants with microalgae extracts reduced the accumulation of these metabolites, while promoting the accumulation of SFA, mainly palmitic and stearic acids. Therefore, microalgae can be a suitable model to promote an efficient and sustainable biorefinery for liquid and gaseous effluents treatment and the production of microalgal biomass intended to produce high value-added bioproducts.

Keys: Microalgae, Wastewater, Biodiesel, Heavy Metals, *Solanum lycopersicum* L., Biostimulant.

ABRÉVIATIONS

- ACE** : *Aphanothece* Crude Extract
- APX** : Ascorbate Peroxidase
- AWW** : Aquaculture Wastewater
- BBE** : Blue Bioeconomy
- BCF** : Bioconcentration Factor
- BRS** : Brassinostéroïdes
- BSA** : Bovine Serum Albumin
- CA** : Carbonic Anhydrase
- CAT** : Catalase
- CBE** : Continuous Bed Extractor
- CCM** : CO₂ Concentrating Mechanism
- Ca** : Calcium
- Cd**: Cadmium
- CE**: Crude Extract
- Chl a**: Chlorophyl a
- Chl b**: Chlorophyl b
- Chl**: Chlorophyl
- CO₂** : Carbon Dioxide
- Cr** : Chromium
- Cu** : Copper
- DHA** : Docosahexaenoic Acid
- DHAR** : Dehydroascorbate Reductase
- DW** : Deionized Water
- DWW** : Domestic wastewater
- EBITDA** : Earnings Before Interest and Taxes Depreciation Amortization
- EPA** : Eicosapentaenoic Acid
- FA** : Fatty Acids
- FAME** : Fatty Acid Methyl Acid
- Fe** : Iron
- FGD** : Flue Gas Desulfurization
- GCMS** : Chromatographie en Phase Gazeuse: Spectrométrie de Masse
- GR** : Glutathione Reductase

H.M : Heavy Metals
H₂O₂ : Hydrogen Peroxide
H₂PO₄ : Di hydrogen Phosphate
HPO₄ : Mono Hydrogen Phosphate
K : Potassium
KI : Potassium Iodine
LOX : Lipoxygenase
MCE : Microalgae Crude Extract
MDA : Malondialdehyde
Mg : Magnesium
Mn : Manganese
MUFAs : Monounsaturated Fatty Acids
N : Azote
NADPH: Nicotinamide Adénine Dinucléotide Phosphate
NH₄⁺ : Ammonia
NO₂ : Nitrite
NO₃⁻ : Nitrate
OD : Optical Density
P : Phosphore
Pb : Plomb
PBR : Photobioreactors
POD : Peroxidase
PUFA : Poly Unsaturated Fatty Acid
PVP : Polyvinyl pyrrolidone
PWW : Piggery wastewater
ROI : Return On Investment
ROS : Reactive Oxygen Species
RuBisCO : Ribulose-1,5-bisphosphate
SFA : Saturated Fatty Acids
SMC : Spent Mushroom Compost
SOD : Superoxide Dismutase
SWE : Seaweed Extract
TCA : Trichloroacetic acid
TEA : Techno-economic analysis

TF : Translocation factor

TN : Total Nitrogen

TP : Total Phosphorus

UE : Union Européenne

UFA : Unsaturated Fatty Acid

VLCFA : Very Long Chain Fatty Acids

WW : Wastewater

WWT : Wastewater treatment

Zn : Zinc

ZnONPs : Zinc Oxide Nanoparticles

PUBLICATIONS

- ✚ **Fal, S., Benhima, R., El Mernissi, N., Kasmi, Y., Smouni, A., El Arroussi, H., 2021.** “Microalgae as promising source for integrated wastewater treatment and biodiesel production”. *International Journal of Phytoremediation* 24, 1–13.
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COMMUNICATIONS

- ✚ ***Aphanothece* sp. as promising biostimulant to alleviate metallic stress in *Solanum lycopersicum* L. by enhancing physiological, biochemical, and metabolic responses.**
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- ✚ **Microalgae as promising biostimulant of plants tolerance against heavy metals stress.** Communication orale aux Journées Doctoriales Interdisciplinaires de l'UM6P organisé le 24 Juin 2022 à l'Université Mohammed VI Polytechniques (UM6P), Benguerir, Maroc.

- ✚ **Wastewater treatment and biodiesel production by Moroccan microalgae strains**
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INTRODUCTION GENERALE

De nos jours, les problèmes environnementaux et énergétiques nous amènent à réfléchir à des nouvelles alternatives économiques et durables (Yen et al., 2013). En effet, la culture des microalgues dans les eaux usées pour la production de biomasse est considérée comme un moyen potentiel pour surmonter ces contraintes (Yen et al., 2013; Kilbane, 2022). Les microalgues sont des microorganismes unicellulaires d'une structure simple dite primitive, de taille allant de 1 à 50 μm et se caractérise par une croissance rapide. Elles utilisent l'énergie solaire pour produire environ la moitié de l'oxygène atmosphérique mondial tout en éliminant le dioxyde de carbone par leur capacité photosynthétique (Aresta *et al.*, 2005; Ben Amor, 2015). En fonction de la structure cellulaire, pigmentation et du cycle de vie, les microalgues sont classés en quatre formes importantes sont : les algues bleues (Cyanophycées), les algues vertes (Chlorophycées), les diatomées (Bacillariophycées) et les algues dorées (Chrysophycées) (Bajhaiya et al., 2012). Les microalgues présentent une large diversité des espèces estimée de 200 000 à 1 000 000. Dans ce nombre juste 10 000 sont identifiées et quelques dizaines exploitées à l'échelle industrielle (Cadoret and Bernard, 2008). Cette diversité permet aux microalgues d'être utilisées en plusieurs domaines d'application à savoir : l'environnement, l'agriculture, L'alimentation humaine et animale, la médecine et la pharmacie etc. À cet égard, la culture des microalgues dans les eaux usées dans une approche intégrée avec la bioraffinerie pour la valorisation de biomasse en chaine, offre une stratégie prometteuse et durable qui aura une viabilité à la fois économique et environnementale (Moreno García *et al.*, 2020 ; Venkata Mohan *et al.*, 2020 ; Huang *et al.*, 2022; Sun *et al.*, 2022).

Les microalgues peuvent éliminer efficacement les polluants et les nutriments présents dans les eaux usées par leurs croissance et leurs métabolisme (Chawla *et al.*, 2020; Leite *et al.*, 2020). Elles les transforment en biomasse biochimiquement riche par des métabolites primaires et secondaires intéressantes qui leurs confèrent un grand nombre d'applications à l'échelle scientifique et industriel (Venkata Mohan *et al.*, 2020; Zhu et al., 2020). De nombreuses études rapportent une productivité élevée des lipides par rapport aux autres métabolites en cas de culture dans les eaux usées (Moreno García et al., 2020; Shahid et al., 2020). En plus des lipides de microalgues qui pourraient potentiellement être convertis en biodiesel. La biomasse de microalgues contient également de nombreux autres composants précieux, notamment des sucres, des acides gras polyinsaturés, des pigments et des protéines etc., qui méritent tous d'être développés en produits raffinés pour diverses applications (Arutselvan *et al.*, 2022; Kilbane, 2022).

A cet égard, nous avons attiré l'attention pour la première fois dans la valorisation des résidus de processus de production du biodiesel pour le développement des biostimulants de la croissance et tolérance des plantes au stress des métaux lourds. Par le biais d'amélioration du système antioxydant des plantes et l'accumulation des métabolites incorporés dans la tolérance/résistance aux stress abiotiques et biotiques y compris les métaux lourds. Récemment, l'utilisation des microalgues et cyanobactéries comme biostimulant prometteur de la croissance et la résistance des plantes aux stress biotiques et abiotiques a été rapportés dans de nombreuses études (Mazepa et al., 2021; Li et al., 2021; Mutale-joan et al., 2021; Rachidi et al., 2021).

Par ailleurs, cela peut être ajouté au développement d'un critère vert de bien à partir des rejets (Goswami *et al.*, 2021). En effet, l'approche intégrée de la bioraffinerie des microalgues pourrait être un bon modèle qui offre une durabilité économique et environnementale (Venkata Mohan *et al.*, 2020 ; Katiyar *et al.*, 2021; Arutselvan *et al.*, 2022). Non seulement pour réduire le coût de la production des microalgues, mais aussi pour achever les objectifs de la purification et de la régénération des eaux usées en réduisant leur coût (Moreno García *et al.*, 2020; Huang *et al.*, 2022; Sun *et al.*, 2022). Puisqu'il se déroule dans une boucle circulaire dans le but de maximiser la récupération des ressources pour établir un processus de zéro déchet (Venkata Mohan *et al.*, 2020).

OBJECTIFS DU TRAVAIL

La présente thèse nous avons choisi d'utiliser les microalgues dans une approche intégrée pour le traitement des eaux usées et la valorisation de la biomasse dans la bioraffinerie afin de produire du biodiesel et des biostimulants des plantes contre le stress des métaux lourds.

Les travaux de la présente thèse sont présentés dans ce manuscrit en trois chapitres :

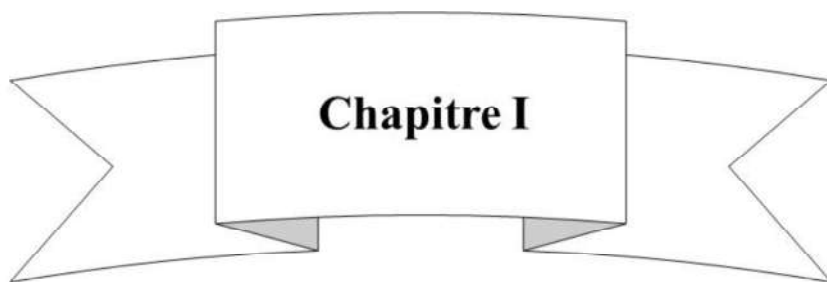
Le premier chapitre consiste une revue bibliographique sous forme d'une « revue critique » sur le potentiel des microalgues pour une approche intégrée de traitement des eaux usées, la séquestration du CO₂ industriel et la valorisation de la biomasse issue en bioraffinerie afin d'avoir plusieurs produits. Dans ce chapitre nous avons présenté aussi notre modèle de bioraffinerie avec une étude techno-économique afin d'assurer la rentabilité de process.

Dans le 2^{ème} chapitre nous mettons le focus sur le potentiel de croissance des microalgues dans les eaux usées, le pouvoir épuratoire et la production élevée de la biomasse avec un taux élevé des lipides de bonne qualité utilisable pour la production du biodiesel. Ensuite, nous avons évalué des souches des microalgues les plus appropriées pour produire du biodiesel après leur croissance dans les eaux usées.

Le 3^{ème} chapitre vise le développement des biostimulants à base des microalgues capable d'améliorer la tolérance des plantes au stress des métaux lourds.

- ✚ Étude de l'effet des biostimulants sur la croissance des plantes de la tomate sous application des deux métaux utilisés Pb et Cd.
- ✚ Étude de l'effet des biostimulants sur les plantes sous Pb et Cd par des voix physiologiques, biochimiques et métabolomiques.

La dernière partie de ce manuscrit comprend une discussion générale de tous les résultats obtenus dans les différentes parties de la thèse et leurs comparaisons avec autres études littérales. Nous avons clôturé notre manuscrit par une conclusion générale dans un contexte générale de l'ensemble des chapitres et perspectives pour la continuité de nos travaux de thèse particulièrement à une échelle industrielle.



Chapitre I

CHAPITRE I. REVUE BIBLIOGRAPHIQUES

A. Article: Integrated biorefinery based microalgae for wastewater and industrial CO₂ treatment with microalgae biomass valorization: A circular bioeconomy approach. A critical Review

I. Introduction et Objectif

La bioéconomie (BE) a été définie en 2017 selon la Commission Européenne par la création de diverses ressources biologiques renouvelables et leurs conversion en produits de valeur ajoutée importants tels que les denrées alimentaires, les fourrages, les produits biochimiques et la bioénergie (European Commission, 2017). De plus, la bioéconomie circulaire (BEC) se concentre sur l'utilisation intégrée des ressources durables, y compris l'utilisation des déchets et la valorisation de la biomasse en sortira avec des produits multiples via un processus en cascade, par ex. bioraffineries (Sherwood, 2020; Stegmann *et al.*, 2020). Il a le potentiel de réduire les effets issus du changement climatique tout en fournissant une source de carbone renouvelable et en générant des opportunités d'affaires et d'emploi, en particulier dans les zones rurales (European Commission, 2017). En effet, ces aspects socio-économiques et environnementaux représentent les trois piliers de la durabilité (Stegmann *et al.*, 2020). La bioraffinerie basée sur des microalgues représente le meilleur modèle qui peut être appliqué pour réaliser un plan de bioéconomie circulaire avec une boucle fermée (Choi *et al.*, 2021).

Les microalgues sont des micro-organismes, photosynthétiques et unicellulaires classés comme micro-organismes procaryotes ou eucaryotes (Tomaselli, 2004). Vivant dans les eaux douces, saumâtres et marines, ainsi que dans les eaux usées agricoles, industrielles et domestiques (Moreno García *et al.*, 2020). De plus, les microalgues sont hautement adaptables, même à des conditions défavorables par ex. salinité, lumière, température, CO₂ et métaux lourds (H.M) (Maity *et al.*, 2014). En tant que, la production des microalgues nécessite de l'eau, les nutriments, de la lumière et du CO₂, cela pourrait être le modèle idéal pour produire de la biomasse à partir des eaux usées et de gaz de combustion, etc.) (Ullmann and Grimm, 2021). L'utilisation de ces sources permet de réduire le coût de la production des microalgues et pourrait également être une stratégie de phycoremédiation permettant de réduire l'impact environnemental de ces effluents (Katiyar *et al.*, 2021; Ullmann and Grimm, 2021). Par conséquent, la réduction des risques environnementaux, l'utilisation d'une matière première renouvelable et l'application du concept de la bioéconomie circulaire qui représentent le cœur

des tendances récentes des agendas politiques de l'Union Européenne (U.E) (Gatto and Re, 2021).

À cet égard, les microalgues pourraient utiliser divers effluents comme source prometteuse de nutriments pour produire une biomasse riche en lipides, glucides, protéines et autres composés bioactifs. Ainsi, l'approche intégrée de la bioraffinerie est basée sur les microalgues qui pourrait être l'une des options durables qui auront une viabilité à la fois économique et environnementale (Katiyar *et al.*, 2021; Venkata Mohan *et al.*, 2020). En outre, l'extraction séquentielle des composés précieux à partir de la biomasse des microalgues rend l'approche de bioraffinerie très attrayante en terme de réduction des déchets, de diversification des revenus et l'utilisation complète des matières premières (Venkata Mohan *et al.*, 2020).

Objectif et contexte

Dans cette revue critique, nous avons fait le focus sur une approche intégrée visant l'utilisation des eaux usées comme source des nutriments et le gaz de combustion comme source de carbone, pour la culture des microalgues. En conséquence, nous nous sommes basés aussi sur une approche de bioraffinerie pour la valorisation de la biomasse et la réutilisation de l'eau traitée pour fermer la boucle et achever la stratégie de bioéconomie circulaire. Nous fournirons quelques points critiques et quelques recommandations pour améliorer la culture des microalgues afin d'achever cette stratégie.

Environmental Advances

Integrated microalgae-based biorefinery for wastewater and industrial CO₂ treatment with microalgae biomass valorization: A circular bioeconomy approach

--Manuscript Draft--

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Article Type:	VSI:Sustainable development
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Abstract:	Microalgae have a huge utilization in different fields such as agriculture, environment, medicine, cosmetics, food and feed. They are taken as the feedstock of sustainably produced chemicals and biofuel. However, the production of microalgal-derived chemicals is economically unprofitable. Integrated biorefinery could be one of the sustainable options making microalgae-derived products economically profitable and environmentally viable with zero waste. In this critical review, we emphasized the integration of wastewater treatment with CO ₂ biosequestration from industrial flue gas and we proposed integrate strategy to biomass valorization using microalgae-based biorefinery. We also proposed our model of integrated biorefinery with techno-economic analysis and the possible microalgae-derived products that can be valorized after domestic wastewater and flue gas use as low-cost inputs for microalgae culture.

ABSTRACT

Microalgae have a huge utilization in different fields such as agriculture, environment, medicine, cosmetic, food and feed. They are taken as feedstock of sustainably produced chemicals and biofuel. However, production of microalgal-derived chemicals is economically unprofitable. Integrated biorefinery could be one of the sustainable options making microalgal derived products economically profitable and environmentally viable with zero waste. In the present study we emphasized the integration of wastewater treatment (WWT) with CO₂ biosequestration from industrial flue gas. In addition, we proposed the possibility of using the integrated biorefinery as a circular bioeconomy approach to make microalgal derived products economically profitable and environmentally viable. We also proposed our model of integrated biorefinery with technoeconomic analysis and the possible microalgal derived products that can be valorized after wastewater and flue gas use as low-cost inputs for microalgal culture.

Keywords: Microalgae, Wastewater treatment, CO₂ biosequestration, Biorefinery, Circular bioeconomy

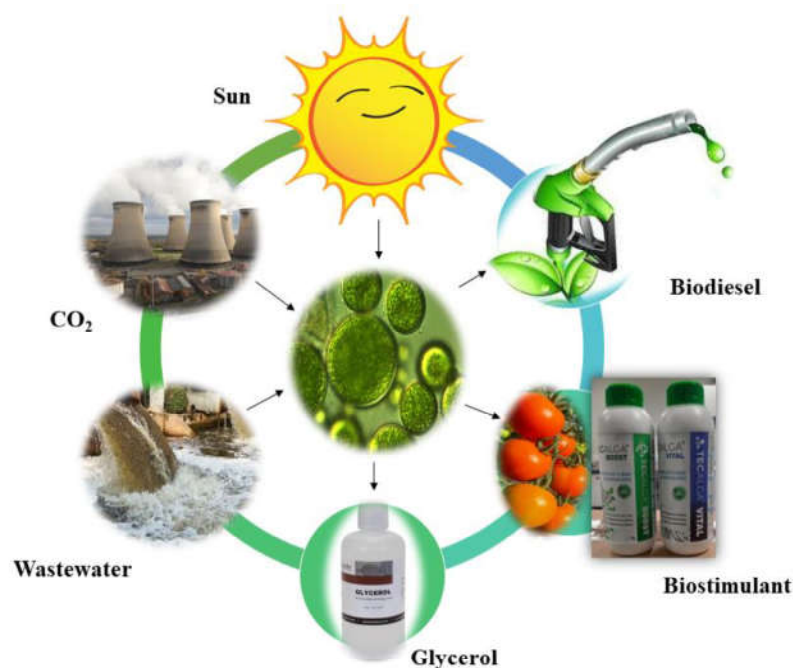


Figure 1: Graphical Abstract

INTRODUCTION

The European Commission in 2017 defined the bioeconomy (BE) as the creation of various renewable biological resources and their conversion into high-value bio-based products such as food, feed, biochemicals, and bioenergy (European Commission, 2017). Furthermore, the circular bioeconomy focuses on the integration of sustainable resource utilization including wastes and biomass valorization with multi-output via cascading process e.g. biorefineries (Sherwood, 2020; Stegmann et al., 2020). It has the potential to reduce the effects of climate change while also providing a renewable carbon source and generating business and employment opportunities, particularly in rural areas (European Commission, 2017). Indeed, these economic, environmental and social aspects represent the three pillars of sustainability (Stegmann et al., 2020). Among the numerous systems that can be applied to achieve circular bioeconomy plan with “the close loop” is microalgae biorefinery (Choi et al., 2021).

Microalgae are microorganisms that classified as procaryotic or eukaryotic microorganisms (Tomaselli, 2004). They can grow in different humid habitats such fresh and marine water, as well as in agricultural, industrial, and municipal wastewaters (Moreno García et al., 2020). Moreover, microalgae are highly adaptable to stress environment and extreme conditions e.g. salinity, light, temperature, CO₂ and heavy metals (H.M) (Maity et al., 2014). Considering microalgae production require water, light and CO₂ it could be the ideal model to produce biomass from some waste sources (WW, flue gas etc.) (Ullmann and Grimm, 2021). The culture of microalgae under these circumstances not only profit from this resource but also could be a phycoremediation strategy that help to reduce the environmental impact of these effluents (Katiyar et al., 2021; Ullmann and Grimm, 2021). In addition, it may be added to the development of a green criterion of “best from waste” (Goswami et al., 2021). Furthermore, the integration of WW with flue gas as source of CO₂ for microalgae culture is advantageous (Razzak et al., 2017). Which may enhance biomass productivity and their valorization for bioenergy and bioactive compounds production (Goswami et al., 2021; Yadav et al., 2019).

Hence, reducing environment hazards, the use of renewable raw materials and the application of circular bioeconomy concepts represent the heart of the recent trends European Union (EU) policy agendas (Gatto and Re, 2021). In this regard, microalgae could use various waste streams as promising source of nutrients to produce biomass rich in highly-lipids, carbohydrates, proteins, and other bioactive compounds. Thus, the integrated approach of microalgae biorefinery could be one of the sustainable options that will have both economic and

environmental viability (Katiyar et al., 2021; Venkata Mohan et al., 2020). It's gone in a circular loop with the goal of maximizing resource recovery to establish a zero-waste process flow (Venkata Mohan et al., 2020). Furthermore, the sequential extraction of precious compounds from microalgae makes the biorefinery approaches very attractive in terms of waste reduction, income diversification, and full feedstock utilization. Indeed, the biorefinery based on microalgae with significant scope and potential as a sustainable platform can be included also as a component of the "Blue Bioeconomy" (BBE). BBE is an economic-based activity associated with aquatic biomass that focuses on developing new innovations, creating models, and providing long-term solutions to environmental issues with the potential to reduce global reliance on the industry (Venkata Mohan et al., 2020). In this critical review we will focus on integrated system to use wastewater as promising source of nutrients and flue gas effluents as carbon source for microalgae cultivation. In consequences, we based in biorefinery approach for biomass valorization and the reuse of treated water to close the loop and achieve the circular bioeconomy strategy. We will provide some critical points and some recommendation to enhance the cultivation of microalgae for this strategy.

MICROALGAE AS PROMISING SOURCE OF WWT AND NUTRIENTS RECOVERY

1. Microalgae based wastewater treatment

Recovery of recyclable nutrients from secondary sources may be critical in meeting the rising population's demands (Nagarajan et al., 2020). Wastewater from various sources is enriched with nutrients and carbon, which can be recovered and reused (Ummalyima et al., 2021). Microalgae can effectively use wastewater nutrients to grow and produce biomass (Abinandan et al., 2018). It may be an effective solution to reduce freshwater use, the cost of fertilizers and energy of wastewater treatment processes (Nagarajan et al., 2020; Ummalyima et al., 2021). In addition, the biomass represents an added value that can be used in production of bioenergy (Biodiesel, bioethanol, biogas etc.), raw chemicals and other products etc. (Beuckels et al., 2015; Ummalyima et al., 2021).

2. Nutrients recovery by microalgae

Wastewater treatment bases on the use of microalgae involves direct consumption of surrounding pollutants or nutrient loads via different biochemical mechanisms; fixation, assimilation, precipitation, and bio-adsorption accumulation (Thakur et al., 2020). Nitrogen is crucial element for microorganism's growth. Nitrogen has in essential role in a wide variety of

biological substances such as genetic materials (DNA, RNA), energy transfer molecules (ATP and ADP), functional elements (proteins, peptides, and enzymes) and structural elements (Chloroplast, mitochondria...) (Rashid et al., 2020). There is many forms of nitrogen in wastewater, while microalgae can assimilate inorganic nitrogen (Gupta et al., 2019). Inorganic nitrogen forms are reduced to ammonium before being incorporated within the intracellular membrane as amino acids. However, nitrate (NO_3^-) is the most oxidized, stable and the most preferred form of nitrogen for microalgae cultivation (Rashid et al., 2020). Generally, the NO_3^- is taken up by microalgae via active transport using NO_3^-/H^+ co-transporters, while is preserved by the proton-motive force generated by H^+ -ATPases and nitrate/nitrite transporter proteins (Figure 2) (Gupta et al., 2019).

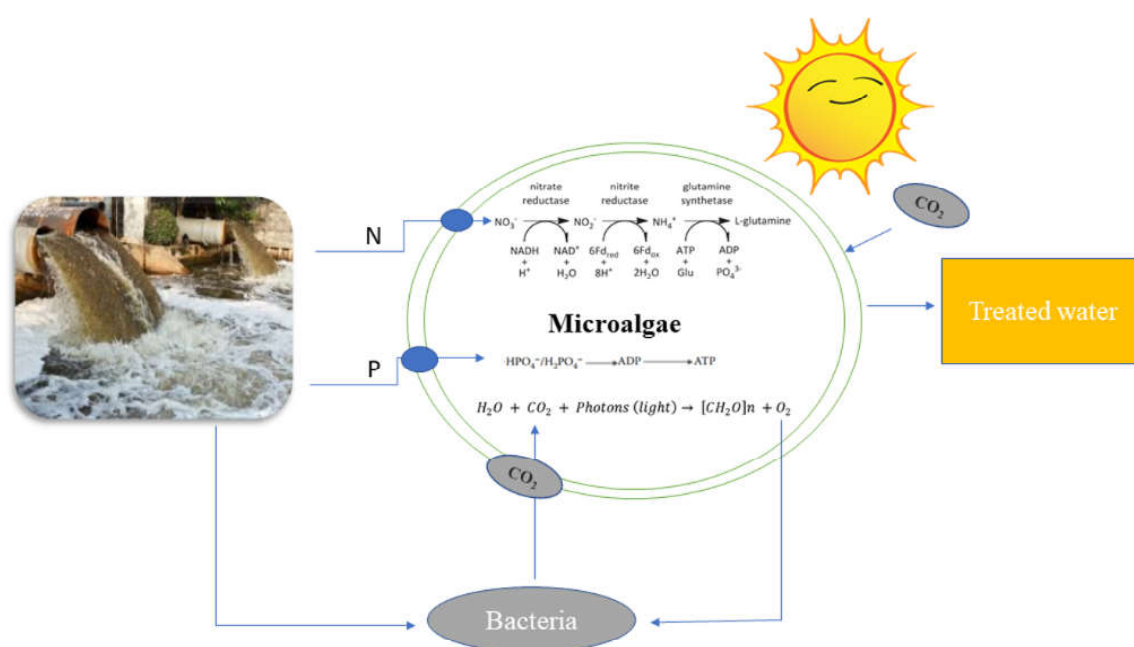


Figure 2: Nutrients recovery from wastewater using microalgae.

Phosphorus is another important element for living organisms. Phosphorus has a vital role in nucleic acids synthesis, energy transfer, lipids, proteins, phospholipids, and carbohydrate metabolism. Indeed, inorganic phosphorus is the most favoured form for microalgae growth and metabolism (Rashid et al., 2020). Wastewater contains a high concentration of phosphorus with the most generous types are HPO_4^- (mono hydrogen phosphate), H_2PO_4^- (di hydrogen phosphate), polyphosphate, and organic phosphate (Goswami et al., 2021). Furthermore, orthophosphate (mono and di hydrogen phosphate) is the most form of phosphate assimilated by microalgae through active transport across the plasma membrane via phosphorylation

process. In phosphorylation process, inorganic phosphate is incorporated into organic compounds involving the generation of ATP from ADP accompanied by energy input (Figure 2). This required energy can be obtained through substrate oxidation, mitochondrial electron transport chain, and transformation of light energy (Gupta et al., 2019; Mehar et al., 2019). Moreover, microalgae have an advantageous character is the storage of excess phosphate in their granules cell when WW contains high concentration of phosphate. It represent a best way for wastewater remediation and phosphorus recovery (Gupta et al., 2019).

Microalgae as photosynthetic microorganisms using carbon dioxide (CO₂) as carbon source to generate their biomass. Microalgae fix CO₂ directly from the atmosphere in the presence of light via autotrophic mechanism or take up from wastewater comprising organic loadings via heterotrophic mechanism detailed information about CO₂ biosequestration be given in the next section. However, microalgae can also take up organic carbon by mixotrophic mechanism (Fuentes-Grünwald et al., 2021; Rashid et al., 2020).

In our opinion mixotrophic mode is the best mode that may be using microalgae in wastewater treatment combined to high biomass production with high biochemical content (Lipids and carbohydrates, etc.). Thus, exploiting their aptitude to use organic matter from wastewater as carbon source and light energy if available. It can be cost effective by favouring microalgae culture in open system with sun as light source. For heterotrophic mode, can be classified as second by incapability of using light to generate biomass and be sufficient in only matter organic in this way can also prolonging the time of phycoremediation.

Heavy metals are taken up by microalgae as oligoelements for their growth. however, the presence of a high concentration in the natural environment could present a real risk to the ecosystem. Indeed, the use of microalgae aptitude for heavy metal removal could be a best alternative to overcome some physicochemical processes limitations (Goswami et al., 2021; Rashid et al., 2020). Microalgae has a common pathway for heavy metals uptake is frequently described as biosorption. It's included the using of living microalgae for metals accumulation or even precipitation in cell space (Goswami et al., 2021). There is also cell-surface sorption in both living and dead microalgae cell. Generally, biosorption take place through ion exchange processes. Firstly, a passive uptake that is a metabolism independent and just occur a membrane plasma permeability to adsorb H.M ions in a short time. On the other hand, in the active uptake that called mechanism dependent metal ions transport across the algal cell membrane into the cytoplasm. Thus, the metal ions can precipitate or bind to intracellular compartment (Gupta et

al., 2020; Rashid et al., 2020). In addition to biosorption there is also other ways for H.M uptake such ion exchange concept, sequestration and compartmentalization in vacuole, chloroplast and mitochondria (Gupta et al., 2020).

Microalgae shows a robustness and efficiency for wastewater treatment eco-friendly and generate in plus biomass with added value. However, we couldn't underestimate the efforts of other microorganisms such as bacteria and fungi that interfere in times in consortium for this process. In addition, biological approaches alone can't be handling this big challenge because of charge funding in wastewater. Therefore, it's necessary to be preceded by physico-chemical approaches. To conclude biological approach, it couldn't be more than alternate and compliment of physico-chemical approaches.

CARBON DIOXIDE BIOSEQUESTRATION AND ASSIMILATION USING MICROALGAE

1. Carbon dioxide biosequestraion by microalgae

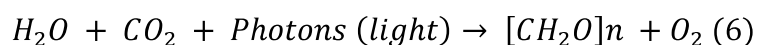
According to the International Energy Agency, (2009) the amount of CO₂ released in 2007 as a result of anthropogenic activities was 28.8 Gt, which is expected to rise to 40.3 Gt by 2030 and 50 Gt by 2050 if proper measures are not realized (International Energy Agency, 2009). As this problem becomes clearer, climate scientists believe that a few more critical decisions must be taken, such as limiting fossil fuel extraction or fully eliminating CO₂ emissions (Venkata Mohan et al., 2016). For this reason, several approaches have been applied for CO₂ biosequestration, physical processes (biochar burial, ocean storage, geological sequestration), chemical processes (mineral carbonization and chemical scrubber) and biological processes (reforestation, agriculture, and photosynthetic microorganisms). However, microalgae could be a promising source for CO₂ biosequestration due the their high photosynthetic efficiency (Cheah et al., 2016; Kassim and Meng, 2017; Zhao and Su, 2020). Microalgae can fix 1.83 kg CO₂/1 kg of biomass (Sudhakar et al., 2011). In contrast to terrestrial plants that can fix about 0.03–0.06 percent v/v of CO₂. Microalgae can capture about 5–15 percent v/v of CO₂ even from flue gas that contains toxic gas that may be harmful for microalgal growth (Katiyar et al., 2021), (Cheah et al., 2016). According to Cheah et al., (2016), some microalgae eg. *Scenedesmus* sp. and *Chlorella* sp. can tolerate high concentration of CO₂ supply as 50% and 70%, even 100% achieving high growth rate (Cheah et al., 2016).

2. Carbon dioxide assimilation

The fixation of CO₂ by microalgae take places on two stages: photosynthesis and CO₂ absorption or incorporation through cells and CO₂ fixation via CO₂ concentrating mechanism (CCM) (Seyed Hosseini et al., 2018).

2.1. Photosynthesis

The most known process of CO₂ capture by microalgae is photosynthesis. In this reaction microalgae use photons to split water giving the first electron donor and release oxygen after hydrolysis. Ultimately, CO₂ converted in lipids and other hydrocarbons molecules (Klinthong et al., 2015). The equation of photosynthesis can be written as follows:



2.2. CO₂ concentrating mechanism (CCM)

Generally, microalgae use CO₂ as first choice of inorganic carbon and bicarbonate HCO₃⁻ as second choice in the absence of CO₂ (Eladel et al., 2019). The transport of these molecules through membrane assured by various transporters, channels, and the enzymes (Mehar et al., 2019). The incorporation of CO₂ across microalgal cells depend to pH value. At low pH levels (5-7) CO₂ enters cell by simple diffusion. However, at high pH values ≥ 7.0 microalgae use bicarbonate HCO₃⁻ as inorganic carbon source that transported actively via carbonic anhydrase (CA) resulting an increasing level of CO₂ at RuBisCO active sites known as CCM (Figure 3) (Gupta et al., 2020; Mehar et al., 2019). Moreover, this increases level of CO₂ favour carboxylation process of RuBisCO resulting an increase of photosynthetic rates and inhibits photorespiration (Beardall and Raven, 2020; Seyed Hosseini et al., 2018). Inside microalgal cells, CA assure the conversion of bicarbonate to CO₂ to be taken up by RuBisCO for fixation into lipids and hydrocarbons molecules (Gupta et al., 2020; Mehar et al., 2019). Generally the RuBisCO embedded in carboxysome for cyanobacteria and for eucaryotes located in pyrenoides (Jensen et al., 2020).

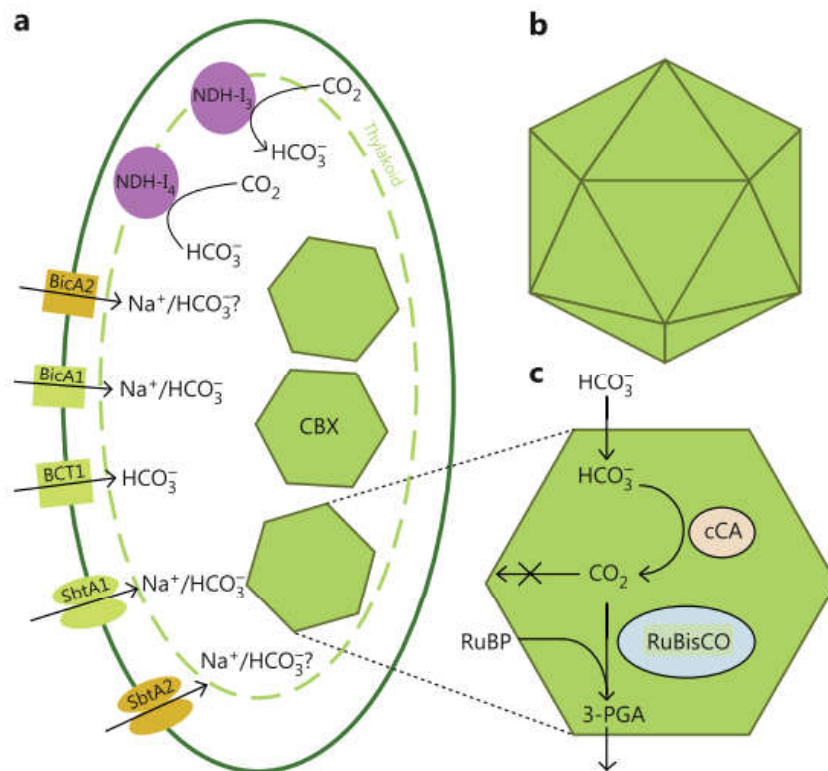


Figure 3: CO₂ concentrating mechanism (CCM) in carboxysome.

a) The bicarbonate ion is accumulated within the cell via a range of active bicarbonate transporters such as BicA1, BicA2, SbtA1, SbtA2 and BCT1, as well as CO₂ uptake and scavenging pumps NDH-I₃ and NDH-I₄. b) carboxysome encapsulate the RubisCo. c) the conversion of bicarbonate ions to CO₂ via anhydrase carbonic (CA) (Rae et al., 2013).

MICROALGAE CULTURE SYSTEMS ADAPTED TO WASTEWATER TREATMENT AND /OR CO₂ BIOSEQUESTRATION

Different culture systems are available to perform microalgae cultivation combined with wastewater treatment in a considerable scale. In present section we detail the features with advantages and disadvantages of open, closed and hybrid systems

1. Open systems

Economically, open pond system is the convenient choice for microalgae cultivation to recover nutrients from wastewater because they are relatively inexpensive to build and easy to scale up (Acién et al., 2016; Cai et al., 2013; Mehar et al., 2019). Raceway pond may build on non-arable lands next to power plants in order to provide carbon dioxide from flue gas. They can also build near wastewater treatment plants station to easily supplied with wastewater nutrients (Abinandan and Shanthakumar, 2015; Cai et al., 2013). Indeed, the raceway pond is the typical open system has been used for microalgae cultivation. To provide sufficient sunlight to microalgae cells for their photosynthesis, it has usually 0.3 m deep. In addition, the raceway is equipped with a paddle way to mixed and circulated well microalgae culture (Figure 4) (Abinandan and Shanthakumar, 2015; Cai et al., 2013). However, the poor light utilization, evaporative losses, diffusion of CO₂ to the atmosphere, contamination by predators, heterotrophs and requirement of large areas of land are major disadvantage in these systems (Eladel et al., 2019; Maity et al., 2014).



Figure 4: Example of open system (Kampman et al., 2013).

Several industrial companies developed some models for biomass production that could be valorized for biofuels production. In Florida, Aurora Biofuels is developing a WWT models using algal ponds to have commercial scale facilities. Moreover, a lot of efforts have been focused on enhancement of microalgae cultivation system to improve biomass production (Cai et al., 2013). Sapphire Energy in Columbus, New Mexico, USA facility use raceways and can produce approximately 5 million liters of biodiesel per year and their aim was to achieve 200 million liters in 2018 (Janssen et al., 2013). PetroSun Biofuels Inc. in the coast of South Texas near South Padre Island, has built saltwater algal ponds in 1,100 acres. Thus, produce a minimum of 16 million L of algal oil refined to biodiesel and jet fuel and 49,896 t of biomass per an (Cai et al., 2013).

2. Closed systems

To overcome the major problems faced in open systems the photobioreactors (PBR) are developed as closed system with engineering aspects. Which could control all the required conditions for microalgae culture. There is an amount of PBR types such airlift, tubular, flat plate, and vertical column (Figure 5) (Abinandan and Shanthakumar, 2015). Generally, Tubular is the most PBR used for microalgae culture with maximum solar capture via changing arrangements of glass tubes vertically or horizontally according to the orientation of the sun (Eladel et al., 2019). They lead to success in culture of one single microalgae with low level of contamination and helping in harvest. PBRs are effective in gas transfer and exchange. Thus, they are used usually for CO₂ sequestration. They don't require a large area for microalgae cultivation in comparison to open systems (Abinandan and Shanthakumar, 2015; Eladel et al., 2019). However, PBRs are limited in biomass production compared to open systems. The installation and maintenance costs are much higher than open ponds system. (Acién et al., 2016; Cai et al., 2013). Indeed, PBRs are not recommended for wastewater management (Eladel et al., 2019). Besides, they are more appropriate for algal products be used in alimentation, medicine, pharmaceutical and cosmetic that require some exigences (Cai et al., 2013). Moreover, closed reactors have a lot of interest from some companies to be scaled up at low cost. Solix BioSystems has developed an algae growth system called Lumian TM AGS4000 with high productivity of 4000-L. In Florida, Algenol Biofuels Inc.'s has 3000 patented photobioreactors in surface of 15-ha. It claimed to have a capacity of 379,000 L of ethanol per year approximately (Cai et al., 2013).

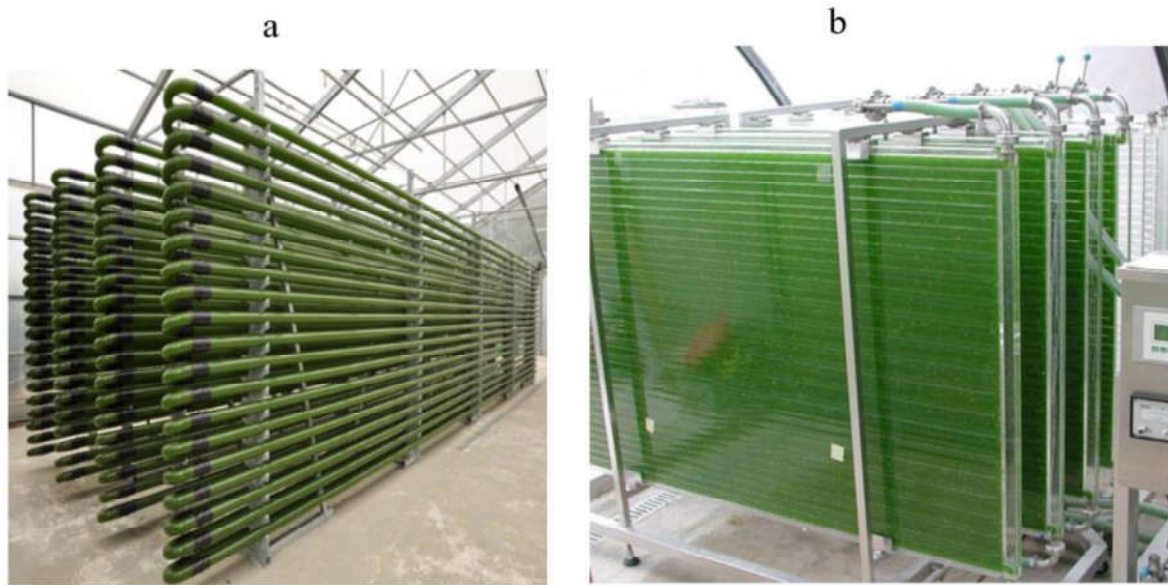


Figure 5: Example of closed system.
a) tubular photobioreactor; b) plate photobioreactor (Jerney and Spilling, 2020).

3. Hybrid system

Between closed and open systems, hybrid system is developed to combine the merits of both in two stages of cultivation. The first one is the culture of microalgae strain in closed system and transferred to open ponds as second stage (Cai et al., 2013; Kadir et al., 2018). Hybrid system could be associated two or more cultivation (Salama et al., 2017). This system can serves to keep microalgae dominance while limiting the contamination by other microorganisms in PBR system at exponential phase before being added to large scale open pond (Kadir et al., 2018). However, the high cost of the first stage still limited the use of this system for commercial application (Cai et al., 2013). An example of using hybrid system in Northwest Europe for wastewater treatment. The MaB-floc SBRs, sequences batch reactors with microalgal bacterial flocs for photosynthetic aerated wastewater treatment based on bioflocculation. They began by indoor PBRs of 4L, and scaled up over 40 and 400L achieving 12, 000 L outdoor raceway pond (Van Den Hende et al., 2014). Every system possess advantages and disadvantages and suitability depends on site-specific conditions and the applied field of microalgae culture and biomass valorization. For wastewater treatment it's clear that open pond is more appropriate in scale up than closed system because of the cost-effectiveness, even that PBR helps in maintaining monoclonal cultivation.

INTEGRATED BIOREFINERY TO PRODUCE MULTI-BASED PRODUCTS AND COST ASSESSMENT

A biorefinery is an infrastructure facility that integrate multiple conversion technologies such as thermochemical, biochemical, combustion, and microorganism growth platforms converting biomass into sustainable bio-based product streams such as biofuels, biochemicals, bioenergy, and other high-valued bio-products (Pereira et al., 2021; Ubando et al., 2020). Therefore, microalgae have gained the most interest among researchers and industries last decades as a potential source of precious products that can be used in the food, feed, biofuels, cosmetics, pharmaceuticals, and nutraceuticals fields (Katiyar et al., 2021; Venkata Mohan et al., 2020). The use of integrated biorefinery approach with sequential extraction to close the loop can meet current circular bioeconomy challenges to produce high-value multiple products at a time (Venkata Mohan et al., 2020). Ultimately, the application of microalgae biorefinery approaches in industrial scale is require a detailed techno-economic, environmental, and lifecycle assessment (Katiyar et al., 2021). The next paragraphs present some examples of cost analysis of integrated biorefinery products in previous studies and our biorefinery model with techno-economic analysis.

The cost of microalgae oils and biodiesel production ranging between 9 \$ to 25 \$ per gallon in ponds and in photobioreactors the cost is estimated to be 15 \$ to 40 \$ per gallon (Klinthong et al., 2015). This price due to the requirement of process steps (i.e., culture, harvesting, drying, pre-treatment, extraction, transesterification, etc.) (Abinandan et al., 2018; Klinthong et al., 2015). To reduce the cost of microalgae oil and biodiesel, a combined process may be involved, i.e the combination of harvesting, pre-treatment and extraction system in one process (Klinthong et al., 2015). Lee et al., (2019), reported that microalgae biodiesel production process by direct transesterification where extraction and transesterification occurred simultaneously may reduce the price of biodiesel compared with conventional process with 18.7 \$ kg⁻¹ and 24.0 \$ kg⁻¹ respectively. Furthermore, the use of wastewater and flue gas as nutrients and CO₂ sources are required to reduce more the cost (Lee et al., 2019). Recently, numerous studies reported that wastewater have been used for microalgae cultivation to reduce the cost of fertilizers and freshwater as mentioned previously (section WW) (Han et al., 2018). The application of this approach may conduct to achieve high lipid productivity as well as high nutrient removal efficiency (Han et al., 2018). Moreover, the use of wastewater as a source of nutrients for microalgae cultivation is advantageous for biofuel yield. It was estimated that using flue gas in wastewater for algal cultivation could produce 1.7 billion litres of oil per year

at a cost of 0.55 \$ L⁻¹, compared to 1.98 \$ L⁻¹ for conventional crude oil (Abinandan et al., 2018; García et al., 2019). It is worth noting that large quantities of biomass can be generated while using microalgae-based wastewater treatment processes. As a result, depending on the nutrient content of the wastewater, up to 1 kg of biomass can be generated per cubic metre of wastewater treated, which is five times more than traditional sludge (Acién et al., 2016; Fernández et al., 2019). In addition, biomass generated using wastewater can cost 1 € kg⁻¹, while ranges from 100 € kg⁻¹ using close reactors to 10 € kg⁻¹ in raceway reactors (Acién Fernández et al., 2019). Kumar et al., (2020), reported that microalgae production combined with simultaneous treatment of dairy effluent using a High-Volume V-shape Pond (HVVP) may be cost-effective. In addition, they achieve an algal production of 504 ton at 0.482 \$ kg⁻¹ with ~240,000 m³ of treated clean water.

However, the production of biodiesel as the main product, even using WW as source of nutrients and freshwater still unprofitable (Han et al., 2018). Hence the application of biorefinery approach with sequential extraction of numerous products at time may satisfy economically and environmentally with zero waste. Moreover, microalgae can derived several products include whole biomass, proteins (food and feed), carbohydrates (bioethanol), lipids (biodiesel), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), fucoxanthin, and astaxanthin (nutrition, medical and cosmetic applications) etc. (Han et al., 2018; Palmer et al., 2021). Numerous models and studies were proposed the integrated approach of biorefinery for multiproduct output using waste as input (Branco-Vieira et al., 2020; Katiyar et al., 2021). Katiyar et al., (2021), proposed an integrated biorefinery approach application using microalgae biomass with 40% lipids, 50% proteins, and 10% carbohydrates. They proposed that 25% of lipids can be sold to the chemical and food industries for 2 € kg⁻¹, while the rest could be used for biodiesel production for 0.50 € kg⁻¹. Proteins has double use after fraction with food value of 5 €kg⁻¹ and feed value of 0.75 € kg⁻¹, respectively. Microalgae carbohydrates are low, thus it can be used for bioethanol production at 1 € kg⁻¹ or can be used as chemical building blocks due to cellulosic part (Katiyar et al., 2021).

The techno-economic analysis in microalgae has been based on cultivation system, modes and regimes or biomass production process. These allowed for estimation of products price, which indicates the financial viability of the manufacturing process and the possibility of integrating microalgae products into the current market (Doshi, 2017). Doshi, (2017), presented a techno-economic analysis (TEA) of the feasibility of a multi-output production using open-pond system due to the lower capital and energy costs for a commercial or large-scale production.

Furthermore, the integration of biodiesel as main product with aquaculture feed and agricultural fertilizer as complimentary products using waste effluents as culture inputs to reduce the cost of production (Doshi, 2017). Doshi, (2017) presented 383,653,000 US\$ as capital cost of investment from the construction of pond to storage of industrial products. In addition, an annual cost operating costs assessed 276,528,000 \$. Ultimately, the output allocation and the cost estimation was 1.17 \$ L⁻¹ for biodiesel, 1.18 \$ L⁻¹ for glycerol, 12 \$ Kg⁻¹ for aquaculture feed and 12 \$ Kg⁻¹ for agriculture fertilizer (Doshi, 2017).

A techno-economic analysis of biodiesel production using *Phaeodactylum tricornutum* in scale-up has been done by (Branco-Vieira et al., 2020). The model takes up an annual production of 1,811 tons of microalgae biomass and 171,705 L of biodiesel from 80,000 m³ of microalgae cultivation in a collection of bubble column photobioreactors built on 15.247 ha of land.

The total operating cost is estimated to be 4,262,857 € year⁻¹ and the total capital cost to be 516,107 € year⁻¹. Total earnings were calculated according to the selling prices for biodiesel (0.75 € L⁻¹), for dry protein-rich residual biomass (2.85 € kg⁻¹) and glycerol (0.10 € kg⁻¹). In view of the economic valorisation of residual biomass and glycerol, microalgae biodiesel production show that it is profitable, and the project's is viable in medium term. The final estimated cost of biodiesel production is 0.33 € L⁻¹ and microalgae biomass is 2.01 € kg⁻¹. In results, it was calculated a ROI of 10%, with a payback period of ten years and an EBITDA of 588,139 € year⁻¹ (Branco-Vieira et al., 2020).

Our scenario for integrated biorefinery approach using microalgae as model is take place in a farmland of 10 ha. Moreover, 70% of this surface is a culture area with 70 pounds with 1000 m² and 300 m³ capacity (Table 1). The other 30% is allowed to the infrastructure for the station operation. The capital investment from the construction to products stockage was estimated 256,717,226.02 \$ and the annual operating cost was estimated 677,163.63 \$ Year⁻¹. The details were presented in table 2 and 3. To minimise the cost of investment to have profitable products we choose to integrate the use of wastewater and the industrial flue gas as source of water, fertilizer and CO₂ source for microalgae culture. Which gives advantage of good yield in biomass with high lipids content in cell. In addition, treated water can be reused for industrial wash, irrigation of green surface, agriculture etc. It can be used also to operate the station and minimise the fees of water. In this regard, the capital investment estimation was reduced to 257,161,483.42 \$. Numerous studies reported the use of biodiesel as the main product, which was very expensive and unprofitable with a cost average of 3.25-4.33 \$ (Campbell et al., 2011; Faried et al., 2017). In our thesis we proposed the valorization of biomass for biodiesel

production for the lipidic fraction but as by-product. Moreover, transesterification also has glycerol as co-product, which has a good price as a second complementary product. The residual of biomass contains a useful compounds as bioactive molecules that can be valorized as plant biostimulant which can be our main product because of its place in the market recently. The leftover undergoes methanization to produce biogas that can be used for operating station and minimised fees of electricity (Figure 6).

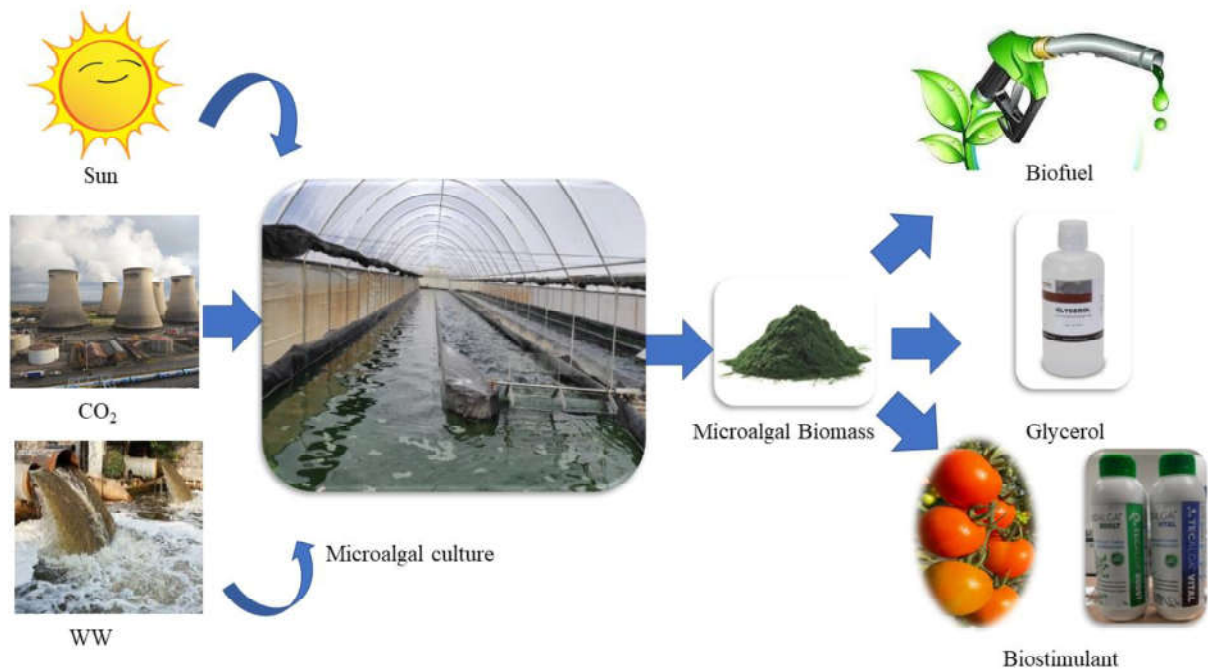


Figure 6: Circular bioeconomy approach-based microalgae for wastewater treatment, CO₂ sequestration and biomass valorisation in biorefinery.

Table 1: Description of the surface allowed to microalgae culture and biomass production

Parameters	Value	Units
Farm Area	10	Ha
Area for Ponds	7	Ha
Area for infrastructure	3	Ha
Number of pounds	70	
Volume capacity/pound	300	m ³

Table 2: Capital investment and operating cost for microalgal biomass production

Fixed capital	Cost for 70 pounds	Unit	References
Pond construction	15,682,955.14	\$	Calculated
Mixing paddle	13,068,655.13	\$	Calculated
Infrastructure for culture	522,955.90	\$	Calculated
Maintenance for all /Year	224,757,672.99	\$	Calculated
Infrastructure for harvest	132,050.51	\$	Calculated
Operating cost	Cost	Unit	Source
Electricity [Dh kWh ⁻¹]	0.17	\$	ONEE
Water [Dh m ⁻³]	0.75	\$	ONEP
Labor LQ [Dh m ⁻¹]/person	542.05	\$	
Labor HQ [dh m ⁻¹]/person	867.19	\$	
DAP Dh/t	739.95	\$	
Urea Dh/t	810.81	\$	
Carbon dioxide [kg ⁻¹]	0.13	\$	
Culture in integration of WW and flue gas as CO ₂ source			
Electricity [Dh kWh ⁻¹]	0.17	\$	ONEE
Labor LQ [Dh m ⁻¹]/person	542.05	\$	
Labor LQ [dh m ⁻¹]/person	867.19	\$	
Flue gas desulfurization (FGD) ≥ 300 MW	349.75	\$	powermag.com 2007

Table 3: The capital investment and operating cost of microalgal biomass products

Equipment or product	Cost	Unit
Centrifuge	352,881.18	\$
Dryer	108,361.96	\$
Continuous bed extractor (CBE)	325,085.87	\$
Chloroform (ton)	449.48	\$
Methanol (ton)	458.57	\$
Sulfuric acid (ton)	199.81	\$
Hexane (ton)	1,298.56	\$
NaOH (ton)	379.58	\$
Installation + Maintenance/year	707,455.30	\$
Tank LPG 5 ton capacity	4,764.50	\$
Bottle (1L)	0.22	\$

CONCLUSION AND OUTLOOK

Microalgae-based wastewater treatment technology can not only purify wastewater and solve environmental pollution problems. But also use the nutrient elements in wastewater to produce algal biomass, which has drawn the attention to be a potential feedstock for sustainable and renewable biofuels and bioproducts production. Moreover, we also showed the potential of microalgae for CO₂ biosequestration and how microalgae can assimilate dioxide carbon in their cell with a focus in the CO₂ concentration mechanism (CCM) including Rubisco and carbonic anhydrase as the key enzymes for CO₂ fixation in microalgae. Biomass production with different compounds makes them as source of integrated biorefinery approach with sequential extraction to close the loop can meet current circular bioeconomy challenges to produce high-value multiple products at a time. In this regard, we proposed our model of integrated biorefinery with technoeconomic analysis and the possible microalgae derived products that can be valorized after wastewater and flue gas use as low-cost inputs for microalgae culture. Microalgae potentialities for integration of CO₂ bio-sequestration, WWT and biorefinery for sustainable circular bioeconomy should be taking seriously in world cities agenda as a great viable solution for economy and environment. The units of culture may be helpful to be near to wastewater and flue gas effluents to minimize the cost of transport. The microalgae derived products should be valorized in max with biorefinery strategy of zero waste and multiproduct at time. Which increased the economics benefits of microalgae culture according to the close loop strategy and circular bioeconomy approach.

II. Synthèse

La bioraffinerie est une infrastructure qui intègre plusieurs technologies convertissant la biomasse en divers produits durables tels que les biocarburants, les produits biochimiques et d'autres bioproduits de valeur ajoutée (Ubando *et al.*, 2020; Pereira *et al.*, 2021). En effet, les microalgues ont suscité l'intérêt des chercheurs et des industries au cours des dernières décennies. En tant que source potentielle de produits précieux pouvant être utilisés dans les domaines de l'alimentation humaine et animale, la bioénergie, les produits pharmaceutiques et cosmétiques (Venkata Mohan *et al.*, 2020; Katiyar *et al.*, 2021). L'utilisation de l'approche de bioraffinerie intégrée avec extraction séquentielle afin d'avoir plusieurs produits de valeur ajoutée à la fois, peut répondre aux défis actuels de la bio économie circulaire (Venkata Mohan *et al.*, 2020). Du coup, la bioraffinerie des microalgues à l'échelle industrielle nécessite une évaluation technico-économique, environnementale et un cycle de vie bien détaillé (Katiyar *et al.*, 2021). A cet égard, l'utilisation des microalgues pour le traitement des eaux usées présente une solution efficace pour réduire l'utilisation d'eau douce, le coût de la production des microalgues et de traitement des eaux usées (Nagarajan *et al.*, 2020; Ummalyima *et al.*, 2021). Par conséquent, le mode mixotrophie est le meilleur mode utilisé par les microalgues dans le traitement des eaux usées combiné avec une production élevée de biomasse à haute teneur biochimique (lipides et glucides, etc.). Il peut être rentable en favorisant la culture des microalgues en système ouvert avec le soleil comme source de lumière. Dans la présente revue nous avons mentionné plusieurs modèles de bioraffinerie en utilisant les microalgues. Par la suite, nous avons proposé notre modèle qui consiste à l'utilisation des eaux usées et de gaz de combustion issus de l'industrie comme source d'eau, des nutriments et de CO₂ pour la culture des microalgues, afin de minimiser le coût d'investissement et d'avoir des produits rentables. Par ailleurs, l'eau traitée peut être réutilisée pour le lavage industriel, l'irrigation des surfaces vertes, l'agriculture, etc. Elle peut également être utilisée pour faire fonctionner la station et minimiser les frais d'eau. Dans notre thèse nous avons proposé la valorisation des lipides de la biomasse pour produire du biodiesel mais comme sous-produit, parce que le biodiesel n'est pas rentable économiquement. En effet, le coût moyen est estimé de 3,25 à 4,33 \$ (Campbell *et al.*, 2011; Faried *et al.*, 2017). De plus, le glycérol résulte de la transestérification comme étant produit complémentaire et avec de bon coût. Les résidus de la biomasse contiennent des molécules bioactives pouvant être valorisées en tant que biostimulant des plantes qui peut être notre produit principal en raison de sa place sur le marché ces dernières années. Le surplus subit une méthanisation pour produire du biogaz qui peut être utilisé pour le fonctionnement de la station afin de minimiser les charges d'électricité.

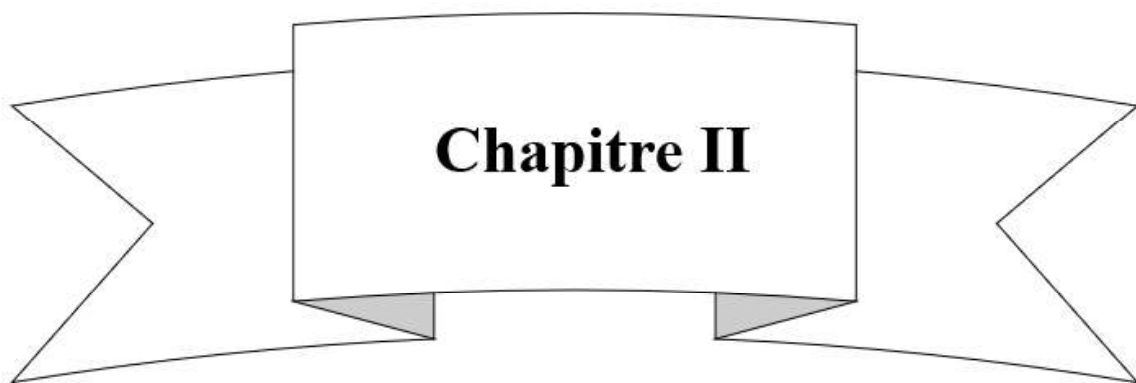
III. Conclusion

Le traitement des eaux usées par des microalgues peut non seulement purifier les eaux usées et résoudre les problèmes de pollution environnementaux. Mais également utiliser les éléments nutritifs des eaux usées pour produire de la biomasse algale, qui attire l'attention sur le fait qu'elle est une matière première potentielle pour la production durable et renouvelable des biocarburants et des bioproduits. Par conséquent, cette biomasse est la source de l'approche de bioraffinerie intégrée avec une extraction séquentielle pour fermer la boucle afin de répondre aux défis actuels de la bioéconomie circulaire pour produire plusieurs produits de valeur ajoutée à la fois. À cet égard, nous avons proposé notre modèle de bioraffinerie intégrée avec une analyse technico-économique des éventuels produits dérivés de microalgues. Les potentialités des microalgues pour l'intégration de la bio-séquestration du CO₂, le traitement des eaux usées et de la bioraffinerie pour une bioéconomie circulaire durable. Cette approche devrait être prise au sérieux dans l'agenda des villes mondiales en tant que solution viable pour l'économie et l'environnement.

B. Plant Biostimulant: definition, composition and place in market

Plant biostimulant have been largely debated last decades regarding composition, sources nature and mode of action (Lau et al., 2022). Numerous definitions were given for biostimulant over the time. Zhang and Schmidt (1997) defined plant biostimulant as materials that promote plant growth in minute quantities. The European Biostimulant Industry Council (EBIC) defined the plant Biostimulant as follow: “Plant biostimulants contain substance(s) and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality” (Calvo et al., 2014). Until this definition it’s hard to distingue Biostimulant to other classes of compounds. Therefore, Yakhin et al. (2017) defined plant biostimulant as “a formulated product from a biological origin that improves plant productivity as a consequence of the novel or emergent properties of the complex of constituents, and not as a sole consequence of the presence of known essential plant nutrients, plant growth regulators, or plant protective compounds.” Moreover, plant biostimulant are any substances or microorganisms used in plants to improve nutrients uptake, environment stress tolerance and crop quality and yield (Del Buono, 2021). By these recent definitions, plant biostimulant were distinguished from other classes of compounds based on their agricultural functions (Del Buono, 2021).

As their definition mentioned they contains microorganisms or bioactive compounds. Among diverse categories of plant biostimulant, Seaweed largely used recently and make marketplace among plant biostimulant around the world (Bulgari et al., 2019). So far, microalgae also have the same interest among researchers and industrial (Mutale-joan et al., 2020). These interest due to microalgae potential in bioactive molecules synthesis like proteins, polysaccharides, PUFA, pigments and phytohormones etc. (Khan et al., 2018; Supraja et al., 2020). Phytohormones and polysaccharides improves plant growth and development. Polysaccharides, PUFA and pigments enhanced plant tolerance to abiotic and biotic stress (Kusvuran, 2021; Mazepa et al., 2021). Plant biostimulant can be applied to plant in different methods: foliar spray, soil drench, and seed priming (Behera et al., 2021). Moreover, the market of plant biostimulant increasing year after year with a compound annual growth rate of 12.1%, which predicted to reach USD 6.2 billion in 2027 (markets and markets, 2022). Indeed, the industrial welcomed plant biostimulant development, which given that plant biostimulant have great potential to replace chemical pesticides and fertilizer (Lau et al., 2022).



Chapitre II

CHAPITRE II: ÉTUDE DU POTENTIEL DES MICROALGUES POUR LE TRAITEMENT DES EAUX USÉES ET LA PRODUCTION DU BIODIESEL

Article: Microalgae as promising source for integrated wastewater treatment and biodiesel production

I. Introduction

Les eaux usées sont l'un des principaux contributeurs à la pollution de l'eau, entraînant un danger réel pour l'environnement et la santé humaine (Tripathi *et al.*, 2019). Elles sont chargées par des micro-organismes, des nutriments organiques et inorganiques, des métaux lourds et des micropolluants (Tripathi *et al.*, 2019). À cet égard, le traitement des eaux usées est indispensable pour une meilleure gestion des ressources en eau, ce qui aide à réduire la pénurie d'eau (Gouveia *et al.* 2016). En effet, le traitement des eaux usées par des procédés innovants et durables et des approches intégrées, a fasciné l'intérêt mondial (Saravanan *et al.*, 2018; Yadav *et al.*, 2019).

Diverses méthodes ont été utilisées pour le traitement des eaux usées, méthodes physiques (filtration, sédimentation, flottation et adsorption, etc.), méthodes chimiques (coagulation, floculation, neutralisation et chloration/dichloration, etc.) et méthodes biologiques (phytoremédiation, lagunes, boues activées, contacteur biologique rotatif et traitement anaérobie, etc.) (Ahmad *et al.*, 2019). Ces méthodes présentent certaines limites technico-économiques telles que l'exigence des grandes surfaces, un apport énergétique intensif et des coûts de maintenance et d'exploitation très élevés (Ahmad *et al.*, 2019; Shahid *et al.*, 2020). Par ailleurs, des approches intégrées et durables de traitement des eaux usées, se focalisent sur le recyclage de l'eau, des nutriments et le carbone organique plutôt que de les éliminer (Shen *et al.* 2015). En effet, la phycoremédiation est une alternative prometteuse, durable, écologique, économique et efficace par rapport aux méthodes conventionnelles (Leite *et al.* 2019; Shahid *et al.* 2020).

Nombreuses études ont prouvé que les microalgues sont très efficaces pour l'élimination des nutriments des eaux usées, en particulier l'azote (N) et du phosphore (P) ainsi que l'élimination des métaux lourds etc. (Hernández-García *et al.*, 2019; Leite *et al.*, 2019; Shahid *et al.*, 2020). De plus, les microalgues peuvent recycler et transformer ces éléments en biomasse riche par des protéines, lipides, carbohydrates, pigments et autres métabolites. Ces métabolites pourraient être valorisés en biocarburants et autres produits de valeur ajoutée (Moreno García *et al.* 2020;

Shahid *et al.* 2020; Zhu *et al.*, 2020). L'utilisation des eaux usées comme milieu de culture des microalgues pourrait être moins coûteux et plus durable. En revanche, la valorisation de la biomasse est limitée dans la bioénergie et l'agriculture selon la source d'eau utilisée (Li *et al.*, 2019; Tripathi *et al.*, 2019). Récemment, la combinaison entre le traitement des eaux usées et la production de biomasse algale est une nécessité conduisant à réduire le coût de production de bioénergie et de traitement des eaux usées (De Francisci *et al.*, 2018; Sureshkumar and Thomas 2019).

Objectif et contexte

La présente étude vise à sélectionner des souches de microalgues robustes qui ont le potentiel d'éliminer les nutriments des eaux usées, et aussi très productives en termes de biomasse et lipides pour la production du biodiesel de bonne qualité.



Microalgae as promising source for integrated wastewater treatment and biodiesel production

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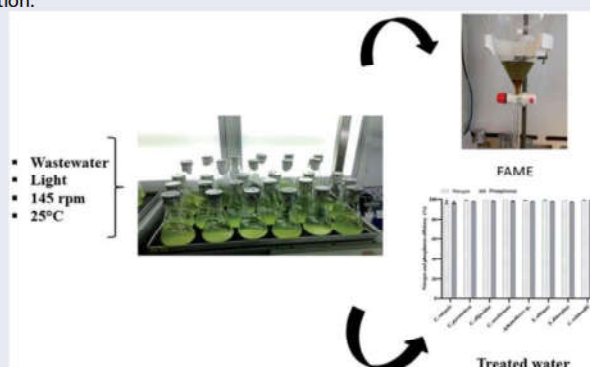
ABSTRACT

Microalgae have been studied for their potential of wastewater treatment as well as a promising source for biodiesel production. This study investigates the potential of microalgae to remove nutrients from domestic wastewater (DWW) while producing lipids-rich biomass for biodiesel production. Eight microalgae were cultivated in (DWW) to evaluate their nutrients removal capacity and biomass production. Total phosphorus (TP) of DWW reduced from 2 mg L⁻¹ to 0.02 mg L⁻¹ with the treatment efficiency of 99.15% and the highest performance was noted in *Chlamydomonas reinhardtii* (*C. reinhardtii*). For total nitrogen (TN), treatment efficiency climbed to 99.07%. It is reduced from 18.35 to 0.17 mg L⁻¹ recorded in *C. reinhardtii* and *Chlorella pyrenoidosa* (*C. pyrenoidosa*). On the other hand, all microalgae showed a high lipids-rich biomass in wastewater compared to BG11. The highest lipid content was 36.93% noted in *Chlorella sorokiniana* (*C. sorokiniana*). Fatty acids methyl ester (FAME) profiles showed a high content of palmitic C16:0, oleic C18:1 and stearic acids C18:0 in studied microalgae strains. In summary, microalgae envisage its potential application in integrated wastewater treatment and biodiesel production. In perspective, the authors focus on the validation of this bioprocess in pilot scale. Furthermore, the use of microalgae for other applications such CO₂ biosequestration and added value products.

KEYWORDS

Biodiesel; fatty acid methyl ester; lipids; microalgae; nutrient removal; wastewater treatment

Novelty statement: The present study investigates the potential of Moroccan microalgae as candidates to wastewater remediation and high biomass production with high lipid rate for biodiesel production.



HIGHLIGHTS

- Wastewater treatment and biodiesel production by microalgae.
- Microalgae showed high potential of nutrients removal from urban wastewater.
- Wastewater enhance growth and biomass production of microalgae strains.
- Wastewater improve lipids accumulation by microalgae strains.
- These lipids used as feedstock for biodiesel production.

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INTRODUCTION

Wastewater is one of the major contributors in water pollution causing severe environmental and health hazards (Tripathi et al. 2019). It contains microorganisms, organics and inorganics nutrients, heavy metals and micro-pollutants (Tripathi et al. 2019). Nowadays, wastewater treatment is indispensable for the best management of water resources. Besides, it may help to reduce water scarcity (Gouveia et al. 2016). In fact, wastewater treatment by innovative and sustainable processes and integrated approaches has fascinated worldwide interest (Saravanan et al. 2018; Yadav et al. 2019).

Wastewater treatment could be conducted by physical (filtration, sedimentation, flotation and adsorption etc.) chemical (coagulation, flocculation, neutralisation and chlorination/dichlorination etc.) and biological methods (oxidation ponds, aerations lagoons, activated sludge, rotating biological contactors and anaerobic treatment etc.) (Ahmad et al., 2019). Although, these methods present some techno-economic limitations such as the requirement of large spaces, intensive energy input, and extensive maintenance and operational costs (Ahmad et al., 2019; Shahid et al. 2020). Moreover, neglecting nutrients removal especially nitrogen and phosphorus from wastewater and generating some gaseous effluents such as CO_2 and NO_x during the process has environmental implications (Katam and Bhattacharyya 2019). Actually, sustainable and integrated approaches for wastewater treatment focus on recycling water, nutrients and organic carbon rather than removing them (Shen et al. 2015). Indeed, phycoremediation promising the most sustainable, eco-friendly, cost-effective, and efficient alternative to conventional methods (Posadas et al. 2015; Leite et al. 2019; Shahid et al. 2020).

Microalgae are potentially diverse, ubiquitous and dominant group of photosynthetic organisms in nature. They are prokaryotic or eukaryotic microorganisms with primitive structure that can be unicellular or simple multicellular (Tomaselli 2004; Andersen 2013; Kamyab et al. 2013). In addition, microalgae have a rapid growth, adaptability to different environmental conditions, require less land, water and nutrients for cultivation (Moreno García et al. 2020). They are highly efficient in CO_2 biosequestration in comparison to terrestrial plants (Approx. 1.8 Kg CO_2 need to make 1 kg of algal biomass) (Sudhakar et al. 2011). According to species and growth conditions microalgae can generate biomass composed of proteins, lipids, carbohydrates and pigments etc. (Velazquez-Lucio et al. 2018). All these assets make microalgae have a large

variety of fields, including aquaculture, food, animal feed, medicine, pharmacy, cosmetic, bioenergy, environment and agriculture etc. (Cardozo et al. 2007).

Several studies reported that microalgae are very efficient in nutrients removal from wastewater especially nitrogen (N) and phosphorus (P) as well as removing minerals, heavy metals and reducing some organic compounds (Leite et al. 2020; Sakarika et al. 2020). Moreover, the phycoremediation has been proved by numerous studies in different sources of wastewater (domestic, industrial and agricultural etc.) and even acid mine drainage (Hernández-García et al. 2019). Likewise, microalgae can recycle organic and inorganic nutrients from wastewater to biomass with proteins, lipids, carbohydrates and pigments. Furthermore, these metabolites could be valorized into biofuels and valuable co-products (Moreno García et al. 2020; Shahid et al. 2020; Zhu et al., 2020). According to Mennaa et al. (2017) microalgae have been proved as bioremediation tool for nutrients removal from wastewater. They are advantageous for this application owing to their capacity to recycle nutrients into biomass that could be valorized in bioenergy, raw chemicals or other products (Beuckels et al. 2015; Zhu et al. 2019).

The use of wastewaters as medium to cultivate microalgae could be inexpensive and sustainable. Although, limited biomass valorization in bioenergy and agriculture (Li et al. 2019; Tripathi et al. 2019). In the fact, microalgae are the most suitable and outstanding feedstock of bioenergy (biodiesel, bioethanol and biogas) production owing to their lipids and sugars content especially under stress conditions (Zhu et al. 2019; Arif et al. 2020). Lipids can be processed to biodiesel by transesterification and sugars to bioethanol after fermentation (Gojkovic et al. 2020). Recently, the combination between wastewater treatment and microalgae biomass production it could be a necessity leading to reduce the cost of bioenergy production and process of wastewater treatment (De Francisci et al. 2018; Sureshkumar and Thomas 2019).

The present study aimed to screen the efficient and robust microalgae strains that have potential to remove nutrients from wastewater and highly productive in terms of biomass and lipids for biodiesel production.

MATERIEL AND METHODS

1. Experimental design

The wastewater used for this study was secondary pre-treated domestic wastewater (DWW) of Khouribga city, Morrocco (32°84'82.21"N, -6°93'4.60"W). The effluent was clarified, filtered and sterilized by autoclave model [paypa stream sterilizer] to be used as culture medium. The characteristics of (DWW) are listed in Table 4.

Table 4: Characterization of domestic wastewater

pH	8.00 ± 0.005
T°C	11.57 ± 0.083
Dissolved oxygen (mg.L⁻¹)	7.34 ± 0.100
Conductivity (ms.cm⁻¹)	3.21± 0.002
Turbidity (NTU)	14.84 ± 0.310
Nitrogen (mg L⁻¹)	18.35 ± 0.005
Phosphorus (mg L⁻¹)	1.96 ± 0.010
Proteins (%)	0.00 ± 0.000
Total Sugar (%)	1.43 ± 0.173

The microalgae strains used for this work were: *Chlorella vulgaris* (*C. vulgaris*), *Chlorella C. pyrenoidosa* (*C. pyrenoidosa*), *Chlorella ellepsoidae* (*C. ellepsoidae*), *C. sorokiniana*, *Aphanothece* sp., *Scenedesmus obliquus* (*S. obliquus*), *Scenedsmus dimorphus* (*S. dimorphus*) and *Chlamydomonas reinhardtii* (*C. reinhardtii*) maintained in Mascir (Moroccan foundation for Advanced Sciences, Innovation and Research) collection. These strains were cultivated in domestic wastewater and in BG11 medium as control (Stanier et al. 1971).

Microalgae strains were cultivated in 250 mL erlenmeyer flasks with an initial concentration equivalent to OD_{680nm} of 0.1 with a volume of 150 mL. The cultures were incubated at 25 ± 1 °C, under shaking (VWR Advanced Digital Shaker) at 145 rpm and continuous illumination 6000 lux, provided by white fluorescent lamps for 26 days. Microalgae growth was evaluated twice-daily by measuring optical density (OD) at 680 using UV/VIS spectrophotometer model [Ultrospec 3100 pro]. The data obtained was converted to log values for developing growth curves and calculating specific growth rates. The specific growth rate was calculated as:

$$\mu = \ln(Nt/No)/\Delta t$$

where N_t and N_0 were the final and initial cell number or OD respectively, and Δt was the time interval (days) of cultivation.

After 26 days of culture, the biomass was harvested by centrifugation (Centrifuge Heraeus Megafuge 40R) at 3500 rpm and 4 °C for 10 min. The supernatant used for physico-chemical analysis. Thereafter, the pellet has been dried in the oven model [Memmert] at 50 °C for 48h and weighted.

1.1. Biochemical characterization of biomass

1.1.1. Protein content

Microalgae proteins were extracted using the modified method described by (Fleurence et al. 1995). Firstly, 10 mg of microalgae biomass powder was suspended in 500 μ L of deionized water. Then, the suspension was heated by Labnet Acc Block Digital Dry Bath at 95 °C for 1 hour and sonicated at 40 KHz for 90 min with sonicator bath model [1510 Branson]. Thereafter, the suspension was centrifuged at 7715 rpm and 4 °C for 20 min by Eppendorf Centrifuge 5424 R. The supernatant was collected and the pellet was re-suspended in 500 μ L of sodium hydroxide 0.1 mol L⁻¹ and treated by the same way. The supernatants were combined and the pellet was discarded. The proteins determined according to Bradford method (Bradford M. 1976). Calibration curves were prepared using bovine serum albumin (BSA) \geq 98%. All measurements were done at 595 nm using UV/VIS spectrophotometer model [Ultrospec 3100 pro].

1.1.2. Carbohydrates content

10 mg of microalgae dry biomass was added to 2 mL of sulfuric acid 2% with purity of 95-97%. Then, the mixture was heated at 90 °C for 2 hours and sonicated. Total sugar was determined according to phenol-sulfuric method with some modifications (Dubois et al. 1956). 2 mL of mixture was mixed with 1 mL of phenol 5% W/V with purity of 99.5-100.5% and homogenized by vortex. Then, 5 mL of sulfuric acid were added quickly and directly to the mixture without touching the wall and homogenized by vortex model [isolab]. We left the tubes in the ambient temperature for 20 minutes. Thereafter, we incubated the tubes at 30 °C in the water bath model [Memmert] for 30 minutes. After that, we left the tubes for 30 min in the darkness. Finally, we measured the optical density at 490 nm against the white using a UV/VIS

spectrophotometer. The concentration of total sugar for each sample determined according to standard range prepared by known concentrations of glucose.

1.1.3. Microalgae lipids analysis

The total lipids were extracted according to Bligh and Dyer method with some modifications (Bligh and Dyer 1959). 100 mg of microalgae dry biomass was added by the following proportions Water/Methanol ($\geq 99.8\%$)/Chloroform (99-99.4%): 1/1/2 (v/v/v) separately. Samples were subsequently placed in an ultrasound bath (40 kHz; 80 W) for 15 min. Thereafter, the samples were centrifuged at 4500 rpm and 4 °C for 10 min. The organic phase contained chloroform was recuperated. We repeated the last step by adding 4 mL of chloroform. We washed the lipids by NaCl 0.9% in a separating funnel. Finally, we evaporated the solvent by nitrogen gas and lipids weighted and kept it at -20 °C for until analysis.

Fatty acid methyl esters (FAME) are obtained via a simple and transesterification process. Therefore, 2 mL of methanol, sulfuric acid 6% were added separately to the lipids extracts. Then, we heated the mixture for 2 hours at 90 °C, sonicated for 1 hour and evaporated by nitrogen gas. Thereafter, we added chloroform and water 2:1 (v/v) for separate the mixture into a separating funnel. We recovered the organic phase that contains chloroform in vials and characterized by gas chromatography (GC) (Agilent 7890A Series GC) coupled to mass spectrometry (MS) equipped with multimode injector and 5 ms column with dimension of 30 m 250 mm 0.25 mm and electron impact ionization.

1.2. Nutrients removal efficiency

The concentration of (TN) and (TP) was measured by taken 50 mL of culture media supernatant for flow ionic analysis by Sakalar scan⁺⁺ system to determine the nutrients removal capacity of microalgae.

Nutrients removal efficiency was calculated as follows:

$$\text{Nutrients removal efficiency (\%)} = (C_i - C_f) / C_i \times 100$$

where C_i is the initial and C_f is the final concentration of target nutrients.

1.3. Statistical analysis

Statistical analyses were performed by SPSS and R software's. Descriptive statistics, correlation and significant differences among the mean values were determined using One-way

ANOVA with a post-hoc Tukey's and duncun's tests at a significant level of 0.05 using SPSS (IBM SPSS statistics 22). Data are presented as means \pm standard deviation of three replicates. For the whole variation of biochemical and metabolic parameters determination, we used Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA). For visualization of the relationship between the variable and treatment, we used the heatmap and hierarchical classification with Euclidean distance matrix as similarity measure and ward link as clustering algorithm using the prcomp function and MixOmics package where each variable was centered by subtracting from the (mean=True), but not by the standard deviation (scale=False). The results visualized in the form of dendrogram and heatmap.

RESULTS AND DISCUSSION

1. Microalgae growth and biomass production

Microalgae strains were grown in DWW and control (BG11) media to select the best and robust candidate that has potential to tolerate DWW and results are presented in Figure 7.a. It can be observed that all strains have shown no significant difference in growth rate between media. Moreover, the highest growth rate reached by *S. obliquus* by 0.114 day^{-1} in BG11 against 0.104 day^{-1} in WW. In accordance, several studies shown that microalgae have the ability to grow well in WW (Mishra and Mohanty 2019; Zhu et al. 2019). Thus, microalgae has the potential and ability to assimilate the nutrients from wastewater and convert them to biomass and organic compounds (Behl et al. 2020).

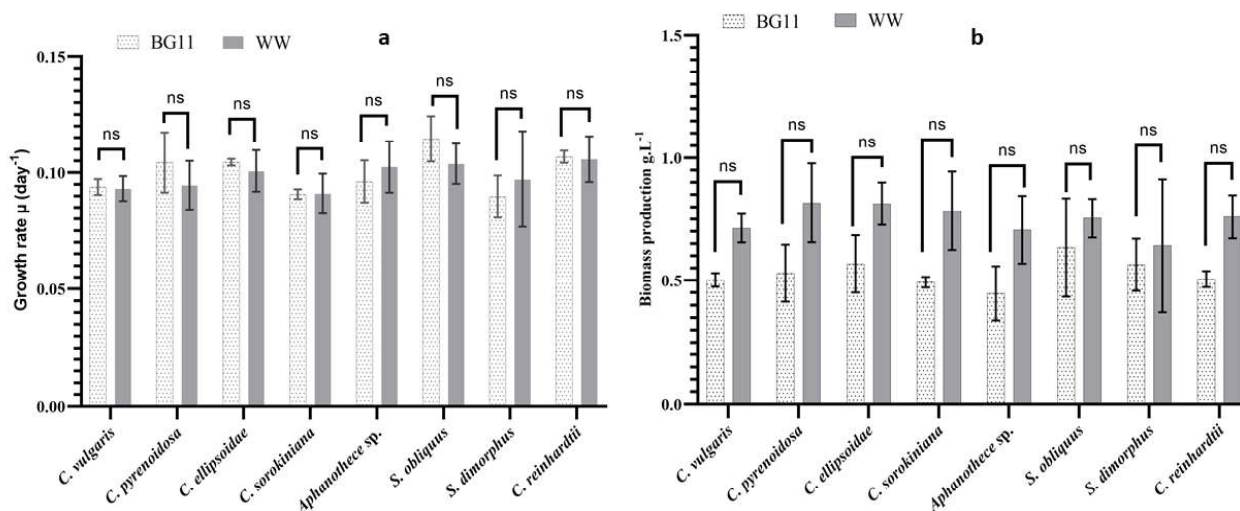


Figure 7: Growth rate (a) and Biomass produced (b) by microalgae strains after 26 days of culture in BG11 and wastewater media under 145 rpm of shaking, 25 °C and continuous illumination of 6000 lux. The error bars indicate the standard deviations from three independent samples. “ns” showed insignificant differences ($p < 0.05$) between BG11 and WW media in each strain according to a Turkey, one-way ANOVA.

Microalgae growth in WW can affect the biomass production, their biochemical composition and valuable compounds productivity (Daneshvar et al. 2019). In the present study, the harvested biomass of microalgae strains cultivated in wastewater was higher than the cultured in BG11 (Figure 7.b). For all studied strains, biomass production fluctuated between 0.64 g L⁻¹ and 0.81 g L⁻¹ in wastewater medium and between 0.44 g L⁻¹ and 0.63 g L⁻¹ in BG11. The highest production was reached by *C. pyrenoidosa* and *C. ellipsoidae* in WW medium by 0.82 g L⁻¹ while *S. obliquus* shown the highest production in BG11 by 0.63 g L⁻¹. These trends are in accordance with Tripathi et al. (2019) study wherein 1.81 ± 0.3 g.L⁻¹ was recorded as maximum of biomass production by *Scenedesmus* sp. ISTGA1 cultured in wastewater compared to control medium (BG11) with 1.0 ± 0.1 g.L⁻¹ after 14 days of culture (Tripathi et al. 2019). The increase of biomass production in WW compared with synthetic medium (BG11) may be attributed to the mixotrophic mode of cultivation in wastewater (Trivedi et al. 2019). Mixotrophy as mode of culture induced enhancement in biomass production have been reported

by several studies (Li et al. 2019; Trivedi et al. 2019). Kabir et al. (2020) reported that *C. vulgaris* and *S. obliquus* were higher biomass producer in WW under mixotrophy mode with the values of $1.81 \pm 0.03 \text{ g L}^{-1}$ and $1.86 \pm 0.1 \text{ g L}^{-1}$ (Kabir et al. 2020). In addition, a maximum of biomass produced by *C. vulgaris* (0.72 g L^{-1}) in raw sewage under mixotrophic conditions (Ge et al. 2018). In consequence, wastewater is used as nutrients source to achieve cost-effective cultivation of microalgae for biorefinery applications (Li et al. 2019; Yadav et al. 2019).

Additionally, there is a negative correlation between biomass productivity and nutrients content in treated wastewater. It's very significant correlation at level 0.01 with -0.887^{**} and -0.889^{**} for nitrogen and phosphorus respectively (Table 5). Indicating nitrogen and phosphorus contents could affect directly the biomass productivity. When nitrogen and phosphorus concentration decreased from WW, microalgae biomass productivity increased. These finding confirms the hypothesis suggested previously of microalgae used WW nutrients for their growth and biomass productivity. The findings of our study shown that wastewater medium (composition in table 4) enhance microalgae strains growth and improving their biomass production.

2. Biochemical characterization of microalgae biomass

At the end of the experiments of microalgae culture, the proteins, carbohydrates and lipids contents of biomass were determined. These are the main components of any microalgae cells. The variation in the biomass composition of microalgae strains used in the present study is shown in Figure 8. The most of studied microalgae showed an increase of lipids content accompanied with decrease of proteins and carbohydrates content in WW medium.

C. sorokiniana showed the highest rate of lipids in WW achieving 36.93% against 15.36% in BG11. While, a little increase in carbohydrates content in WW with 7.53% compared with 6.42% in BG11. Decreased protein content of 8.57% for WW cultivation against 13.66% in BG11 was observed. Microalgae under stress have the ability to accumulate more lipids and carbohydrates to high-reduced state as storage compounds and were stocked in cells for the microalgae survival (Ansari et al. 2017; Malvis et al. 2019). In accordance to our results, Ansari et al. (2017) reported that lipid contents of *S. obliquus*, *C. sorokiniana* and *A. falcatus* were 25%, 26% and 25.20% respectively in synthetic medium. While, increased to 30.85%, 31.85% and 35.9% respectively when using aquaculture wastewater (AWW) as culture medium. Conversely to our finding, carbohydrates contents for *S. obliquus*, *C. sorokiniana* and *A.*

falcatus were 32.53%, 29.74% and 29.40% respectively when cultured in BG11 medium. While, increased to 35.05% for *S. obliquus*, 35.43% for *C. sorokiniana* and 33.88% for *A. falcatus* when cultivated in AWW medium. In agreement to our results, proteins content of *S. obliquus*, *C. sorokiniana* and *A. falcatus* cultivated in BG11 medium were 26.16%, 33.26% and 36.94% respectively. While, decreased to 19.52%, 28.81% and 30.59% respectively in AWW medium (Ansari et al. 2017).

The biochemical composition of microalgal biomass generated in wastewater medium is regulated by the nutrients source and composition (Molinuevo-Salces et al. 2016). Lipids content of *C. vulgaris*, *S. obliquus* and *C. reinhardtii* cultured in piggery wastewater (PWW) was varied between 35% and 40%, much higher than determined in synthetic medium 19%. The carbohydrate content of these microalgae in PWW were ranged between 35% - 40%, were much better in comparison to values found in synthetic medium went to 22.4%. They found that proteins content was further increased to 23%-25% in PWW besides 58.1% noted in synthetic medium (Molinuevo-Salces et al. 2016). In fact, it can be concluded that lipids accumulation in microalgae biomass was ruled to stress condition using WW as medium with low nitrogen concentration (18.35 mg L^{-1}) compared to BG11 (1094.9 mg L^{-1}). Chokshi et al. (2016) reported that lipids content 25.05% of *Acutodesmus dimorphus* cultured in DWW for 8 days was higher in comparison to BG11 medium 21.85. Indeed, they concluded that the biomass of *A. dimorphus* grown in DWW can be used as a biofuel feedstock as well as animal feed (Chokshi et al. 2016). In addition, Silambarasan et al. (2021) reported that the lipids content of consortium (*Chlorella* sp. + *Scenedesmus* sp.) grown in 75% wastewater was 34.83 %, which significantly higher than individual algal strains with 22.34–29.50% and 23.86–30.0% respectively. Moreover, the enhancement of lipids content in WW due to their deficiency in TN ($14.85\text{--}61.47 \text{ mg L}^{-1}$) compared to BG11 (1094.9 mg L^{-1}) (Silambarasan et al., 2021).

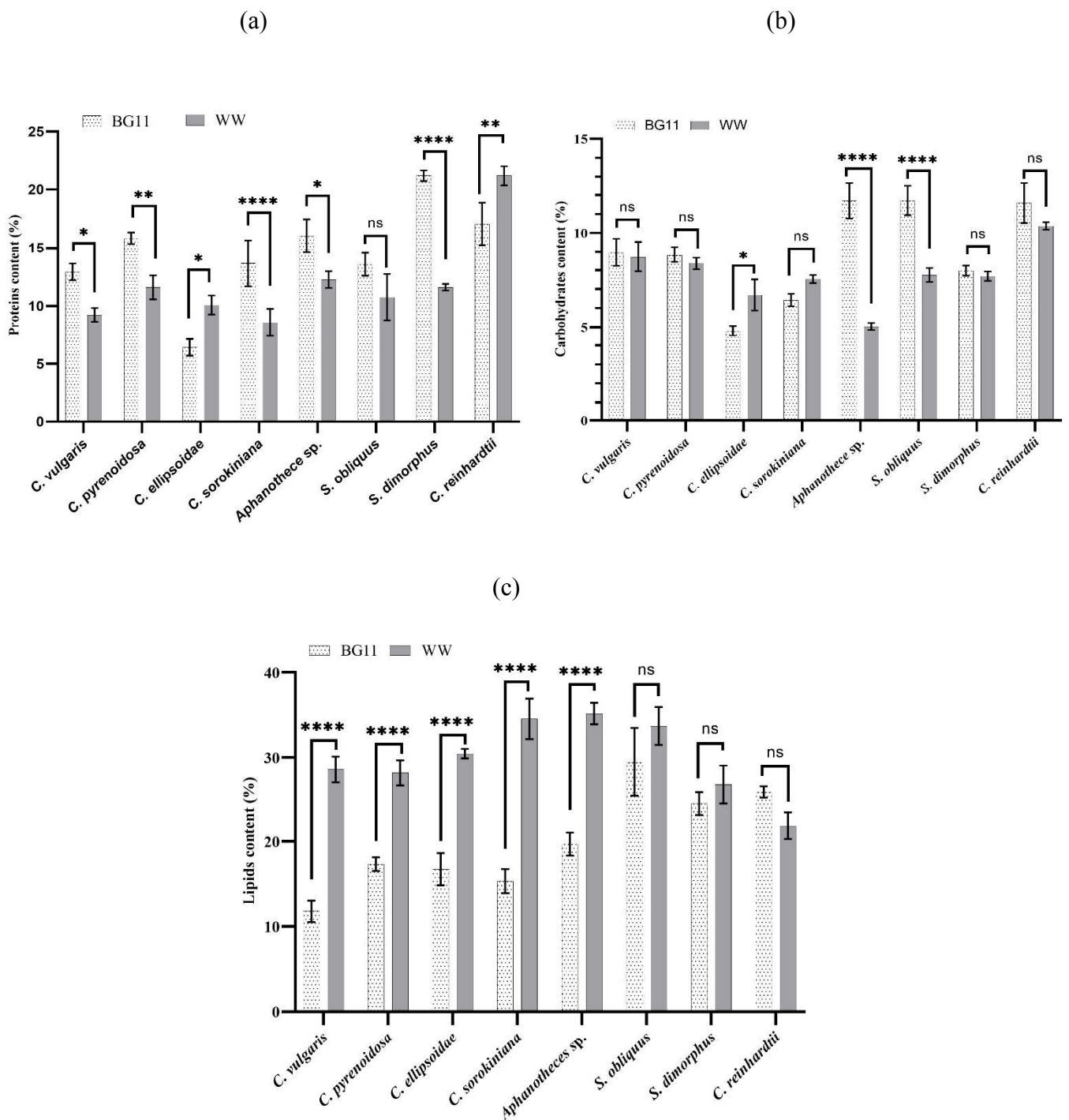


Figure 8: Proteins content (a); carbohydrates content (b) and lipids content (c) in microalgae which was cultured in BG11 and WW media under 145 rpm of shaking, 25 °C and continuous illumination of 6000 lux. The error bars indicate the standard deviations from three independent samples. “ns” showed insignificant differences, while “*” indicates statistical significance ($P < 0.05$); “***” indicates statistical significance ($P < 0.01$); “****” indicates statistical significance ($P=0$) between BG11 and WW media in each strain according to a Turkey, one-way ANOVA.

To understand the relationship between macromolecules accumulation in microalgae biomass, some previous studies shown there are an accumulation of carbohydrates and proteins in the log phase of culture (Wang et al. 2019). However, there is an accumulation of lipids in stationary phase for all the treatments (Wang et al. 2019). In the other hand, some earlier studies found that the accumulation of lipids in microalgae biomass besides carbohydrates and proteins was regulated mostly with stress conditions (illumination, Nutrients limitation, salinity) (Shen et al. 2015; Rinna et al. 2017; Hemalatha et al., 2019).

Moreover, a positive correlation was found between biomass productivity and proteins; carbohydrates and lipids content with 0.641^{**}; 0.792^{**}; 0.889^{**} respectively and significant at level 0.01 (table 5). Nevertheless, there is a negative correlation between nitrogen and phosphorus content in wastewater and biochemical compounds production in studied microalgae. Thus, the nutrients assimilation could impact also biochemical composition of microalgae. In the present study, we found more accumulation of lipids besides proteins and carbohydrates by studied microalgae strains after wastewater remediation. Therefore, it can be concluded that wastewater improved our strains capacity for biomass and lipids production. Finally, *C. sorokiniana*, *Aphanothece* sp. and *S. obliquus* were the species with the greater aptitude to be used as feedstock of lipids from urban wastewater source.

Table 5: Correlation between biomass production, their biochemical characterization and nutrients removal

		Nitrogen	Phosphorus	Biomass	Lipids	Carbohydrates	Proteins
Nitrogen	Pearson Correlation	1	1,000**	-0.887**	-0.913**	-0.865**	-0.725**
	Sig. (2-tailed)		0000	0.000	0.000	0.000	0.000
	N	27	27	27	27	27	27
Phosphorus	Pearson Correlation	1,000**	1	-0.889**	-0.911**	-0.868**	-0.731**
	Sig. (2-tailed)	0.000		0.000	0.000	0.000	0.000
	N	27	27	27	27	27	27
Biomass	Pearson Correlation	-0.887**	-0.889**	1	0.851**	0.792**	0.641**
	Sig. (2-tailed)	0.000	0.000		0.000	0.000	0.000
	N	27	27	27	27	27	27
Lipids	Pearson Correlation	-0.913**	-0.911**	0.851**	1	0.646**	0.477*
	Sig. (2-tailed)	0.000	0.000	0.000		0.000	0.012
	N	27	27	27	27	27	27
Carbohydrates	Pearson Correlation	-0.865**	-0.868**	0.792**	0.646**	1	0.794**
	Sig. (2-tailed)	0.000	0.000	0.000	0.000		0.000
	N	27	27	27	27	27	27
Proteins	Pearson Correlation	-0.725**	-0.731**	0.641**	0.477*	0.794**	1
	Sig. (2-tailed)	0.000	0.000	0.000	0.012	0.000	
	N	27	27	27	27	27	27

** . Correlation is significant at the level 0.01 (2-tailed).

* . Correlation is significant at the level 0.05 (2-tailed).

3. Fatty acids methyl ester profile of microalgae lipids

The FAME profile presented significant difference among microalgae strains and media used in this study (table 6). Palmitic acid C16:0, stearic acid C18:0 and oleic acid C18:1 constituted the dominant fatty acids in the microalgae. Moreover, *C. vulgaris* presented the maximum of stearic and palmitic acids content in wastewater by 9.73% and 13.33% respectively compared to BG11 medium with 25.88 % and 14.63% respectively. Indeed *C. ellipsoidae* and *C. sorokiniana* showed the maximum of oleic acid in WW medium by 28.96% and 28.95% respectively compared to BG11 (0% and 0% respectively). Saturated fatty acids constituted the major fatty acid in microalgae strains and ranging between 24.07% and 45.03%. Monounsaturated fatty acids (MUFAs) were the second most dominant type of fatty acid recorded ranging among 0% and 29.49%. Finally, polyunsaturated fatty acids were present in small quantity ranging among 0% and 4.14%.

Table 6: Fatty acids methyl ester profile of lipids extracted from microalgae strains growing in WW and BG11 media

	<i>C. vulgaris</i>		<i>C. pyrinoidosa</i>		<i>C. ellipsoidae</i>		<i>C. sorokiniana</i>		<i>Aphanothece</i> <i>sp.</i>		<i>S. obliquus</i>		<i>S. dimorphus</i>		<i>C. reinhardtii</i>	
	BG11	WW	BG11	WW	BG11	WW	BG11	WW	BG11	WW	BG11	WW	BG11	WW	BG11	WW
C12:0	-	-	-	-	-	-	-	-	-	-	-	-	0.08	-	-	-
C14:0	0.63	0.62	0.44	0.67	0.45	0.61	0.42	0.47	0.39	0.45	0.42	0.68	0.42	0.47	0.43	0.51
C15:0	1.07	0.78	0.63	1.88	0.78	1.62	0.98	1.61	1.33	1.55	0.67	2.36	1.87	2.44	1.1	1.75
C16:0	14.63	13.3	8.48	13.2	9.29	11.9	8.43	12.09	9.36	9.77	10.84	12	9.16	11.11	-	9.37
C17:0	0.48	0.81	-	-	-	-	-	-	-	-	0.36	-	-	-	-	-
C18:0	25.88	9.73	15.83	11.1	15.98	11.89	17.8	12.33	15.47	15.97	22.54	14	14.97	16.27	19.53	17.25
C19:0	-	0.1	-	0.1	0.12	0.14	-	-	-	-	-	-	-	-	-	0.16
C20:0	1.43	0.63	0.84	0.86	0.83	0.78	0.82	0.8	0.82	0.92	1.62	0.94	0.69	0.7	0.99	0.86
C22:0	0.67	0.83	0.45	0.43	0.44	0.44	0.41	0.46	0.45	0.5	-	0.61	0.38	0.52	0.49	0.72
C24:0	-	0.28	0.49	0.29	0.38	0.28	0.3	0.27	0.34	0.35	0.35	0.61	0.21	0.67	0.56	0.51
C26:0	-	-	-	-	-	-	-	-	-	0.49	-	0.75	-	0.59	-	-
C28:0	0.24	0.65	0.51	0.54	0.68	0.59	0.48	0.48	1.14	1.14	1.7	1.39	0.87	1.12	0.97	0.8
C30:0	-	-	-	-	-	-	-	-	0.46	-	0.68	0.26	0.41	0.26	-	-
∑ SFA	45.03	27.73	27.67	29.07	28.95	28.25	29.64	28.51	29.76	31.14	39.18	33.6	29.06	34.15	24.07	31.93
C16:1	-	-	-	-	-	-	-	-	-	-	-	1.46	-	-	8.71	-
C18:1	3.84	27.44	16.9	28.72	-	28.96	-	28.95	20.94	21.2	4.4	21.95	-	24.14	-	17.31
C20:1		0.55		0.51	-	0.53	-	0.52	0.25	0.29	-	0.67	-	0.24	-	0.23
∑ MUFA	3.84	27.99	16.9	29.23	-	29.49	-	29.47	21.19	21.49	4.4	24.08	-	24.38	8.71	17.54
C16:3	-	5.73	4.62	6.34	4.97	6.2	4.77	6.37	5.72	5.85	-	3.71	5.38	4.32	4.21	4.23
C18:3	-	-	-	1.08	17.04		15.71	-	-	-	-	-	18.39	-	13.77	1.83
C16:4	-	1.62	1.56	1.8	-	2.08	1.44	1.82		1.77	-	1.73	-	-	-	-
C18:4	-	-	2.58	-	3.18	1.28	2.53	1.66	2.23	2.17	-	1.33	2.63	1.98	1.85	-
∑ PUFA ≥4	-	1.62	4.14	1.8	3.18	3.36	3.97	3.48	2.23	3.94	-	3.06	2.63	1.98	1.85	-
unknown FAMES	3.24	1.04	0.85	1.9	0.57	0.86	0.41	1.18	0.52	1.06	0.82	0.75	0.65	1.45	0.56	0.07
Total FAME	52.11	64.11	54.18	69.42	54.71	68.16	54.5	69.01	59.42	63.48	44.4	65.2	56.11	66.28	53.17	55.6

When microalgae strains compared on FA composition, two major groups were identified by cluster analysis (Figure 9 a. b). The first cluster aggregate all studied microalgae strains cultured in BG11 in addition to *C. sorokiniana* in WW. The second cluster gathers the studied microalgae strains when cultured in WW medium. The difference between both culture groups resulted in a clear separation of two different cluster clouds. That confirmed the result suggested by the phylogenetic tree with two major groups (Figure 9 b). One includes microalgae strains cultured in BG11 and the second contain the strains cultured in WW medium with a difference of 35.5% in the first axis and 24.1% in the second axis. In the PCA plotting, the two axis explained 59.6% of total variation in microalgae fatty acid composition (Figure 9 c). The first

axis showed the effect of variations on FA mostly associated with our strains cultured in WW in the right side of diagram. In the left side of diagram was associated with the strains cultured in BG11. Finally, the microalgae strains *C. vulgaris* and *S. obliquus* cultured in BG11 appeared isolated in the bottom of PCA diagram.

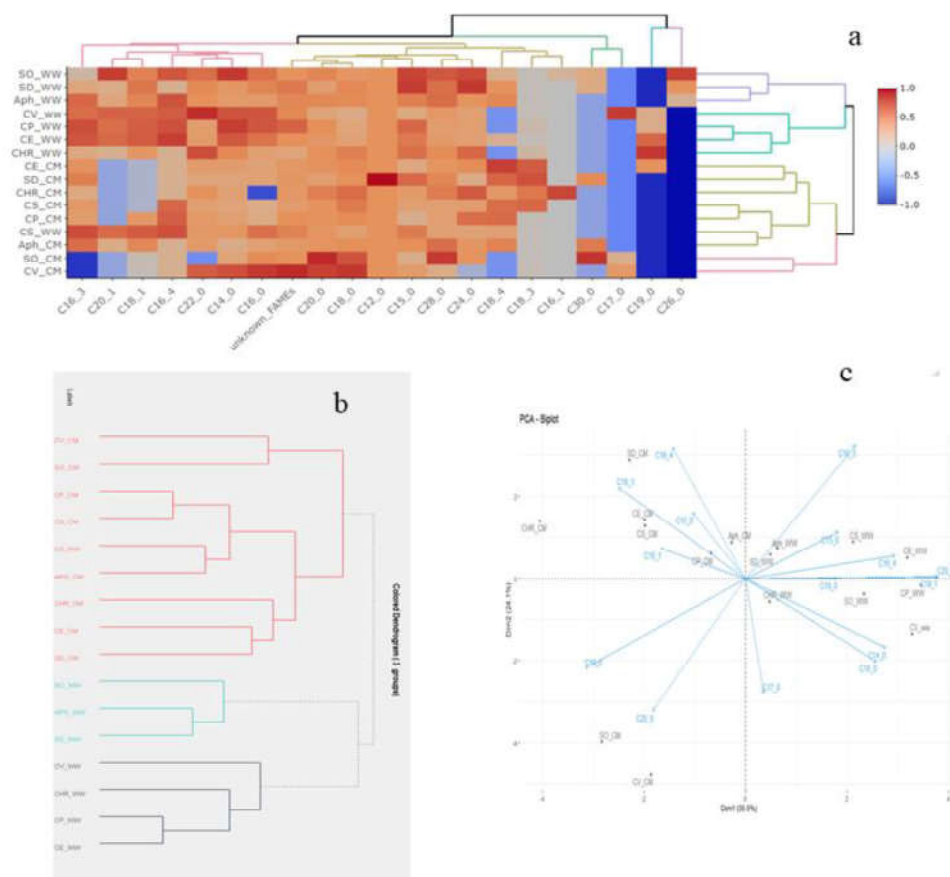


Figure 9: Distribution of microalgae strains based on fatty acids richness (a) Heat mapping; (b) Cluster analysis of fatty acids richness (c): Principle component analysis of the variables and scores for the cultures (PCA – Biplot visualization). CV: *Chlorella vulgaris*; CP: *Chlorella pyrenoidosa*; CE: *Chlorella ellipsoidea*; CS: *Chlorella sorokiniana*; Aph: *Aphanothece* sp.; SO: *Scenedesmus obliquus*; SD: *Scenedesmus dimorphus*; CHR: *Chlamydomons reinhardtii*.

Microalgae lipids represent the biodiesel feedstock. They can be converted to FAMES by transesterification (Li et al. 2020). FAMES constitute the main compound of biodiesel and are very important in evaluation of biodiesel properties (Daneshvar et al. 2019). In the present study, FAMES profile presented a rather difference among microalgae strains and culture media and ranged from C12 to C30. All strains presented abundance on palmitic C16:0, oleic C18:1 and stearic acids C18:0 in both media. *C. ellipsoidea* and *C. sorokiniana* showed a maximum

of stearic acid in BG11 medium by 28.96% and 28.95% respectively. Indeed, *C. vulgaris* presented a maximum of stearic and palmitic acids in wastewater by 25.88% and 14.63% respectively. Rinna et al. (2017) reported that the FAME composition was represented by the fatty acids with the chain-length from 4 to 24 carbon atoms (Rinna et al. 2017). Additionally, the most abundant fatty acids were palmitic and stearic acids in the microalgae lipids under all conditions (Fernández-Linares et al. 2017; Daneshvar et al. 2019; Hernández-García et al. 2019; Malvis et al. 2019). They were common with oleaginous plant-crops, and represented the most suitable fatty acids for biodiesel production (Ji et al. 2015; Ge et al. 2018).

All microalgae strains used in this study showed a total of FAMES in wastewater medium superior to BG11. The maximum FAMES content noted in *C. pyrenoidosa* with (69.42%) in WW compared to BG11 (54.11%). Moreover, we noted the appearance of some FAMES in wastewater that absent in BG11 such as C16:4, C16:3, C19:0, C20:1 and C24:0 in *C. Vulgaris* and C16:0, C18:1, C19:1 and C20:1 in *C. reinhardtii*. Similar, Shen et al. (2014) reported that FAMES of *C. vulgaris* was enhanced from 74% in synthetic medium to 84% under phosphorus and nitrogen deficiency conditions (Shen et al. 2014). Palmitic acid was the fatty acid with the highest concentration in microalgal cells cultured in WW, followed by oleic acid. The consortium *Chlorella* sp. + *Scenedesmus* sp. produced more palmitic acid (44.38–51.75%) and oleic acid (31.11–35.45%) as compared to individual consortium algal strains (Silambarasan et al. 2021).

The fatty acids dominant in *S. quadricauda* and *T. suecica* cultured in BBM and F/2 media respectively were in respect, C16:0 (46.43%) and C18:3n-3 (49.61%) (Daneshvar et al. 2019). The oleic acid percentage of *S. quadricaudan* increased from BBM medium 10.96% to 32.13% and 52.26% for the first and second cycle of mixotrophic cultivation and 29.16% for the heterotrophic cultivation in DWW respectively. However, a significant decrease of palmitic acid from 46.43% in BBM medium to 25.99%, 24.48% for the first and second cycle of mixotrophic cultivation and 13.82% for the heterotrophic cultivation in DWW respectively (Daneshvar et al. 2019). The most abundant fatty acids of *P. tricornutum* were palmitoleic acid (C16:1, 40.33–43.63% in total) and palmitic acid (C16:0, 29.47–37.80%), followed by C14:0 (8.28–11.38%), C20:2 (3.61–7.44%), and C16:2 (2.20–3.55%) in both mixed wastewater and F/2 media (Wang et al. 2019). Tripathi et al. (2019), reported that wastewater can be used as microalgae culture medium to enhance biomass, lipids and FAMES yields. The FAME content of *Scenedesmus* sp. *ISTGAI* demonstrated 90.1% and 95.5% in BG-11 and WW respectively (Tripathi et al. 2019). This FAME distributed between saturated (45.4%) and unsaturated fatty

acids (50.1%) with palmitic acid (23%) and stearic acid (22.4%) were the dominant of the total saturated fatty acid in in case of WW (Tripathi et al. 2019).

According to European standards of biodiesel quality, the common composition of fatty acids required ranges between C16 and C18 chain length (Rai and Gupta 2017). To attain a good biodiesel with less risk of combustion and ignition interruption, it's recommended to have high percentage of saturated fatty acids and less of polyunsaturated fatty acids. They are suitable for higher cetane number and better oxidative stability (Fernández-Linares et al. 2017; Hernández-García et al. 2019; Wang et al., 2019).

In the present study, C16 and C18 carbons chain length were the predominant of the total FAME composition. As example *C. sorokiniana* showed a C16-C18 percentage of 63.22% in wastewater medium besides 50.68% in BG11. The second parameter of biodiesel quality that is verified is linolenic acid (C18:3) percentage that is required below 12% (Rutz and Janssen 2006; European Standard EN 14214 2009; Rai and Gupta 2017). Our results showed a percentage of linolenic acid (C18:3) inferior to 12% ruling out *C. reinhardtii* (13.77%), *S. dimorphus* (18.39%), *C. sorokiniana* (15.71%) and *C. ellipsoidosa* (17.04%) in BG11. Generally, a high content of PUFA is not desirable for biodiesel quality due to its susceptibility by free radical attack (Rai and Gupta 2017). According to European standards EN 14214 (2009), the PUFA should not be increased by 1%. For this reason, three candidates can be retained from the eight strains tested. They were *C. reinhardtii* in wastewater, *S. obliquus* and *C. vulgaris* that show 0% on PUFAs in BG11. Finally, the FAME profile evaluated *C. reinhardtii* as a suitable candidate for biodiesel production using WW as culture medium. For the others strains with low concentration ranged between 1.62 % and 4.14 %. Even superior than 1% it can be fixed and enhanced by hydrogenation, culture methods and others processes (Ge et al. 2018). Hence, the low PUFA content, high oleic and palmitic acid compositions indicate that the FAME obtained from WW culture may satisfy the fuel specifications in these three parameters. In agreement, *C. vulgaris* even grown under mixotrophic conditions has high levels on C16:0 and C18:1 as the major fatty acids compounds (Ge et al. 2018). As these produce a biodiesel with a high cetane number, but still the suitable fatty acids for a best biodiesel with high quality (Ge et al. 2018). Generally, high levels of PUFAs are unsuitable to biodiesel production because they have more double bonds that difficult for biological degradation (Xu et al., 2020). Ge et al. (2018), mentioned that is necessarily to correct the PUFAs percentage by a partial catalytic hydrogenation (Ge et al. 2018). In the present study, the eight Moroccan microalgae strains cultured in WW showed an abundance in saturated fatty acid especially C16-

C18 with less PUFA. In fact, the FAME obtain from WW culture are more appropriate for biodiesel production according to European standard EN 14214 (European Standard EN 14214. 2009). According to this standard, *C. reihardtii* cultured in wastewater, *S. obliquus* and *C. vulgaris* in BG11 qualified the suitable strains for biodiesel production for this study.

To our knowledge, we noted for the first time in microalgae, the appearance of a very long chain fatty acid (melissic or tricantanoic acid) C30:0. We recorded 0.46% of C30:0 in *Aphanothece* sp. cultivated in BG11, 0.68%-0.26% in *S. obliquus* cultivated in BG11 and WW media respectively and 0.41%-0.26% in *S. dimorphus* in BG11 and WW media respectively. This melissic acid has important industrial significance application as surfactant, emulsifier and additive of industrial bitumen (Barreto and Gonzalez Leo 2014). Indeed, it's used as additive for formation of several new cyclodextrin structures which may be used as polymer compounds like soaps or detergents, plasticizers for various resins (Stanley et al. 1971). It can be used also to enhance nutritional value and clinical application (Shukla et al. 2018).

When microalgae strains compared on FA richness, two major groups were identified by cluster analysis. The first cluster includes all strains in BG11 added by *C. Sorokiniana* in WW. The second cluster contains our strains cultured in WW medium. WW medium enriched microalgae biomass in fatty acids. Similarly, The FA composition distribution using a hierarchical cluster analysis shown that the both culture groups deviate from each other within the majority of parameters studied (Sandmann et al. 2018). In addition, the difference noted in FAME profiles among strains used to recover the distribution of different FA among Phyla, classes and genera of microalgae. Therefore, it has been found some difference in FA patterns for groups at lower taxonomic rank, between species of the same genus or even among multiples isolates of the same species (Jeong et al. 2011).

Francisco et al. (2010) showed a multivariation distribution of Fatty acids based on unsaturated degree between six microalgae species and five vegetable sources. The dendrogram of unsaturated degree parameter clearly showed two separate clusters with the first cluster group (sunflower, soybean, peanut, rapeseed and *D. tertiolecta*) and the second one (*P. tricornutum*, palm, *S. obliquus*, *Phormidium* sp., *C. vulgaris* and *A. microscopica N`ageli*). There was also the formation of subclusters distributed as: subcluster 1 (soybean and sunflower), subcluster 2 (peanut, rapeseed and *D. tertiolecta*), subcluster 3 (*P. tricornutum*) and subcluster 4 (palm, *S. obliquus*, *Phormidium* sp., *C. vulgaris* and *A. microscopica N`ageli*) (Francisco et al. 2010).

Component analysis helps to visualize differences between the control and the test applied in the cultures within the combined dataset (Sandmann et al. 2018). The difference between both culture groups resulted in a clear separation of two different cluster clouds (Sandmann et al. 2018). That confirms the result suggested by the phylogenetic tree with two major groups. One includes the strains cultured in BG11 and the second contain the strains cultured in WW medium with a difference of 35.5% in the first axis and 24.1% in the second axis. In the PCA plotting, the two-axis explained 59.6% of total variation in microalgae fatty acid composition. The first axis showed the effect of variations on FA mostly associated with our strains cultured in WW in the right side of diagram. The left side of diagram associated the strains cultured in BG11. The microalgae strains *C. vulgaris* and *S. obliquus* cultured in BG11 appeared isolated in the bottom of PCA diagram and were associated with the different composition of FA types; in particular, the absence of PUFA (0%). Accordingly to Nascimento et al. (2013) the comparison among algae species based on the level of saturation and unsaturation FA, identified three groups by cluster analysis. They showed that the total of variation in algae fatty acid was 100% explained in two-axis. The first one presented the effect of MUFA variations, generally linked with *D. brasiliensis*, *P. subcapitata*, *C microporum* and *B. terribilis*. Increases in saturated FA were noted in the left side of diagram and included *Chlamydomonas* sp., *S. obliquus*, and *C. vulgaris*. The PUFA composition was increased and associated with the second axis. But the decrease of PUFA composition frequently was related to the bottom part of diagram (Nascimento et al. 2013). In conclusion, WW enhances microalgae strains richness in Fatty acids with the abundance of palmitic, stearic and oleic acids. Moreover, microalgae cultivated in WW has also less PUFA content and qualified suitable for biodiesel production according to European standards.

4. Nutrients removal and wastewater treatment efficiency

Wastewater treatment efficiency was studied in this study by following the nitrogen and phosphorus recovery. The result presented in (Figure 10) show that all studied microalgae species widely assimilated the N and P from WW medium. Nitrogen is one of the most important nutrients for microalgae growth and has a crucial role in protein, nucleic acid and phospholipids synthesis (Daneshvar et al. 2019). Although, nitrogen in wastewater is present in the form of NH_4^+ (ammonia), NO_2^- (nitrite) and NO_3^- (nitrate). The removal of nitrogen conducted by several pathway including microalgal assimilation and nitrification processes by nitrifies (Daneshvar et al. 2019; Behl et al. 2020; Li et al. 2020). Therefore, the studied microalgae showed an efficiency up than 99% of nitrogen removal (Figure 10). The total

nitrogen present in WW medium was found reduce from 18.35 mg L⁻¹ to 0.17 mg L⁻¹. As well, the highest performance for N elimination noted in *C. reinhardtii* and *C. pyrenoidosa*. These results are found comparable with previous studies where 90-100% of TN recovered from WW (Daneshvar et al. 2019; Mishra and Mohanty 2019). In addition, Li et al. (2020) reported that attained a removal efficiency of NH⁴⁺-N above 90% using *Desmodesmus* sp. (Li et al. 2020).

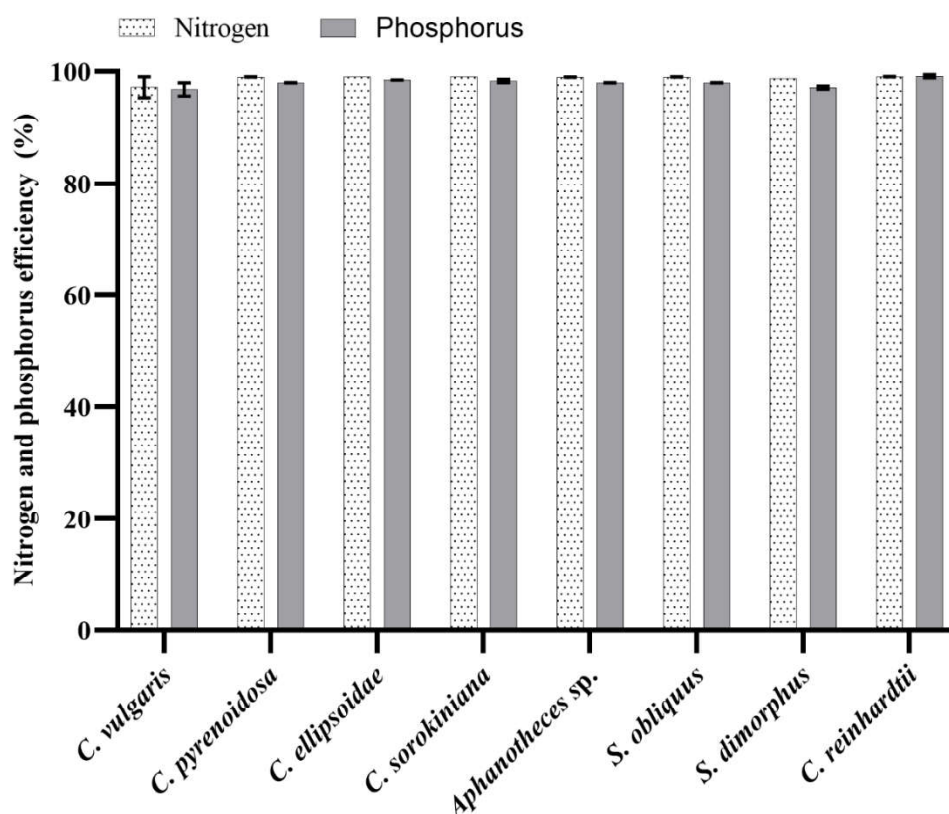


Figure 10: Nitrogen and phosphorus removal efficiency from wastewater medium by microalgae strains after 26 days of culture under 145 rpm of shaking, 25 °C and continuous illumination of 6000 lux. The error bars indicate the standard deviations from three independent samples. Based in statistical analysis there is no significant difference between strains for N and P removal efficiency.

The phosphorus played a pivotal role in microalgae metabolism processes such as ATP, phospholipids and nucleic acids (Mishra and Mohanty 2019). Even though, the direct discharge of phosphorus into water body without incomplete treatment may cause eutrophication that still leads to huge environmental and economic losses every year (Maroušek et al. 2020). Furthermore, orthophosphate is the preferable form of phosphate for microalgae (Mehtar et al. 2019). The removal of phosphorus from WW by microalgae can be carried out by assimilation across the plasma membrane via phosphorylation process using active transport (Mehtar et al. 2019). Moreover, microalgae has an advantageous character is the storage of excess phosphate

in their volutin granules cell via a luxury uptake pathway (Suvidha Gupta et al. 2019; Bahman et al. 2020). These reserves can be used for prolonged growth of microalgal cell (Thakur et al. 2020). Consequently, all microalgae strains used in the present study showed more than 99% of phosphate removal efficiency (Figure 10). The total phosphorus present in WW was found reduce from 1.96 mg L⁻¹ to 0.02 mg L⁻¹. Moreover, *C. reinhardtii* showed the highest performance for T and P elimination. These results are found to be in accordance with previous studies where 90-100% of phosphate removal was achieved (Chen et al. 2018; Daneshvar et al. 2019; Mishra and Mohanty 2019). In other study, the removal efficiency of PO₄³⁻-P reached approximatively 100% (Li et al. 2020).

Microalgae studied strains have shown a very significant potential for remediation of wastewater and the production of a lipids-rich biomass which could be used in biodiesel production. In addition, a positive correlation was found between nitrogen and phosphorus assimilation by microalgae strains used in the present study. This correlation is highly significant at level 0.01 (1.000**) (table 5). Indicating that nitrogen and phosphorus were assimilates together one depend to other not separately. In agreement, Beuckels et al. (2015) reported that *Chlorella* and *Scenedesmus* have a potential in adjusting the concentration of N and P in their biomass and the supply of the both nutrients in wastewater. Even with variable loading of N and P, they can achieve an important simultaneous removal of both N and P in wastewater. Indeed, they shown that the uptake of P was depended on the supply of P and N together not just P supply and vice versa for N (Beuckels et al. 2015). In respect of phycoremediation ability, all microalgae strains used in this study were successfully achieved nutrients removal from wastewater according to Directive 98/15/CE with 80% and 70-80 % removal for total phosphorus and nitrogen respectively.

CONCLUSION

Our study reveals the aptitude of microalgae for combination between wastewater treatment and biodiesel production. Our results showed a high potential for removal of nutrients that meets the standard of European directives for water quality. Additionally, wastewater was appropriate for microalgae growth generating an important biomass yielded 0.82 g L^{-1} by *C. pyrenoidosa* and *C. ellipsoidae*. WW also enhances the biochemical composition by accumulation of more lipids besides proteins and carbohydrates. Therefore, *C. sorokiniana*, *Aphanothece* sp. and *S. obliquus* yielded the highest lipids content with 36.93%, 35.16% and 33.7% respectively suggesting their possible use as feedstock of lipids from urban wastewater source. The microalgae strains showed abundance in saturated fatty acid, especially palmitic and linolenic acids, with less PUFA for both BG11 and WW culture media with remarkable superiority in WW. This kind of FAME is the most suitable for biodiesel production. According to FAMES profile, *C. reihardtii*, *S. obliquus* and *C. vulgaris* strains qualified as candidates for biodiesel production according to European standard EN 14214. Hydrogenation could be used to reduce PUFA from other strains. Other studies would be necessary to validate these results in the pilot scale. Microalgae strains used for this study qualified the combination between wastewater treatment and biodiesel production. As perspectives of our study, the aptitude of these microalgae strains in urban wastewater can be combined also with the biosequestration of CO_2 for industrial gaseous effluents treatment, and the production of biodiesel and high added-value can be valorized in a biorefinery approach.

II. Synthèse

Des souches de microalgues ont été cultivées dans les eaux usées domestiques et le milieu conventionnel (BG11) en tant que témoin pour sélectionner les meilleures candidates qui sont capables à tolérer et traiter efficacement les eaux usées. Par conséquent, toutes les souches testées ont montré une bonne croissance dans les eaux usées avec un potentiel d'assimilation des nutriments N, P et de la matière organique (Figure 10). Elles ont montré une efficacité d'assimilation supérieure à 99% pour les deux nutriments majeurs N et P qui sont responsables de la pollution d'eau et l'eutrophisation, toute en accordance avec les Directive 98/15/CE avec 80% et 70-80% d'élimination pour le P et le N total, respectivement. Ces résultats s'avèrent conformes avec des études précédentes qui ont montré l'aptitude des microalgues dans l'assimilation d'N et P à partir des eaux usées avec une efficacité qui dépasse 90% et arrive même à 100% (Daneshvar *et al.* 2019; Mishra and Mohanty 2019; Behl *et al.* 2020; Li *et al.* 2020).

La plupart des microalgues étudiées ont montré une augmentation de la teneur en lipides accompagnée d'une diminution de la teneur en protéines et en glucides dans les E.U. Nos résultats sont en cohérence avec plusieurs études précédentes qui rapportent que les microalgues cultivées dans les eaux usées ont accumulé des teneurs élevées en lipides et en sucres par rapport au milieu conventionnel (Chokshi *et al.* 2016; Hemalatha *et al.*, 2019; Wang *et al.* 2019; Silambarasan *et al.*, 2021). En effet, les microalgues sous stress ont la capacité d'accumuler plus les lipides et les sucres comme molécules de stockage qui assurent leur survie et leur résistance au stress (Ansari *et al.*, 2017; Malvis *et al.*, 2019). Ces lipides ont l'objet d'une conversion en FAMES par trans-estérification.

Le profil lipidique des FAMES a présenté une différence significative entre les souches de microalgues et les milieux de culture utilisés. Les acides palmitiques, stéariques et oléiques constituaient les acides gras dominants chez les microalgues surtout dans les E.U. En accord, plusieurs études ont rapporté que les acides palmitiques et stéariques sont les plus abondant dans les microalgues sous toutes les conditions (Fernández-Linares *et al.*, 2017; Daneshvar *et al.*, 2019; Hernández-García *et al.*, 2019; Malvis *et al.*, 2019). En effet, ce genre des FAMES représente les acides gras les plus appropriés pour le biodiesel (Daneshvar *et al.*, 2019; Li *et al.*, 2020). Selon les normes Européennes, pour avoir un bon biodiesel avec moins de risque de combustion et interruption dans l'inflammation, il est recommandé d'avoir un pourcentage élevé des acides gras saturés et moins d'acides gras polyinsaturés (European Standard EN

14214, 2009; Fernández-Linares *et al.* 2017; Hernández-García *et al.* 2019; Wang *et al.*, 2019). Par ailleurs, les PUFAs ne doivent pas dépasser 1 %. Pour cette raison, *C. reinhardtii* a été retenu comme la candidate appropriée pour la production du biodiesel en utilisant les E.U comme milieu de culture. Pour les autres souches, le pourcentage des PUFAs peut être régulé et amélioré par hydrogénation, méthodes de culture et autres processus (Ge *et al.* 2018).

III. Conclusion

Notre étude révèle l'aptitude des microalgues dans la combinaison entre le traitement des eaux usées et la production du biodiesel. Nos résultats ont montré un fort potentiel d'élimination des nutriments surtout N et P conformes aux normes des directives Européennes pour la qualité d'eau. De plus, les eaux usées étaient appropriées pour la croissance de microalgues générant une productivité élevée en biomasse et lipides. Les souches de microalgues ont montré une abondance en acides gras saturés, en particulier les acides, palmitique et linoléique, avec moins de PUFA surtout dans les eaux usées. En effet, ce type de FA est le plus approprié pour la production du biodiesel. Après analyse de profile lipidique et en accordance avec les standards Européen (EN 14214), *C. reinhardtii* est qualifiée comme candidate pour la production du biodiesel à parti des eaux usées. Par ailleurs, d'autres études seraient nécessaires pour valider ces résultats à l'échelle pilote. La combinaison avec autres types de stress paraît possible et faisable comme la culture des microalgues pour le traitement des eaux usées et la séquestration du CO₂ avec valorisation de la biomasse dans une démarche de bioraffinerie.

Dans ce chapitre les souches des microalgues utilisées ont montré leur potentiel pour la combinaison entre le traitement des eaux usées et la production du biodiesel. Dans le cadre de notre thèse nous suivons l'approche de bioraffinerie afin de bénéficier de tous les bienfaits de la biomasse des microalgues. En effet, après extraction des lipides qui ont été convertis en biodiesel par trans-estérification, les résidus qui restent ont l'objet pour le développement d'un extrait brut qui aura utilisé comme biostimulant des plantes pour enlever le stress métallique.



Chapitre III

CHAPITRE III: EFFET DES EXTRAITS BRUTES DES MICROALGUES EN TANT QUE BIOSTIMULANT CONTRE LE STRESS DES METAUX LOURDS CHEZ LES PLANTES DE TOMATE

I. Introduction

La contamination des sols par les métaux lourds constitue une menace sérieuse pour les organismes vivants, y compris les plantes, les animaux et les êtres humains (Malecka et al., 2021). Les métaux sont naturellement présents dans l'environnement, mais leur prévalence est augmentée par les activités anthropiques (Nigam et al., 2021). Les métaux non essentiels tels que Pb et Cd, qui sont toxiques, affectent négativement la croissance et le développement des plantes en altérant les processus physiologiques et biochimiques (Meena *et al.*, 2018; AbdElgawad *et al.*, 2020). Généralement, les métaux lourds induisent la génération des espèces réactives de l'oxygène (ROS) entraînant un déséquilibre redox et une peroxydation des lipides (Jamla *et al.*, 2021). Tout en provoquant un effet novice sur les fonctions ultra structurales, physiologiques et biochimiques des plantes (Huang *et al.*, 2019; Khare *et al.*, 2022). En réponse, les plantes déploient leur système antioxydant comprenant des molécules antioxydantes tels que: la proline, les polyphénols, le glutathion, l'ascorbate, les bêtaïnes etc. et des enzymes antioxydants comme superoxyde dismutase (SOD), catalase (CAT), peroxydase (POD), ascorbate peroxydase (APX), glutathion réductase (GR) et déhydroascorbate réductase (DHAR) (Cherif *et al.*, 2011; Jamla *et al.*, 2021).

De nombreuses méthodes ont été utilisées pour remédier les sols contaminés par les H.M, par exemple le lavage des sols, l'excavation etc. Cependant, la plupart de ces outils sont coûteux ou n'offrent pas une solution permanente (Bhandari et al., 2007). Récemment, l'utilisation des biostimulants a montré une amélioration de la tolérance des plantes à travers l'activation du système antioxydant de ces dernières face aux différents stressés (Nigam et al., 2021). Le biostimulant des plantes est défini comme "une formulation des produits d'origine biologique qui améliorent la productivité des plantes en raison de nouvelles propriétés ou émergentes d'un complexe des constituants, et non comme une seule conséquence de la présence d'éléments nutritifs essentiels pour les plantes, des régulateurs de la croissance des plantes, ou composés phytosanitaires." (Yakhin et al., 2017). Les composés humiques (acide humique, acides fulviques et humines), les extraits d'algues, les hydrolysats des protéines (peptides de signalisation et acides aminés libres) et les microbes (bactéries, levures, champignons filamenteux et microalgues) sont les types les plus courants des biostimulants des plantes

(Bulgari *et al.*, 2019). Parmi ces catégories, les algues se sont révélées efficaces pour la création des biostimulants des plantes. Cependant, les microalgues et les cyanobactéries ont reçu plus d'attention dans ce domaine au cours des dernières décennies (Mutale-joan *et al.*, 2020).

Les microalgues (eucaryotes) et les cyanobactéries (procaryotes) sont un groupe potentiellement diversifié, omniprésent et dominant d'organismes photosynthétiques dans la nature (Tomaselli, 2004; Andersen, 2013). Ils ont une grande variété de domaines d'application, y compris l'alimentation, l'alimentation animale, l'aquaculture, la médecine, l'agriculture, la bioénergie, l'environnement et l'application aussi aux domaines cosmo-pharmaceutiques, etc. (Cardozo *et al.*, 2007). Par ailleurs, de nombreuses études ont montré le potentiel des microalgues pour améliorer la tolérance aux stress abiotiques et biotiques chez les plantes. L'extrait des microalgues-cyanobactéries a amélioré le système antioxydant de la plante de tomate et de l'absorption des nutriments sous stress salin (Mutale-joan *et al.*, 2021). De plus, Kusvuran, (2021) a montré que l'extrait de *C. vulgaris* a atténué le stress hydrique en stimulant le système antioxydant et par l'amélioration de l'absorption des nutriments dans la plante de brocoli. Les extraits des microalgues en tant que biostimulant agissent comme des substances stimulant la croissance, qui peuvent fournir des substances bioactives telles que des acides aminés, des vitamines, des acides gras, des glucides, des polyamines, des bêtaïnes, des phytohormones, en particulier des cytokinines et des auxines, etc. (Supraja *et al.*, 2020a). Ces molécules bioactives améliorent, la croissance et le développement des plantes, l'absorption des nutriments, le système antioxydant contre les stress abiotiques et biotiques et la productivité des cultures (Kusvuran, 2021; Mazepa *et al.*, 2021). De plus, l'application du biostimulant a montré l'accumulation de plusieurs métabolites d'intérêt impliqués dans les stress abiotiques et biotiques tels que les VLCFA, PUFA, alcanes/alcènes et stérols. Par ailleurs, l'activation de diverses voies impliquées dans la tolérance des plantes aux stress (El Arroussi *et al.*, 2018; Rachidi *et al.*, 2021; Mutale-joan *et al.*, 2021).

Le présent chapitre met en évidence l'effet d'extrait brute des microalgues en tant que biostimulant pour atténuer le stress des métaux lourds chez les plantes de tomates par l'amélioration du système antioxydant, assimilation des nutriments et l'accumulation des métabolites.

Article 1: Microalgae as biostimulant improving antioxidant system and metabolic pathways to alleviate metal stress in tomato plant

Algal Research

Microalgae as biostimulant improving antioxidant system and metabolic pathways to alleviate heavy metals stress in tomato plant

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Microalgae as biostimulant improving antioxidant system and metabolic pathways to alleviate metal stress in tomato plant

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ABSTRACT

Heavy metals (H.M) have harmful impact on plant productivity and pose a serious risk to humans and animals health. In the present study, we investigated the effect of Microalgae Crude Extract (MCE) on growth, antioxidant system, and metabolic responses of tomato plant exposed to 2 mM Pb and Cd. The results showed a significant reduction of tomato plant weights root (32.85%; 27.84%) and shoot (21.05%; 18.50%) and photosynthetic pigments Chl a and Chl b with 55.06% and 58.17% under 2 mM Pb and Cd conditions, respectively. Moreover, MCE treatment showed a significant enhancement of plant biomass compared to plants under Pb and Cd. On the other hand, MCE treatment significantly enhanced antioxidant system in plant under Pb and Cd stress such as scavenging enzymes and molecules: POD (49.16%; 75.01%), CAT (40.22%; 42.58%), SOD (33.91%; 34.16%), proline (29.96%; 47.36%) and polyphenols (8.82%; 5.42%) etc. Furthermore, MCE treatment showed remodulation of metabolic pathways related to plant tolerance such as wax construction mechanism, particularly SFA, UFA, VLCFA, alkanes, alkenes, and sterols biosynthesis to enhance tolerance to H.M stress. In the present study, we emphasized that MCE alleviates heavy metal stress by enhancement of antioxidant system and remodulation of metabolites related to stress tolerance.

Keywords: Antioxidant System, Heavy Metal Stress, Metabolomic analysis, Microalgae Crude Extract, *Solanum lycopersicum* (Tomato).

INTRODUCTION

Soil contamination by heavy metal raises worldwide concerns. Metals are naturally present in environment, but their prevalence are increased by anthropogenic activities (Nigam et al., 2021). Thus, the accumulation of these metals in soil poses a serious threat to living organisms including plants, animals and humans (Małecka et al., 2021). Non-essential metals such as Pb and Cd, which are toxic affect negatively the plant growth and development altering physiological and biochemical processes (Meena *et al.*, 2018; AbdElgawad *et al.*, 2020). Generally, Toxic metal like Pb and Cd trigger Reactive Oxygen Species (ROS) generation leading to redox imbalance and lipid peroxidation (Jamla *et al.*, 2021). While, cause novice effect on ultrastructural, physiological and biochemical function in plants (Huang *et al.*, 2019; Khare *et al.*, 2022). To detoxify H.M effect, plants deploy their antioxidative system including non-enzymatic antioxidants such as: proline, polyphenols, glutathion, ascorbate, betaines etc. and scavenging enzymes like superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR) and dehydroascorbate reductase (DHAR) (Cherif *et al.*, 2011; Jamla *et al.*, 2021).

The activation of plant antioxidative system can be a good strategy to enhance performance in tolerance to stresses (Jamla et al., 2021). Recently, the use of plant biostimulant showed improvement of plants tolerance via antioxidative system activation to different stresses including H.M (Nigam et al., 2021). Plant biostimulant is defined as “a formulated product from a biological origin that improves plant productivity as a consequence of the novel or emergent properties of the complex of constituents, and not as a sole consequence of the presence of known essential plant nutrients, plant growth regulators, or plant protective compounds.” (Yakhin et al., 2017).

Recently, natural biostimulant have gained prominence including those developed from algal biomass. They act as growth stimulating substances, which can provide bioactive substances such as amino acids, vitamins, fatty acids, carbohydrates, polyamines, betaines, phytohormones especially cytokinins and auxins etc (Supraja *et al.*, 2020a). These bioactive molecules enhance plant growth and development, enhance nutrient uptake, improve antioxidant system against abiotic and biotic stresses, and improve crop productivity (Kusvuran, 2021; Mazepa *et al.*, 2021). Furthermore, plants treatment by microalgae extract showed the accumulation of several metabolites implicated in abiotic and biotic stresses such as VLCFA, PUFA, Alkanes, Alkenes, Sterols. Moreover, the activation of various pathways involved in plant tolerance to stress (El

Arroussi *et al.*, 2018; Rachidi *et al.*, 2021; Mutale-joan *et al.*, 2021). The present study aimed to highlight the effect of microalgae extract in heavy metal stress alleviation in tomato plants by improvement of antioxidant system and enhancement of metabolic pathways and metabolites accumulation.

MATERIALS AND METHODS

1. Microalgae culture conditions and growth

The all-microalgae strains studied in this study were maintained in Algal Biotechnology Center at MAScIR. Eight strains (*Aphanothece* sp., *Chlorella vulgaris*, *Chlorella pyrenoidosa*, *Chlorella ellipsoidea*, *Chlorella sorokiniana*, *Scenedesmus obliquus*, *Scenedesmus dimorphus* and *Chlamydomonas reinhardtii*) were grown in BG11 medium (Stanier *et al.*, 1971). The culture performed in triplicates using a 250 mL erlenmeyer flasks containing 150 mL of culture with a initial concentration of OD₆₈₀ 0.1. The cultures were incubated at 25 ± 1 °C, 145 rpm of agitation (VWR Advanced Digital Shaker) at continuous illumination 100 µmol.m⁻².s⁻¹ provided by white fluorescent lamps for 26 days. Microalgae growth was evaluated twice-daily by measuring optical density (OD) at 680 nm using UV/VIS spectrophotometer brand Ultrospec 3100 pro.

2. Microalgae crude extract preparation

1.5 g microalgae dry biomass was ground and suspended in 2% HCl. The mixture was heated for 2 h at 95 °C with stirring, alternated by sonication for 15 min each 30 min and autoclaved at 121 °C for 20 min. The crude extract (composition Annexe S1) was recovered as supernatant by centrifugation for 10 min at 4000 rpm and 4 °C and stored at -20 °C. The pH of supernatant was adjusted to 6.5 by KOH. The effect of Microalgae Crude Extract (MCE) on plants under heavy metals stress (2mM of Pb and Cd) at three concentrations 0.1%, 0.5% and 1% was investigated. Furthermore, the concentration 1% of *Aphanothece* Crude Extract (ACE) was selected based on their positive effect on agronomic parameters (root and shoot length and root and shoot weight) to deepen the studies of the effect of this extract on physiologic, biochemical and metabolomic response of plants under heavy metals stress.

3. Plant material and culture conditions

The seeds of the plant model *Solanum lycopersicum* L. var. JANA were sown on 24 cell trays filled in commercial peat (composition in table Annexe 2) incubated in phytotron with a day/night cycle of 16/8 h at 25 °C, 240 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and 69-72% relative humidity. Uniform plants were selected and used for the present study. The experiment was organized as follow: 1. Control (without treatment), 2. Treated by MCE, 3. Treated with 2 mM Pb NO₃, 4. Treated with 2mM PbNO₃ + MCE, 5. Treated with 2mM Cd (NO₃)₂·4H₂O, 6. Treated with 2 mM Cd (NO₃)₂·4H₂O + MCE. The plants in first stage irrigated by 10 ml of any treatment dissolved in D.W for each plant in cell 3 times in week alternated with DW if necessary. In the last stage when plants were growth and require more water plants were irrigated daily by D.W. The experiment is repeated twice time for confirmation. 40 days after transplantation, the plants were harvested and washed well a repeated time with Deionized Water (DW), and the agronomic and physiologic parameters were evaluated. In addition, just for *Aphanathece* extract we chose to do the experiment in the air space to meet real conditions, which uniform plants were selected and transported into 6 bacs with 10 plants per bac. The experiment was organized as follow: 1. Control (without treatment), 2. Treated by ACE, 3. Treated with 2 mM Pb NO₃, 4. Treated with 2mM PbNO₃ + ACE, 5. Treated with 2mM Cd (NO₃)₂·4H₂O, 6. Treated with 2 mM Cd (NO₃)₂·4H₂O + ACE.

4. Physiological parameters analysis

4.1. Photosynthetic pigments

Photosynthetic pigments extracted and determined according to Xiong *et al.*, (2017) method. 0.1 g of fresh tomato leaves were grinded in azote liquid using a mortar and pestle. The concentration of pigments was calculated according to the following formulae (Lichtenthaler, 1987):

$$\text{Chlorophyll } a \text{ (mg L}^{-1}\text{)} = 16.82 \cdot A665 - 9.28 \cdot A652 \text{ (1)}$$

$$\text{Chlorophyll } b \text{ (mg L}^{-1}\text{)} = 36.92 \cdot A652 - 16.54 \cdot A665 \text{ (2)}$$

$$\text{Carotenoids (mg L}^{-1}\text{)} = (1000 \cdot A470 - 1.91 \cdot Ca - 95.15 \cdot Cb)/225 \text{ (3)}$$

4.2. Heavy metals and mineral nutrients analysis in tomato tissues

0.4 g of each ground sample in triplicates was placed in individual tubes and digested with 2.5 mL of H₂SO₄ at 100°C for 2H, followed by adding 2 mL of 30% hydrogen peroxide (H₂O₂) carefully to each tube to complete digestion at 330°C for 2H. Finally, after the sample solutions

were cooled down, the volume was adjusted to 75 mL, and then filtered. The concentrations of NPK were determined by flow ionic analysis, Sakalar scan++ system at the Green Biotechnology Center, Mascir, Morocco. 0.4 g of dry root and shoot were put it into digestion tube placed on aluminium heating block digester in triplicate. The digestion and mineralization of samples was done according to Gupta et al., (2016) method. Heavy metals and mineral nutrient concentrations were estimated using inductively coupled plasma-optical emission spectroscopy (ICP-OES); (iCAP-7000 Duo, Thermo Fisher Scientific) at the Cereal and Legume Quality Laboratory, ICARDA, Morocco. Bioconcentration factor (BCF) and translocation factor (TF) were used to evaluate the potential of tomato to accumulate H.M within their root and the translocation to the above part. BCF and TF were determined following equation publishes in Baruah et al., (2019):

$BCF = \text{Heavy metal content (mg/kg) in the roots} / \text{Heavy metal content (mg/ kg) in the soil}$ (4)

$TF = \text{Heavy metal content (mg/kg) in a certain above-ground tissue} / \text{Heavy metal content (mg/kg) in the roots}$ (5)

5. Biochemical parameters analysis

5.1. Proteins, Soluble sugar and Polyphenols

Soluble proteins in tomato leaves were extracted according to Fleurence et al., (1995) and determined following Bradford, (1976) method by recording absorbance at 595 nm by Spectra Max Plus Molecular Devices spectrophotometer using Bovine Serum Albumin (BSA) as standard. Total sugar was determined according to phenol-sulfuric method (Dubois et al., 1956). Total phenols were colorimetrically determined using Folin-Ciocalteu reagent as described by (Velioglu et al., 1998).

5.2. Determination of stress biomarkers H₂O₂, MDA and proline content

The content of Hydrogen peroxide (H₂O₂) was determined according to Velikova *et al.*, (2000) method with slight modifications. 0.1 g of fresh tomato leaves was homogenized with 1 mL of 0.1% (w/v) trichloroacetic acid (TCA) on ice and centrifuged at 12, 000 ×g for 15 min. 0.5 mL of the supernatant was mixed with 1 mL of 10 mM potassium phosphate buffer (pH 7) and 1 mL of 1 mM Potassium Iodine (KI). H₂O₂ content was calculated using a standard curve after recorded absorbance at 390 nm. For proline content determination we followed Bates, (1973) method. Malondialdehyde (MDA) content determined according to Heath and Packer, (1968) protocol.

5.3. Scavenging enzymes assays

The enzyme extract was prepared by homogenizing 0.1 g of tomato leaves in 1 mL of 100 mM sodium phosphate buffer (pH 7.8), with 0.1 mM EDTA, 1% (w/v) polyvinyl pyrrolidone (PVP) and 0.5% (v/v) triton X-100. The crude protein concentration in supernatant was estimated by Bradford method using BSA as standard for protein quantification (Bradford, 1976). SOD activity was determined according to Beauchamp and Fridovich, (1971) method. CAT activity was determined according to Aebi, (1974) method. Then, POD activity was measured following guaiacol oxidation method (Kar and Mishra, 1976).

5.4. GC-MS Metabolomic analysis

To explore the tomato plants metabolomic responses under heavy metals (Pb and Cd) stress and MCE treatment, following the described GC–MS method by (Rachidi *et al.*, 2021). Metabolomic analysis carry out by gas chromatography (GC) (Agilent 7890 A Series GC) coupled to mass spectrometry (MS) (Agilent 5975C) equipped with multimode injector and HP-5MS column with dimension of 30 m 250 mm 0.25 mm and electron impact ionization.

6. Statistical analysis:

Statistical analyses were performed using SPSS (IBM SPSS statistics 22). Descriptive statistics and significant differences of the mean values were determined using one-way ANOVA with a post-hoc Tukey's and duncun's tests at a significant level of 0.05. While "*" indicates statistical significance ($P < 0.05$); "***" indicates statistical significance ($P < 0.01$); "****" indicates statistical significance ($P < 0.001$); "*****" indicates statistical significance ($P=0$). Graphs were generated by GraphPad prism 9 software. All experiments in the work were performed in three replicates; the results were represented as arithmetic mean \pm standard deviation.

RESULTS AND DISCUSSION

1. Effect of MCE on plant growth under Pb and Cd stress

In preliminary experiment studies, different concentrations of PbNO_3 and $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (0.1, 0.2, 0.4, 0.8, 1, 1.5 and 2 mM) were set to investigate the changes of morphological symptoms in tomato plant. Interestingly, no remarkable morphologic differences were found among individuals when exposed to low dose of heavy metals treatment (0 to 1 mM) for PbNO_3 and $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, while the plants were seriously hampered and grew abnormally when exposed to 2 mM PbNO_3 and $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (Figure 11). Therefore, we selected the 2 mM PbNO_3 and

Cd (NO₃)₂·4H₂O to study the effect of metal on physiological, biochemical and metabolomic response of tomato plant on heavy metals and in combination with microalgae extracts. Moreover, for Microalgae crude extract three concentration were tested 0.1%, 0.5% and 1 %. 1% showed a positive effect and well recovered plants under Pb and Cd stress. Furthermore, based on agronomic results *Aphanothece* extract as a cyanobacteria against Pb and Cd stress, *C. vulgaris* extract against Pb and *C. pyrenoidosa* against Cd stress showed a high positive effect under metallic stress. In this regard, these extracts were selected for physiological biochemical and metabolic studies.

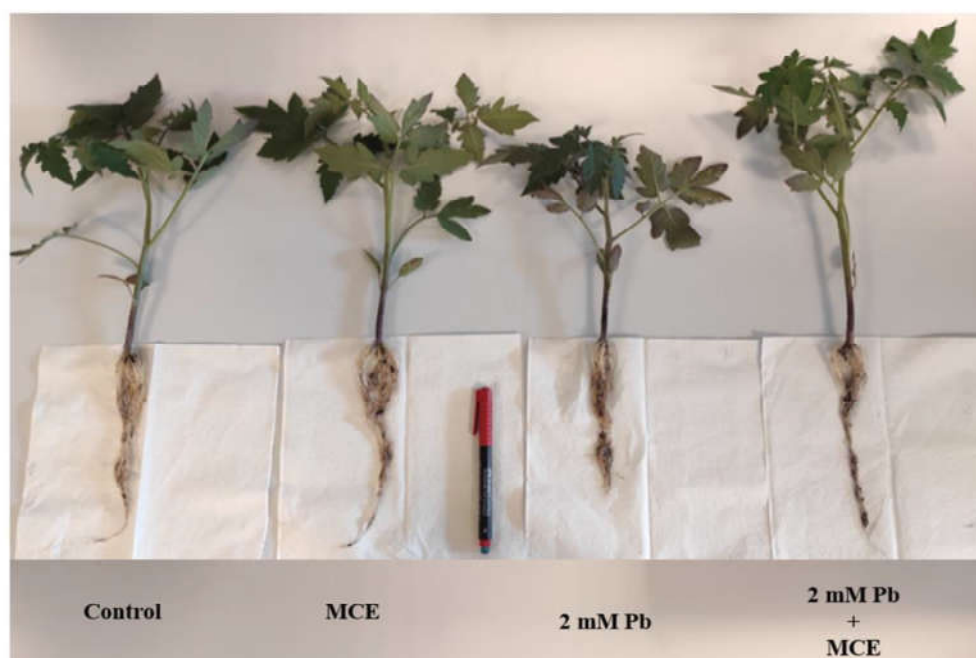


Figure 11: Effect of MCE on agronomic parameters of tomato plants under Pb stress.

Growth inhibition is the most prominent effect of H.M on plants (Kısa *et al.*, 2016; Baruah *et al.*, 2019). Under 2 mM Pb and Cd, tomato plants showed significant reduction of root weight (32.85%; 27.84%) and shoot (21.05%; 18.50%) compared to control (Figure 12 A, B, C and D). In accordance with our results, *Pisum sativum* L. exposition to 0.9 mM of Pb showed reduction of root and shoot dry weight by 0.58 g and 1.48 g compared to control with 0.89 g and 2.25 g, respectively (Galal *et al.*, 2021). In addition, tomato plants showed significant reduction in shoot and root weight by 36% and 51.61%, respectively in hydroponic culture exposed to 0.025 mM of Cd and 232 ppm of potassium (K) (Naciri *et al.*, 2021). Roots are the

first part of plant in contact with H.M thus, in response to H.M stress plants reduce the number of lateral roots, root elongation and root hair density in the order to limit H.M uptake (Feng *et al.*, 2021). Moreover, the reduction of intracellular space and the strong binding between pectin molecules in cell wall may be the cause of growth reduction. E.g. Cd incorporate the lignin to cell-wall, which increase cell-wall rigidity and reduce expansion (Pál *et al.*, 2006). MCE supplementation to plant under Pb and Cd stress showed a significant enhancement of tomato root and shoot weight (Figure 12). Moreover, the highest improvement of plant weight under Pb stress noted in *Aphanothece* sp. by 102.86 % and 30.27% in root and shoot, respectively. Followed by *C. vulgaris* by 93.68% and 72% in root and shoot, respectively. For Cd, the highest amelioration noted in *Aphanothece* sp. by 90.51% in root and 66.78% in shoot. Followed by *C. Pyrenoidosa* with 86.52% in root and 66.78% in shoot. Thus, we selected ACE to alleviate Pb and Cd stresses, *C. vulgaris* for Pb and *C. Pyrenoidosa* for Cd stress. In the light of this point, the use of microalgae as biostimulant showed positive effect on plant growth and yield by reducing stress and restoring previous damages (Ronga *et al.*, 2019). The use of seaweed extract (SWE) was reported to alleviate H.M stress on tomato plant growth. Under 6 mM Pb, SWE enhanced the dry weight of root, stem, leaves and whole of tomato plant with 7.01%, 9.71%, 13.91% and 11.79%, respectively compared to control. Moreover, SWE treatment of tomato plants exposed to 1.6 mM Cd showed significant enhancements of the dry weight of root, stem, leaves and whole of tomato plant with 32.55%, 13.09%, 42.10% and 31.90%, respectively (Abd El-All *et al.*, 2021). The stimulation effect of microalgae extract was assimilated to the presence of plant growth promoting substances such as: macro-and micro nutrients, amino acids, fatty acids, polysaccharides, phytohormones etc., which can affect cellular physiology of plants enhancing plant growth and productivity (Ronga *et al.*, 2019; Behera *et al.*, 2021; Ferreira *et al.*, 2021; Li *et al.*, 2021).

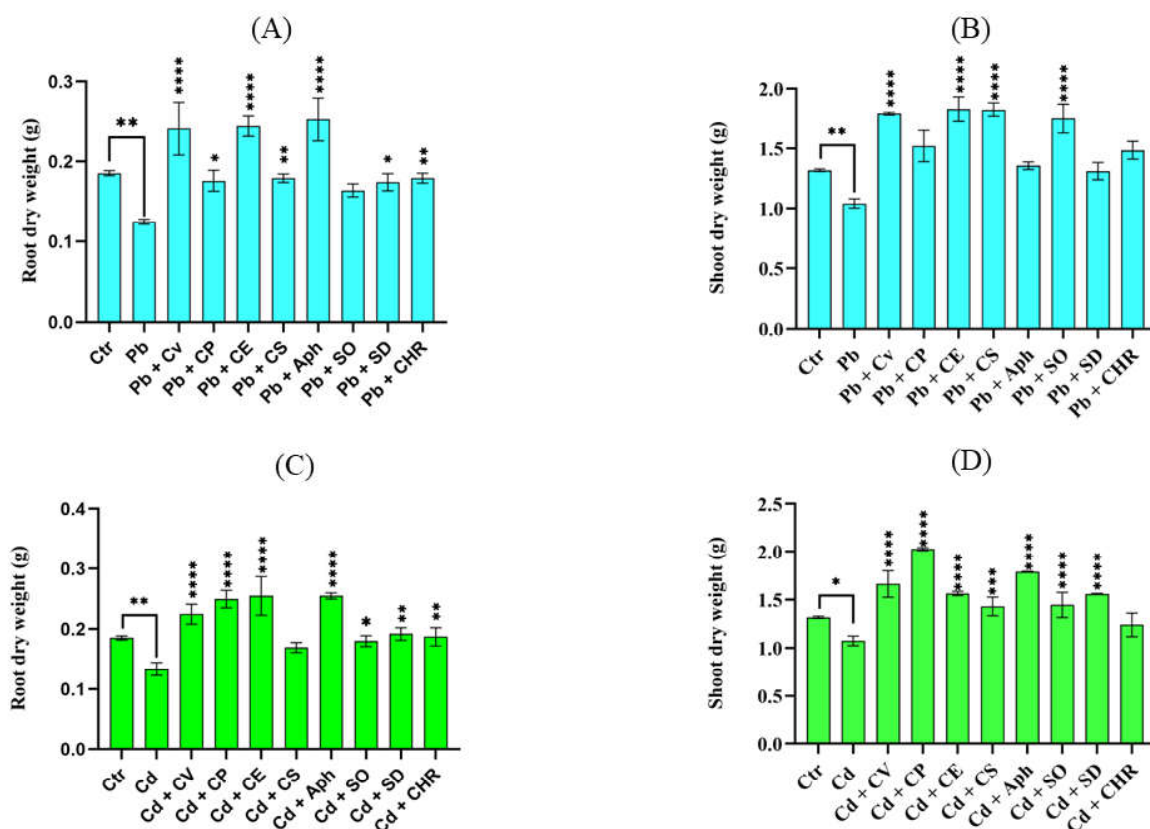


Figure 12: Effect of MCE on tomato root (A, C) and shoot (B, D) dry matter under Pb and Cd stress. CV: *Chlorella vulgaris*, CP: *Chlorella pyrenoidosa*, CE: *Chlorella ellipsoidae*, CS: *Chlorella sorokiniana*, APH: *Aphanothece* sp., SO: *Scenedesmus obliquus*, SD: *Scenedesmus dimorphus* and CHR: *Chlamydomonas reinhardtii*.

2. Effect of *C. vulgaris* and *C. pyrenoidosa* extracts on photosynthetic pigments under Pb and Cd stress

The effect of H.M on photosynthetic pigments was investigated in the present study (Figure 13). All photosynthetic pigments measured were decreased significantly under 2 mM Pb and Cd. 2mM-Pb stress showed reduction in Chl a, Chl b, total chl, and carotenoid with 55.06%, 58.17%, 56.24% and 38,38%, respectively. In addition, 2 mM Cd showed reduction in the same pigments with 57.95%, 73.25%, 63.45%, and 41.49%, respectively. In accordance, Abd El-All *et al.*, (2021), showed that Pb at 6 mM of soil reduced Chl a, Chl b and carotenoid with 60.51%, 68.25% and 67.80%, respectively. In addition, Cd at 1.6 mM of soil reduced the same pigments with 69.68%, 70.25%, 74.49%, respectively (Abd El-All *et al.*, 2021). Moreover, Cd at 0.025 mM showed significant reduction of Chl a and Chl b content in tomato leaves by 70% and 44.44%, respectively (Siddiqui *et al.*, 2022). Photosynthetic pigments reduction may be related to the inhibition of Chlorophylls biosynthesis and increase in chlorophyllase activity

(Siddiqui *et al.*, 2022). It may be due also to ROS generation and inhibition of essential nutrients uptake for photosynthesis such as Mg^{2+} , K^+ , P, Fe^{2+} , Mn^{2+} (Faizan *et al.*, 2021; Jimenez *et al.*, 2020). Furthermore, 0.6 mM Pb and 1.2 mM Cd reduced significantly Chl stability index in tomato leaves by 55% and 25%, respectively (Baruah *et al.*, 2019). Sarah *et al.*, (2021) related the reduction of total chl content and carotenoids to deficiency on K. Similarly, Naciri *et al.*, (2021), showed that K has an important role in photosynthetic pigments formation and prevents formed photosynthetic pigments degradation. However, *C. vulgaris* and *C. pyrenoidosa* extract supplementation showed significant amelioration of this pigment under 2 mM Pb and Cd. *C. vulgaris* extract significantly enhanced Chl a, Chl b, total Chl and carotenoids under 2 mM Pb with 97.49%, 214.98%, 139.91%, and 2.19%, respectively. In addition, *C. pyrenoidosa* treatment under 2 mM Cd showed significant enhancement of Chl a, total Chl, and carotenoids by 26.62%, 18.69%, and 11.52%, respectively compared to plants under 2 mM Cd and 2 mM Pb. In accordance, SWE spray in leaves under Pb at 6 mM of soil showed significant increase of Chl a, chl b, total Chl and carotenoids content by 6.81%, 0.63%, 4.77%, and 8.69%, respectively. Moreover, foliar application of SWE under Cd at 1.6 mM of soil showed significant augmentation of the same pigments by 8.87%, 6.04%, 20.12%, and 7.81%, respectively (Abd El-All *et al.*, 2021). The cocultivation of tomato plant with mixed consortium of microalgae consisting mainly of *Chlorella* sp., *Scenedesmus* sp., *Synechocystis* sp., and *Spirulina* sp., exhibited ameliorative effect to plant chlorophyll with Chl a (562.5%), Chl b (200%) and carotenoids (266.66%) (Supraja *et al.*, 2020a). Furthermore, microalgae biomass used as organic fertilizer showed a positive effect in photosynthetic pigments contents (Jimenez *et al.*, 2020). The treatment by cellular extract of microalgae at 40% concentration showed enhancement of Chl a, Chl b, and carotenoids with 28.57%, 160% and 100%, respectively (Supraja *et al.*, 2020b).

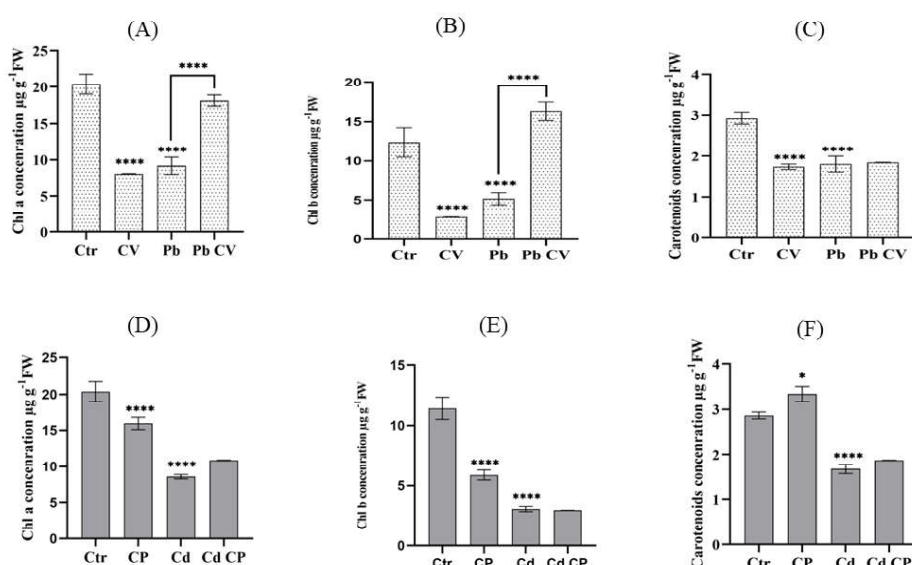


Figure 13: Effect of *C. vulgaris* and *C. pyrenoidosa* extract on tomato leaves photosynthetic pigments Chl a (A, D), Chl b (B, E), and Carotenoids (C, F) under Pb and Cd stress. CV: *Chlorella vulgaris*, CP: *Chlorella pyrenoidosa*.

3. Effect of *C. vulgaris* and *C. pyrenoidosa* extract on biochemical parameters under Pb and Cd stress

In the present study, we investigate the effect of *C. vulgaris* and *C. pyrenoidosa* extract on oxidative stress pathways in tomato plant under metallic stress. 2mM Pb and Cd significantly increased H₂O₂ (60%; 100.88%) and MDA (41.26%; 18.90%) in tomato leaves (Figure 14 A, B, C and D). This elevation was accompanied by accumulation of several molecules which involved in response to stress and to keep cell homeostasis. Indeed, 2 mM Pb and Cd significantly increased proline (67.97%; 67.97%), soluble sugar (19.59%; 22.49%), proteins (27.37%; 48%) and polyphenols (11.64%; 12.46%) (Fig. 15 A, B, C, and D) and (Fig. 16 A, B, C and D). In line of our results, 0.2 mM Cd induced significant increase in H₂O₂ and MDA content by 202.2% and 421.4%, respectively. This oxidative stress in tomato plant was accompanied with elevation of proline content by 89.9% (Rahmatizadeh et al., 2019). Moreover, Cd stress at 0.025 mM significantly increased tomato leaves in H₂O₂ and MDA content by 175% and 75%, respectively (Siddiqui et al., 2022). The growth of soybean at 2.5 mM Pb showed significant increase in MDA by 41.09%, which induce lipid peroxidation. This elevation is followed by starch and proteins content elevation with 16.20% and 30.58% as molecules responses to metal stress (Blanco et al., 2021). In addition, proteins and soluble

sugars were increased in tomato shoot under 0.2 mM Cd, which were 228.1% and 121% , respectively (Rahmatizadeh *et al.*, 2019). Furthermore, Plants used soluble and reduced sugar from starch as source of energy under abiotic stress (Blanco *et al.*, 2021). Moreover, proteins content under Cd stress synthesis might be due to antioxidative activities especially scavenging enzymes (Baruah *et al.*, 2019). 1% MCE from *C. vulgaris* treatment under 2 mM Pb and *C. pyrenoidosa* under 2 mM Cd showed significant reduction in H₂O₂ (14.72%; 28.31%) and MDA (11.34%; 20.78%) compared to tomato plants under 2 mM Pb and Cd, respectively. In addition, *C. vulgaris* and *C. pyrenoidosa* extract showed also significant decrease in proline (29.96%; 47.36%), polyphenols (8.82%; 5.42%), proteins (22.20%; 21.91%) and sugar (2.40%; 16.82%). Previous studies showed the potential of microalgae extract to modulate oxidative stress in plants (Rachidi *et al.*, 2021; Mutale-joan *et al.*, 2021). In addition, selenium application as biostimulant at 0.001 mM under Cd stress reduced proline accumulation in tomato leaves from 105 $\mu\text{mol g}^{-1}$ FW to 67 $\mu\text{mol g}^{-1}$ FW at 0.5 μM CdCl₂ (Alves *et al.*, 2020). Furthermore, microalgae contain antioxidant molecules such ascorbic acid, carotenoids, tocopherols, phycocyanin, and phenolic compounds. Which has an important role in redox homeostasis (El-Baky and El-Baroty, 2013; Chokshi *et al.*, 2017).

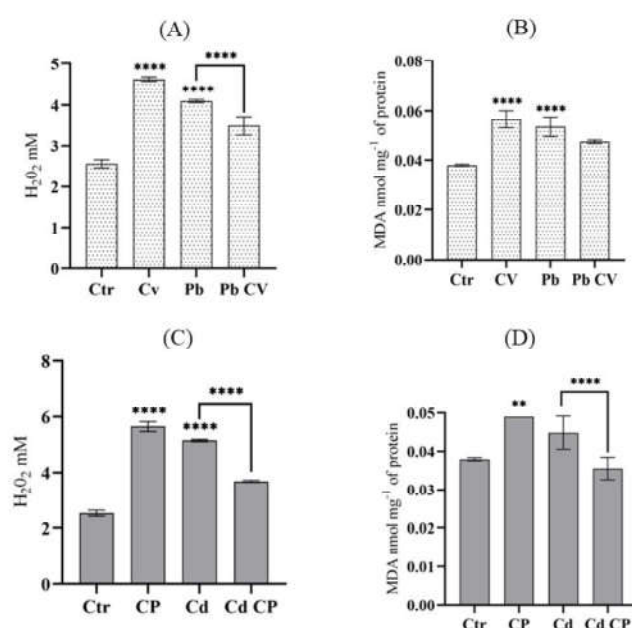


Figure 14: Effect of *C. vulgaris* and *C. pyrenoidosa* extract on H₂O₂ (A, C) and MDA (B, D) in plants exposed to Pb and Cd stress. CV: *Chlorella vulgaris*, CP: *Chlorella pyrenoidosa*.

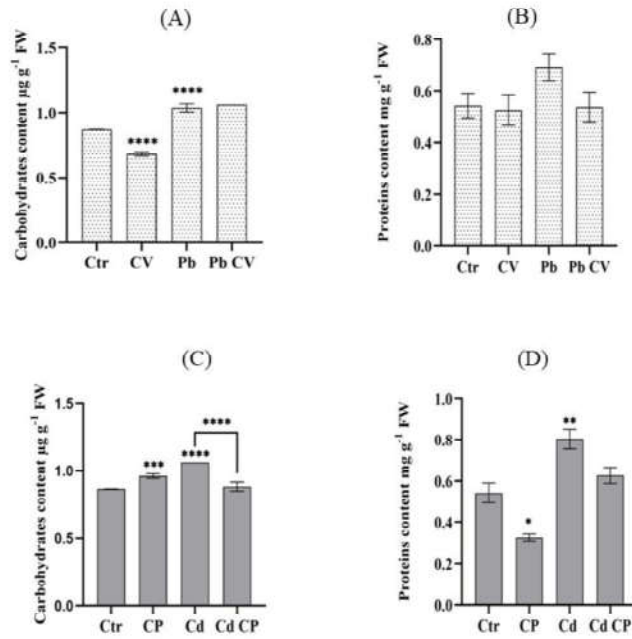


Figure 15: Effect of *C. vulgaris* and *C. pyrenoidosa* extract on proteins (A, C) and soluble sugar (B, D) in plants under Pb and Cd stress. CV: *Chlorella vulgaris*, CP: *Chlorella pyrenoidosa*.

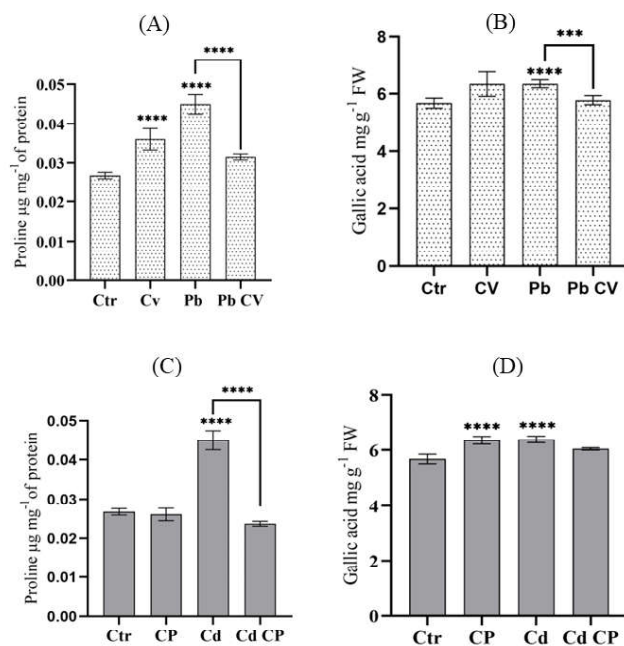


Fig. 16: Effect of *C. vulgaris* and *C. pyrenoidosa* extract on proline (A, C) and polyphenols (B, D) in plants exposed to Pb and Cd stress. CV: *Chlorella vulgaris*, CP: *Chlorella pyrenoidosa*.

Plants use the scavenging enzymes to reduce free radicals under metallic stress (Kumar et al., 2020). In the present study, 2 mM Pb and Cd increased significantly SOD with 68.91%; 53.81%, CAT with 23.64%; 89.37% and POD with 143.50%; 264.30%, respectively (Figure 17 A, B, C, D, E and F). In accordance, 6 mM Pb in soil showed increase of POD by 51.39%, while 1.6 mM Cd in soil showed increase of 70% compared to control (Abd El-All et al., 2021). Moreover, CAT activity in tomato leaves increased by 170.58% and 235.29% at 0.5 mM Pb and 1.2 mM Cd, respectively (Baruah et al., 2019). Siddiqui *et al.*, (2022), showed the increase of SOD activity by 100% under 0.025 mM Cd and other scavenging enzymes (GR, APX, DHA, MDHAR) in the range of 1.7–2.6 fold. SOD is the first line of plant defenses, which dismutase H_2O_2 to H_2O and O (Zhang et al., 2020). The excess of H_2O_2 result from SOD or other free radicals were detoxified by other enzymes CAT, POD, G.R and APX (Alves et al., 2020). On the other hand, *C. vulgaris* extract application under 2 mM Pb showed decrease of SOD, CAT, and POD by 33.91%, 40.22%, and 49.16%, respectively. *C. pyrenoidosa* extract supplementation under Cd reduced SOD, CAT, and POD activities by 34.16%, 42.58%, and 75.01%, respectively (Figure 17). In line with our results, Venkatachalam *et al.*, (2017) reported that the treatment of *Leucaena leucocephala* by zinc oxide nanoparticles (ZnONPs) prepared from *Ulva lactuca* biomass at 25 mg^{-1} under (0.3 mM Pb and 0.27 mM 50 mg L^{-1} Cd stress showed reduction of scavenging enzymes. It showed significant reduction of SOD activity which were 97.4% and 95.7% under Pb and Cd, respectively. While, CAT activity reduced by 90.9% and 85.3% under Pb and Cd, respectively (Venkatachalam et al., 2017). In accordance, foliar spray of SWE to tomato under metallic stress reduced significantly POD activity with 11 % and in result recovered the deleterious effect of H.M (Abd El-All et al., 2021). Moreover, the treatment of tomato plants by Ca (10 mmol L^{-1}), salicylic acid ($100\text{ }\mu\text{mol L}^{-1}$) and epibrassinolide (1 mol L^{-1}) in combination showed reduction of SOD, CAT, and POD by 137.64%, 62.91% and 74.08%, respectively after their elevation under 5 mg L^{-1} of Cd by 151.89%, 155.18% and 99.49%, respectively (Guo et al., 2018). Microalgae biostimulant contains bioactive molecules such as proline, betaines, phytohormones which can regulate plant redox homeostasis leading to stress resistance and tolerance (Ferreira et al., 2021). Indeed, MCE act as bio elicitor, which enhance plant tolerance to metallic stress by activation of antioxidative system.

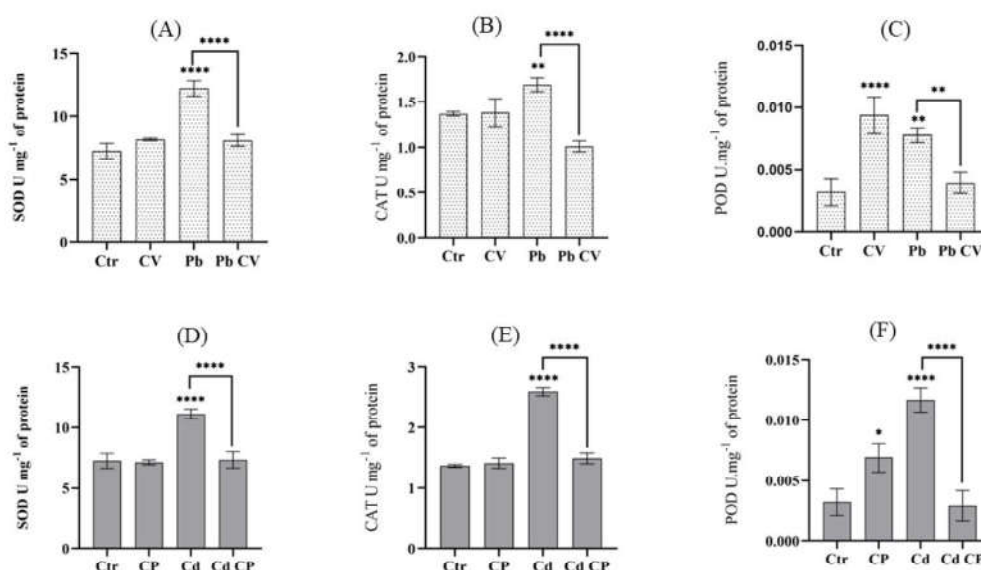


Figure 17: Effect of *C. vulgaris* and *C. pyrenoidosa* extract on scavenging enzymes SOD (A, D), CAT (B, E) and POD (C, F) in plants under Pb and Cd stress. CV: *Chlorella vulgaris*, CP: *Chlorella pyrenoidosa*.

4. Effect of *C. vulgaris* and *C. pyrenoidosa* extract on metabolomics responses of tomato plants under Pb and Cd stress

In this study, we report the effect of MCE on metabolomics changes in tomato responding to heavy metal (Pb or Cd) stress using a GC-MS-based metabolomics approach. 2 mM Pb and Cd showed increase in SFA, PUFA, VLCFA, Alkanes and alkenes, while decreased MUFA especially, C16:1 and C18:1 (table 7). The accumulation of these metabolites acts as plant response to metallic stress to prevent membrane permeability, fluidity, and integrity alteration. 2 mM Pb and Cd showed increase of SFA by 287.27% and 152.15%, respectively such as palmitic, stearic, and arachidonic acids. In accordance, plants treated with Cd showed a significant increase of SFA such as palmitic and stearic acids with 65.11% and 68.25%, respectively (Kumar *et al.*, 2012). Indeed, C16 and C18 FAs are the main precursors of cuticular wax synthesis. In addition, the elongation of these FA to VLCFA, desaturation to PUFA or decarboxylation to form alkanes leading to mature cuticle which has role major in plants defences to abiotic and biotic stress and to prevent water loss (Lim *et al.*, 2017). In addition, 2 mM Pb and Cd showed increase in VLCFA with 102.85% and 32.60%, respectively. Moreover, 2 mM Pb and Cd exhibited increase of PUFA with 163.67% and 1151.11%, respectively. Generally, VLCFA and PUFA are the major component of membranes, which keep their permeability, fluidity, and integrity (Kumar *et al.*, 2012; Kazaz *et al.*, 2022). They can also act

as signalling molecules (He and Ding, 2020; Kazaz *et al.*, 2022). Thus, the increase in these metabolites is a sign of plants response to metallic stress to maintain membrane fluidity and integrity. Furthermore, MCE supplementation showed remodulation of metabolites accumulated under Pb and Cd stress. The treatment with *C. vulgaris* extract showed reduction in VLCF, MUFA, PUFA, Alkanes and Alkenes with 20.49%, 67.67%, 100%, 24.05% and 100% compared to plants exposed to 2 mM Pb (table 7 and 8). Moreover, *C. pyrenidosa* extract supplementation showed reduction of SFA, VLCF, MUFA, PUFA, Alkanes and Alkenes with 99.98%, 99.99.98%, 99.98%, 99.99% and 99.98%, respectively compared to 2 mM Cd (table 7 and 8). In the present study 2 mM Pb and Cd showed significant accumulation of butanedioic acid (malate), citric acid and glutaric acid (table 9). Wang *et al.*, (2015) reported that gluconate, citrate, malate, and oxalate have a central role in tolerance to H.M. Furthermore, MCE enhanced plant tolerance to metallic stress via remodulation of metabolites, especially those related to stress as PUFA, VLCFA, Alkanes, Alkenes and sterols.

Table 7: Effect of *C. vulgaris* and *C. pyrenoidosa* extract on FAMES changes under Pb and Cd stress

Metabolites ($\mu\text{g g}^{-1}$)	Ctrl	Pb	Pb + CV	Cd	Cd + CP	CV	CP
C5:0			53495.515				
C9	47375.293	31622.698	47617.527	25072.957	3.343	0.507	3.572
C10:0			349828.104				
C11	31988.704		16129.239				
C12	6669.917	12486.435		90541.266	1.027		1.672
C14	36453.970	29588.574	33042.548	18468.502	3.093		3.464
C15	29242.785			26109.945	1.611	1.383	256.898
C16:0	187803.440	2643317.759	2443212.271	1781536.524	275.156	84.734	31.629
C17:0	63316.523	118837.736	61897.679				3.943
C18:0	409417.898	583741.903	561975.788	321431.819	78.447	22.436	50.929
C19:0	21079.425						
C20:0	85976.231	140115.004	110495.540	68389.830	9.761	1.983	6.169
C21:0	5313.388	21200.007	19878.782		1.709		
SFA	917967.656	3580910.115	3697572.994	2331550.843	374.146		358.276
C22:0	82087.987	129972.815	68105.306	60267.539	8.712	1.066	4.844
C23:0			22378.379	17041.792	2.194		1.426
C24:0	61499.911	159998.641	127698.690	126520.079	17.317	1.164	8.263
C25:0	15740.599	38884.641	27346.230	13903.330	3.069		
C26:0	22892.840	40780.113	48362.062	23897.844	5.126		3.103
VLCFA	182221.337	369636.210	293890.667	241630.584	36.416		17.636
C16:1	1677293.884	114449.415	119651.457	71909.953	10.466	2.291	
C18:1	39198.356	397373.567	49996.738		2.956	8.891	32.214
C20:1		13001.374					
MUFA	1716492.240	524824.356	169648.195	71909.953	13.421		32.214
C16:2				20644.618			
C16:3				118303.469			
C18:2	143116.526	377367.390		673219.595	5.467	12.975	
C18:3				978376.996		1.841	
PUFA	143116.526	377367.390		1790544.678	5.467		

Ctrl, Control; CV, *C. vulgaris*; CP, *C. Pyrenoidosa*.

Table 8: Effect of *C. vulgaris* and *C. pyrenoidosa* extract on Alkanes, Alkenes and Sterols changes under Pb and Cd stress

Metabolites ($\mu\text{g g}^{-1}$)	Ctr	Pb	Pb + CV	Cd	Cd + CP	CV	CP
Decane		167290.150	33436.202	98508.443	27.895	0.858	
Tridecane		26950.200		8056.357	1.625		
2-Pentadecane			25731.358				
E-14-Hexadecenal			19890.916				
Heptadecanal			8021.989				29.354
Octadecane		28069.224			0.780	2.534	
9,17-Octadecadienal			40653.317				
Eicosane	7701.081	31968.033	373976.153	33212.207	2.950	8.416	22.469
Heneicosane,		18013.793	12867.451	190457.839		0.474	1.313
Tetracosane	174268.972	458244.952	26761.840	70985.852	39.579	13.764	13.289
Hexacosane						1.687	
Heptacosane				16924.013		1.641	2.020
Octacosane		77670.256	32784.357		3.172		
Nonacosane	69563.090	40285.028	54268.052	24494.877	3.855	2.069	
2-Methyltriacontane	170046.312		74569.544	117110.469	11.340	3.460	7.266
2-Methyltriacontane		196174.359	75782.908	61656.209	13.211		6.893
Dotriacontane, 2-methyl-		62976.220	40858.569	40164.172	5.558		4.363
Tetracontane							2.835
Alkanes	421579.455	1107642.214	841268.811	661570.438	109.964		89.804
2-Octene		9174416.883			290.209		762.988
1-Pentadecene	5352.002						
1-Octadecene	13954.504	54505.662					
9-Tricosene,		17002.951					
1-Hexacosene	14520.845						
2-Pentene, 2,3-dimethyl						8.086	
3-Penten-2-one, 4-methyl						98.634	
2-Pentanone, 4-hydroxy-4-methyl-						21.018	
7-Pentadecyne						1.405	
Alkenes	33827.351	9245925.496			290.209	129.144	762.988
16,22-Epoxycholestane			21666.156				
Estra-1,3,5(10)-trien-6-one, 3,1						2.520	
7-Chloro-2,3-dihydro-3-(4-N,N-di						1.037	

Ctr, Control; CV, *C. vulgaris*; CP, *C. Pyrenoidosa*.

Table 9: Effect of *C. vulgaris* and *C. pyrenoidosa* extract on other metabolites under Pb and Cd stress

Metabolites ($\mu\text{g g}^{-1}$)	Ctrl	Pb	Pb + CV	Cd	Cd +CP	CV	CP
Butanedioic acid,	8404.518	30265.504	24459.720				
Succinic acid, 3-methylbutyl 2-n	37872.111					0.853	6.489
Valeric acid, 2,6-dimethylnon-1-	13804.244						
Citric acid, trimethyl ester	38732.247	23734.324	461666.462	42209.639	7.029	3.767	
Benzeneethanamine, N-[(pentafluoroethyl)amino]-	7207.137						
Glutaric acid, isobutyl 5-methoxy						1.576	
Glutaric acid, hex-2-en-1-yl dec.				46684.728			
Benzo[h]quinoline, 2,4-dimethyl	23785.089	69044.519	18183.359		5.578		2.260
1H-Indole-2-carboxylic acid, 6-(1-methyl-2-propyl)-	11936.501	14854.726	6510.006				
2-Ethylbutyric acid, 2,7-dimethyl	15795.085						
Vitamin E	20922.668	46858.320		34366.924	2.364		
4-Chloro-6-methoxy-2-methylquinoline	482851.382						
1H-Pyrazole-1-acetamide, 4-iodo	26531.890						
1,2-Benzenediol, 3,5-bis(1,1-dimethyl-2-propyl)-	6820.789						
Pyrimidine, 4-amino-2-methoxy-5-methyl	210654.588						
4-Ethyl-1,3-thiazole	3640830.235						
Benzenamine, 4-methoxy-N-(2,4-dimethylphenyl)-	109093.726	33542.065	55995.606				
Thiophene-2-thiocarboxamide, 5-(1-methyl-2-propyl)-	18314.319						
6-Chloro-2-(3,4-dimethoxyphenyl)quinoline	80702.214						
1-[3,4-Dichlorophenyl]-3-[4-[[1-(2-propyl)oxy]phenyl]phenyl]piperazine	12560.809						
2(3H)-Furanone, dihydro-5-tetradecyl	212237.088		378668.551	47.516			
1-[3-(Trifluoromethyl)phenyl]piperazine		213404.281					
2-(4-Nitrophenylamino)-4-(N-methyl-2-propyl)pyridine		107646.981	134596.280				
1-[3,4-Dichlorophenyl]-3-[4-[[1-(2-propyl)oxy]phenyl]phenyl]piperazine		13373.073	21302.389				
1-Pyridineacetonitrile, 2-ethyl		20990.593					
Methanethiol, (N-cycloheptylmethyl)-		8730.241					
6,6-Dimethyl-cyclooct-4-enone		31856.970					
4-Chloro-6-methoxy-2-methylquinoline		17051.192	5650.868	8728.742			
2-Bromo-4,5-dimethoxycinnamic acid		16722.953					
2-(n-Propyl)oxybenzylidene acetone		13548.848					
1,2-Benzenediol, 3,5-bis(1,1-dimethyl-2-propyl)-		51063.260		2164.254			
4-Amino-5-(5-bromothiophen-2-yl)pyridine		20790.098		7447.303			
2-Nitroso-1-naphthol-4-sulfonic acid			54204.986			0.428964904	
1-(2-Aminobenzylidene)-1,2,3,4-tetrahydroquinoline			273994.242				
1-naphthalenol, 4-[2-[4-nitro-2-(2-propyl)oxybenzylidene]acetyl]phenyl			32804.002				
2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro			8962.425				
(Z)-14-Tricosenyl formate			12167.368				
2-Propanol, 1-(isopropylamino)-3-(2-propyl)pyridine			19526.785				
2H-Pyran-2-one, tetrahydro-6-undecyl			23609.638				
4-(3,4-Dimethoxybenzylidene)-1-(2-propyl)pyridine			9265.505				
2,5-Dihydroxybenzoic acid, 3-TMS			9422.607				
2-[5-(4-Chlorophenyl)-1H-1,2,4-triazol-3-yl]pyridine			7285.231		2.680		
1-(4-Chlorophenoxy)-1-(1H-imidazol-2-yl)pyridine			6309.104				
3-Quinolinecarboxylic acid, 6,8-dimethyl			5143.897				
Indole, 3-(4-nitrophenylamino)-			15046.950	1.014			
Benzothiophene-3-carboxylic acid			3419.661				
4-Dehydroxy-N-(4,5-methylenedioxyphenyl)quinoline			5265.985				
2,4-Cyclohexadien-1-one, 3,5-bis(2-propyl)pyridine			12460.970				
gamma-Tocopherol, O-methyl			15173.243				
1,2-Benzenediol, 3,5-bis(1,1-dimethyl-2-propyl)-			42441.141	31743.333	14.203	0.611	
1-methyl-4-phenyl-5-thioxo-1,2,4-triazol-3-amine			14250.736	9559.536			
1,2-Benzisothiazol-3-amine			24979.828				
4-Chloro-6-methoxy-2-methylquinoline			13497.718				6.560

1,2-Benzenediol, 3,5-bis(1,1-dim.	79656.783	9197.570	15.579
1,4-Bis(trimethylsilyl)benzene	12956.468	11.416	
Benzenamine, 4-methyl-N,N-bis(4-		6719.117	
benzenamine, 4-methyl-N-(4-methy		47908.136	
3-Oxo-1,8-octanedicarboxylic aci.		15020.060	
1-(4-Fluorophenyl)-2-[(4-hydroxy		36601.765	
1H-1,2,3,4-Tetrazole-1-propanoic		85963.436	
2-Pyrimidinamine, 4,6-dimethyl		109536.700	
Benzenepropanoic acid, 3,5-bis(1		10763.216	
phenol, 4-methoxy-2-[2-(5-nitro		68444.525	
4-Fluorobenzoic acid, nonyl ester		21391.680	
2-[2-[2-(4-Chloro-phenoxy)-ethyl		23299.478	
Tris(2,4-di-tert-butylphenyl) ph		4424.891	
2,4-Cyclohexadien-1-one, 3,5-bis.		4910.798	8.148
3-Acetoxy-3-hydroxypropionic aci		2.720	
2-[Ethyl(methylamino)ethyl cycl		2.592	
3-Oxo-1,8-octanedicarboxylic aci.		6.557	
1H-indole, 5-nitro-1-(4-nitrophe		2.221	
Thiophen-2-methylamine, N-(2-flu		0.759	
(R)-(-)-14-Methyl-8-hexadecyn-1-ol		2.161	
Methyl dehydroabietate		5.042	2.465
Dibutyl phthalate			2.465
2,12-Dichloro-7,7-diethyl-6H,1			1.201
Benzenesulfonamide, 4-(2-hydroxy			1.253
6-Chloro-4-phenyl-2-propylquinoline			6.863
7-Chloro-3a-ethyl-3-methyl-3a,4			8.476
2-Phenylcyclopropionamide, N-(4			7.320
2-Butenal, 2-ethyl-			150.979
Butylated Hydroxytoluene			0.898
Benzamide, N,N-dinonyl-3-methoxy-			6.101
Tetrazolo[1,5-b]pyridazine, 6-ch		0.265	
1,2,4-Dithiazolidine-3,5-dione		4.016	
4,6-Diamino-5-pyrimidinyl hydrog		0.668	
2,6-Dimethoxybenzotrile		1.307	
Benzothiazole, 5-chloro-2-methyl-		2.200	
Benzaldehyde, 3-[(2-bromophenoxy		4.960	
2-Butenoic acid, 3-methyl-, ethy.		0.641	
2-Furancarboxamide, N-methyl-		6.006	
2,2-Dimethylbutanedioic acid		13.482	
5H-Naphtho[2,3-c]carbazole		1.221	
Naphthalene, 1,1'-(1,2-ethanedi		4.067	
N,N'-Bis(2,6-dimethyl-6-nitrosoh		1.628	
7H-Dibenzo[b,g]carbazole, 7-methyl-		0.879	
gamma.-Terpinene		0.465	
3(2H)-benzofuranthione, 2-(3-met		0.988	
1,2-Benzenediol, 3-[[3,5-dichlo		1.233	
1H-Pyrazole, 4,5-dihydro-5,5-dim.		2.352	
1H-Trindene,		1.317	
2,5-Dihydroxybenzaldehyde,		1.072	
trans-4-(2-(5-Nitro-2-furyl)viny		1.549	
Octamethyl-7-(2-		2.428	
Quinazolin-4(3H)-one, 2-[2-(3-br		0.360	
Phthalic acid, heptyl 2-(methylt		1.091	
2-Nitro-4-trifluoromethylsulfony		0.953	
Benzenepropanoic acid, 3,5-bis(1		0.465	
L-Lysine, N6-acetyl-N2-[N-[N-[N		1.184	

Ctr, Control; CV, *C. vulgaris*; CP, *C. Pyrenoidosa*.

CONCLUSION

2 mM Pb and Cd stress reduced tomato growth and photosynthetic pigments through the generation of ROS and metabolic changes. However, MCE application showed corrective effect which increased tomato growth and photosynthetic pigments under Pb and Cd stress. Tomato exposure to Pb and Cd showed increase in H₂O₂, which in results increase lipids peroxidation (MDA). In response, tomato accumulated proline, polyphenols and increased scavenging enzymes as SOD, CAT, and POD against heavy metal stress. Furthermore, MCE can improve plant tolerance to H.M stress by stimulation of plant oxidative system at early stage. *C. vulgaris* and *C. pyrenoidosa* extract treatment alone showed elevation of H₂O₂. However, *C. vulgaris* and *C. pyrenoidosa* extract supplementation showed reduction of H₂O₂, MDA and scavenging enzymes activities after their elevation under Pb and Cd stress. Metal stress application showed that 2 mM Pb and Cd increased SFA, PUFA, VLCFA, alkanes and alkenes, while decreased MUFA compared to intreated plants. However, *C. vulgaris* and *C. pyrenoidosa* extract supplementation showed remodulation of metabolites accumulated under Pb and Cd stress. In plants exposed to 2 mM Pb and Cd the treatment with *C. vulgaris* and *C. pyrenoidosa* extract showed reduction in VLCFA, MUFA, PUFA, alkanes and alkenes. MCE can alleviate metal stress by enhancing antioxidative system and metabolites remodulation in plants.

II. Synthèse

Dans la présente étude le traitement des plantes de tomate sous stress de Pb et Cd par des extraits brutes des microalgues (MCE) a amélioré la croissance et la photosynthèse des plantes. En accordance, l'utilisation des microalgues comme biostimulant a montré un effet positif sur la croissance, la photosynthèse et le rendement des plantes en réduisant le stress et en restaurant les dommages antérieurs (Ronga *et al.*, 2019; Abd El-All *et al.*, 2021). L'effet stimulant de l'extrait des microalgues peut être dû à la présence des substances favorisant la croissance des plantes telles que : macro et micronutriments, acides aminés, acides gras, polysaccharides, phytohormones, etc., qui peuvent affecter la physiologie cellulaire des plantes en améliorant la croissance et la productivité des plantes (Behera *et al.*, 2021; Ferreira *et al.*, 2021; Li *et al.*, 2021).

De plus, le traitement des plantes de tomate par MCE a montré la réduction de la réponse du système oxydatif après son augmentation sous 2 mM de Pb et Cd. À cet égard, MCE a amélioré la tolérance et la résistance des plantes de tomate au stress métallique. L'application de MCE tout seul a montré une élévation de H₂O₂ et MDA. Ce qui laisse les plantes synthétisent des molécules et enzymes antioxydantes. Du coup, une immunisation précoce des plantes. En effet, le traitement des plantes par MCE a montré une réduction de H₂O₂, MDA, enzymes et molécules antioxydantes après leur élévation sous Pb et Cd stress. En cohérence, des études précédentes ont montré que l'utilisation des biostimulants a réduit les enzymes et molécules du système oxydatif après leur élévation sous stress des métaux lourds (Alves *et al.*, 2020; Abd El-All *et al.*, 2021). Par conséquent, le MCE a enlevé le stress des métaux lourds par stimulation du système antioxydant des plantes.

L'effet du MCE sur les changements métaboliques de la tomate répondant au stress des métaux lourds (Pb ou Cd) a été envisagé dans la présente étude. En effet, les plantes de la tomate exposées aux Pb et Cd ont montré la diminution des MUFA surtout C16:1 and C18:1, tandis qu'une augmentation des SFA, VLCFA, PUFAs, les alcanes/alcènes et les stérols. L'accumulation de ces métabolites agit comme réponse de la plante au stress métallique pour empêcher l'altération de la perméabilité, de la fluidité et de l'intégrité de la membrane. En effet, les AG C16 et C18 sont les principaux précurseurs de synthèse de la cuticule. De plus, l'élongation de ces FA en VLCFA, la désaturation en PUFAs ou la décarboxylation pour former des alcanes conduisant à une cuticule mature qui joue un rôle majeur dans la défense des plantes contre les stress abiotiques et biotiques et pour prévenir la perte d'eau (Lim *et al.*, 2017). Par

ailleurs, les stérols des plantes peuvent agir comme des précurseurs des Brassinostéroïdes (BRS) (Kour et al., 2021). De plus, le traitement des plantes sous stress de Pb et Cd en MCE a montré une redistribution des métabolites accumulés avec diminution des SFA, VLCFA, PUFAs, alcanes/alcènes et stérols.

III. Conclusion

L'application des métaux lourds Pb et Cd a réduit la croissance de la tomate et les pigments photosynthétiques grâce à la génération des ROS et de changements métaboliques. L'exposition de la tomate au Pb et Cd a montré une augmentation de H₂O₂, à son tour induit la peroxydation des lipides (MDA). En réponse, la tomate a accumulé de la proline, des polyphénols et des enzymes antioxydantes comme SOD, CAT et POD contre le stress des métaux lourds. De plus, le traitement par MCE a amélioré la tolérance des plantes au stress des métaux lourds en stimulant le système oxydatif des plantes à un stade précoce. À cet égard, Le traitement par extrait de *C. vulgaris* et *C. pyrenoidosa* seul a montré une élévation de H₂O₂. Pourtant, l'application en MCE a montré une réduction des activités de H₂O₂, MDA, des molécules et des enzymes antioxydantes après leur élévation sous stress de Pb et Cd. Par ailleurs, l'application de stress métallique aux plantes a montré que Pb et Cd augmentaient les SFA, les PUFAs, les VLCFA, les alcanes/alcènes, tout en diminuant les MUFA par rapport aux plantes témoins qui sont connus incorporés face au stress. Ensuite, le traitement par MCE a réduit tous ces métabolites. Par conséquent, le traitement des plantes par MCE peut atténuer le stress métallique en améliorant le système antioxydant et la redistribution des métabolites chez les plantes

Article 2: *Aphanothece* sp. as promising biostimulant to alleviate metallic stress in *Solanum lycopersicum* L. by enhancing physiological, biochemical, and metabolic responses.



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***Aphanothece* sp. as promising biostimulant to alleviate metallic stress in *Solanum lycopersicum* L. by enhancing physiological, biochemical, and metabolic responses.**

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ABSTRACT

Heavy metals (H.M) are a major environmental concern around the world. They have harmful impact on plant productivity and pose a serious risk to humans and animals health. In the present study, we investigated the effect of *Aphanothece* Crude Extract (ACE) on physiological, biochemical, and metabolic responses of tomato plant exposed to 2 mM Pb and Cd. The results showed a significant reduction of tomato plant weights and perturbation in nutrients absorption under 2 mM Pb and Cd conditions. Moreover, ACE treatment showed a significant enhancement of plant biomass compared to plants under Pb and Cd. On the other hand, ACE application favored H.M accumulation in root and inhibited their translocation to shoot. In addition, ACE treatment significantly enhanced several stress responses in plant under Pb and Cd stress such as scavenging enzymes and molecules: POD, CAT, SOD, proline, and polyphenols etc. Furthermore, ACE treatment showed remodulation of metabolic pathways related to plant tolerance such as wax construction mechanism, particularly SFA, UFA, VLFA, alkanes, alkenes, and sterols biosynthesis to enhance tolerance and resistance to H.M stress. In the present study, we emphasized that ACE alleviates H.M stress by minimizing metal translocation to above-part of plant and enhancing plant growth, nutrients absorption, and biochemical responses.

Keywords: Microalgae crude extract, Heavy metal stress, Nutrient uptake, Metabolomic analysis, *Solanum lycopersicum* (Tomato).

INTRODUCTION

Heavy metal (H.M) pollution is a major environmental and human health concern (Ishaq et al., 2020). They can be found naturally in soil, but their prevalence are increased by anthropogenic activities such as mining, urbanisation, industrialisation, smelting or the overuse of fertilizers, pesticides and sewage irrigation (Tauqeer et al., 2016; Feng et al., 2021). Soil contamination with H.M has been widely reported (Lamhamdi et al., 2013; Rahmatizadeh et al., 2019; Tauqeer et al., 2016). In small quantities, some metals are required for some plant physiological functions. However, some others like Pb and Cd even at low level are highly toxic (AbdElgawad et al., 2020). Plants exposed to these metals exhibited significant changes on morphological, physiological, biochemical, and ultrastructural parameters (Meena et al., 2018). Among H.M, Pb and Cd are the most toxic causing a high level of damage to plants as well as human health through food chain (Lamhamdi et al., 2013; Rahmatizadeh et al., 2019). Pb and Cd can reduce plants growth, preventing photosynthesis, organelles deterioration, generate ROS which activate antioxidative system and perturbate nutrient uptake and translocation (AbdElgawad et al., 2020; Alves et al., 2020; Alves et al., 2020). Numerous methods were used to remediate H.M contaminated soils e.g., soil washing, excavation, chemical/physical and biological methods. However, many of these tools are costly or do not offer a permanent solution (Bhandari et al., 2007).

The use of plant Biostimulant represent a good strategy to enhance tolerance and resistance to heavy metal stress (Abd El-All et al., 2021; Venkatachalam et al., 2017). Plant Biostimulant is defined as “a formulated product from a biological origin that improves plant productivity as a consequence of the novel or emergent properties of the complex of constituents, and not as a sole consequence of the presence of known essential plant nutrients, plant growth regulators, or plant protective compounds.” (Yakhin et al., 2017). Humic compounds (humic acid, fulvic acids, and humins), algal extracts, protein hydrolysates (signalling peptides and free amino acids), and microbes (bacteria, yeast, filamentous fungi, and microalgae) are the most common types of plant biostimulants (Bulgari et al., 2019). Among these categories, seaweeds have shown to be effective for the creation of plant growth biostimulants. However, microalgae and cyanobacteria received more attention in this field in last decades (Mutale-joan et al., 2020).

Microalgae (eukaryotic) and cyanobacteria (procaryotic) are potentially diverse, ubiquitous and dominant group of photosynthetic organisms in nature (Andersen, 2013; Tomaselli, 2004). They have a large variety of applications fields, including aquaculture, food,

animal feed, medicine, pharmacy, cosmetic, bioenergy, environment, and agriculture, etc. (Cardozo et al., 2007). Furthermore, numerous studies showed the potential of microalgae to improve tolerance to abiotic and biotic stresses in plants. microalgae-cyanobacteria extract enhanced antioxidative system of tomato plant and nutrients uptake under salt stress (Mutalejoan et al., 2021). In addition, Kusvuran, (2021) showed that 5% *C. vulgaris* extract alleviates drought stress by lowering the accumulated MDA content by 10%-39% accumulated under drought stress and enhance the nutrients uptake in the broccoli plant by (7%-182%). In the present study, we investigate the effects of ACE as biostimulant on physiologic, biochemical, and metabolic responses of tomato plants to Pb and Cd stress.

RESULTS

1. Effect of microalgae ACE extract on tomato growth under Pb and Cd stress

To investigate the effect of ACE on tomato growth under metallic stress, we applied firstly ACE in germination stage in magenta boxes, but without significant difference in germination parameters (data not provided here). However, old seedling plants has a remarkable difference between treatments, thus we used to apply our ACE on vegetative stage. In results, tomato plants exposed to 2 mM Pb and Cd stress showed no significant difference in tomato dry weights even for root or shoot under 2 mM Pb. However, a significant reduction was noted in tomato dry weight of shoot and root under 2 mM Cd by 29.25% and 39.48%, respectively compared to control (Figure 18). Furthermore, the treatment of tomato under 2 mM Cd by ACE showed a significant recovery of dry weights shoot and root by 26.11% and 40.35%, respectively compared to plants under 2 mM Cd.

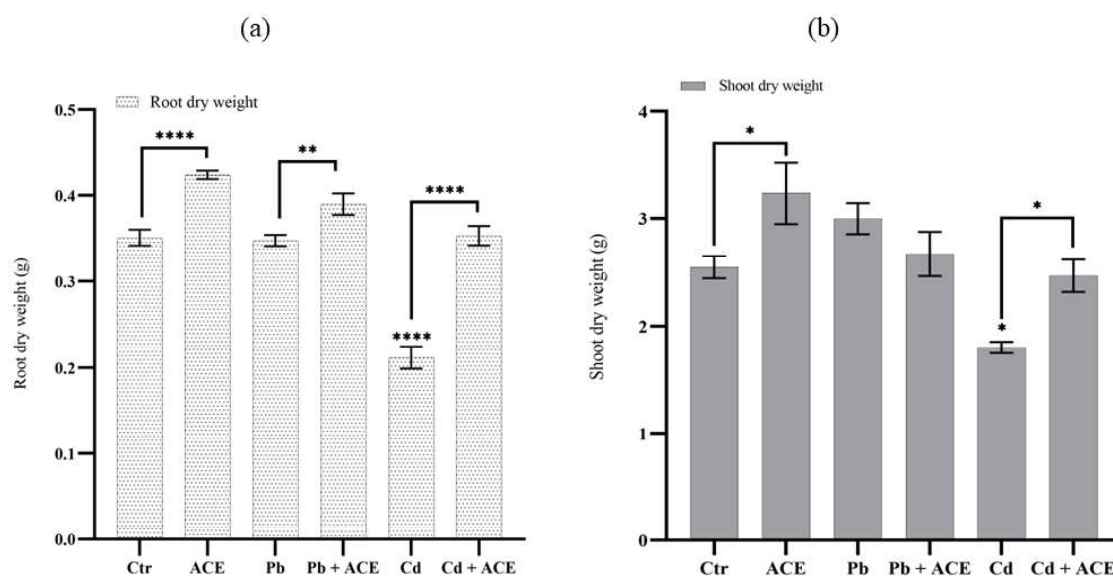


Figure 18: Effect of ACE on tomato root (a) and shoot (b) dry matter under Pb and Cd stress.

2. Effect of ACE on photosynthetic pigments under Pb and Cd stress

The result presented in Figure 19 (a), (b), (c), and (d) demonstrates the effect of Pb and Cd stress alone and in combination with ACE on Chl a, Chl b, total Chl and carotenoids in the leaves of tomato plants. 2 mM Pb and Cd decreased significantly Chl a (49.97 % and 41.92 %, respectively), Chl b (44.73 % and 41.37 %), total Chl (48.73 % and 41.79 %) and significantly increased carotenoids (39.26 % and 24.15 %), respectively in tomato leaves. However, ACE supplementation normalized the content of chlorophyll pigments increasing significantly Chl a (23.70 % and 24.15 %), Chl b (14.70 % and 30.74 %), total Chl (21.57 and 25.62 %) and significantly decreased carotenoids content (3.15 % and 4.18 %).

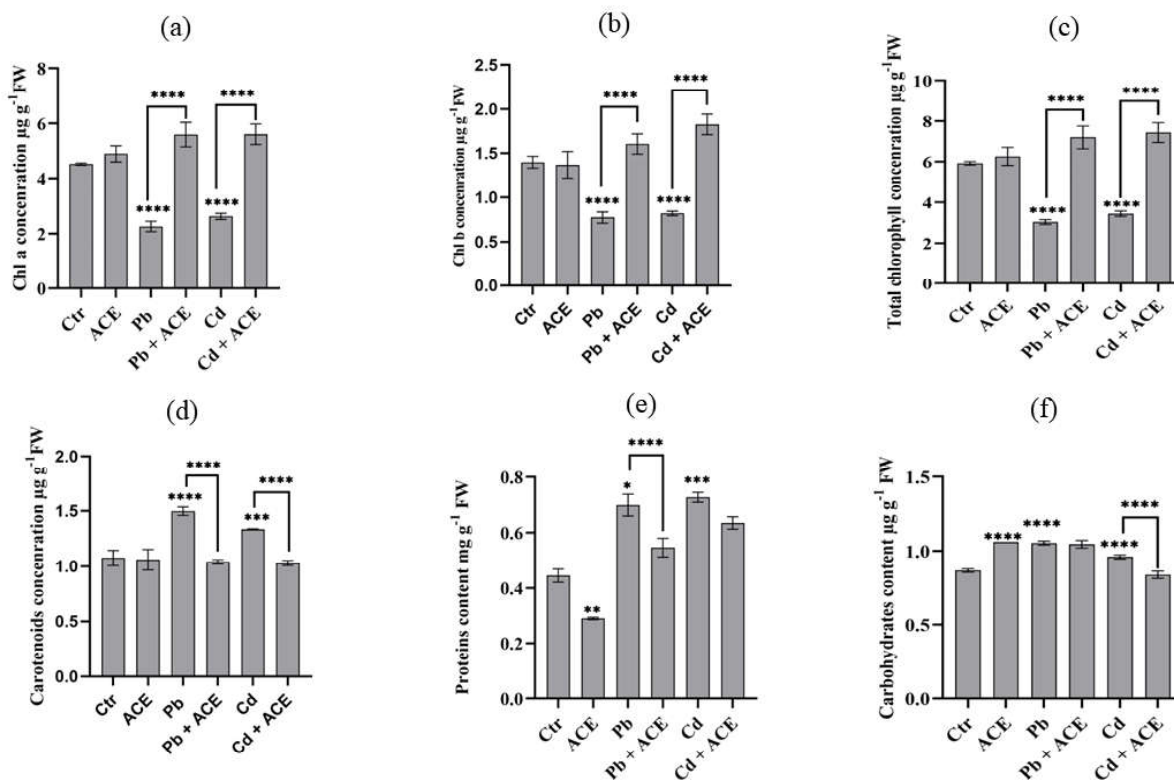


Figure 19: Effect of ACE on tomato leaves photosynthetic pigments (a) Chl a, (b) Chl b, (c) Total Chl and (d) Carotenoids and Soluble Proteins (e) and sugar (f).

3. Effect of ACE on plant antioxidant system against Pb and Cd stress

In the present study, we investigate the effect of ACE on plant antioxidative system under 2 mM Pb and Cd. Tomato leaves showed significant elevation of H_2O_2 level with 63.45% and 110.15% under 2 mM Pb and Cd stress, respectively. Moreover, 2mM Pb and Cd showed an increase of MDA level with 98.67 % and 66.24 %, respectively (Figure 20 (a), (b)). Moreover, 1% ACE supplementation reduced significantly H_2O_2 and MDA level by 15.41% and 64.79 % for Pb and 56.34% and 45.11% for Cd, respectively. Furthermore, the exposure to 2 mM Pb and Cd showed an accumulation of proline and polyphenols by 43.01% and 8.20% under 2 mM Pb, respectively and 58.21% and 9.26% under 2 mM Cd, respectively (Figure 20 (c), (d)). In addition, ACE supplementation showed reduction in proline and polyphenols accumulation under stress by 31.46% and 7.56% under Pb +ACE in comparison to 2 mM Pb. For Cd, ACE application showed reduction of 35.98% and 15.16% in proline and polyphenols, respectively. 2 mM Pb and Cd exhibited significant increase of soluble sugar and proteins content in tomato leaves (Figure 20 (e), (f)). However, ACE treatment normalized proteins and carbohydrates content in tomato leaves to meet control content. In parallel, we noted a significant increase of

scavenging enzymes SOD (68.23%; 33.28%), POD (759.25%; 150.63%), and CAT (93.14; 1.34%) under 2 mM Pb and Cd, respectively except CAT under Cd (Figure 20 (e), (f) and (g)). Furthermore, ACE supplementation showed neutralization of all enzymes after their elevation under H.M stress (Figure 20 (e), (f) and (g)). The lowest reduction recorded in SOD under 2 mM Cd + ACE with 25.65%.

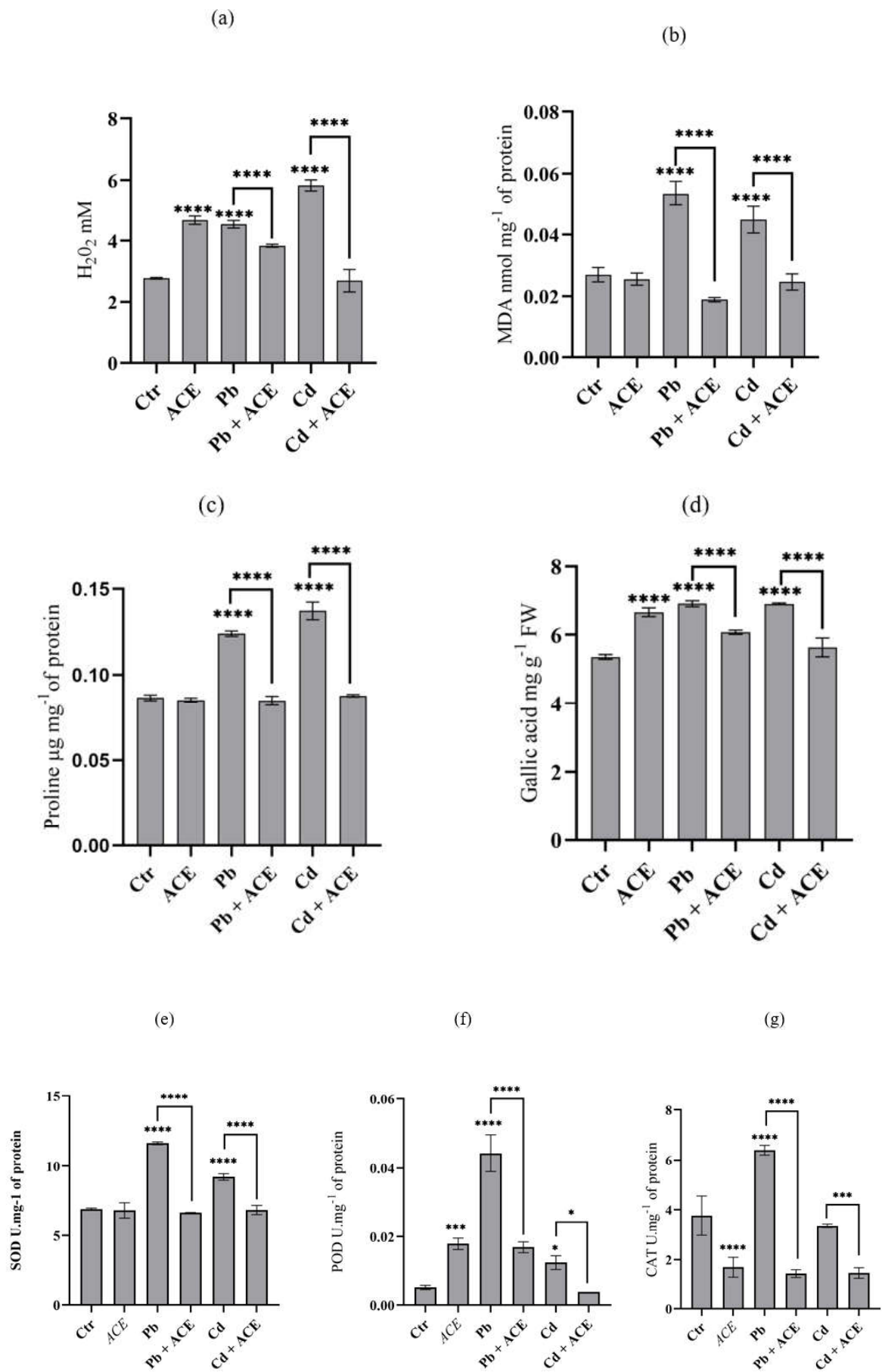


Figure 20: Effect of ACE on antioxidative system of tomato against Pb and Cd stress (a) H₂O₂, (b) MDA, (C) Proline, (d) Polyphenols, (e) SOD, (f) POD, (g) CAT.

4. Effect of ACE on tomato plant metabolomic under H.M stress

Metal stress application showed that 2 mM Pb and Cd reduced SFA by 43.50%, and 66.16% and MUFA with 100% and 454.01%, respectively, while increased PUFA, alkanes and sterols compared to control (Figure 21 and table 10, 11). However, ACE supplementation under Pb and Cd showed increase of SFA and MUFA, while decrease PUFA, alkanes and sterols (Figure 21 and table 10, 11). Moreover, ACE application alone showed increase of PUFA, alkanes and sterols.

Table 10: Effect of ACE on FAME changes under Pb and Cd stress

Metabolites	Ctr	ACE	Pb	Pb +ACE	Cd	Cd + ACE
($\mu\text{g g}^{-1}$)						
C8:0	44.835	-	-	-	-	-
C9:0	-	2.973	-	-	-	-
C14:0	20.636	16.000	14.130	15.913	10.030	13.464
C16:0	1655.103	1388.564	1096.607	1199.354	673.447	977.848
C17:0	-	-	-	14.936	-	-
C18:0	672.034	287.399	233.162	272.759	127.847	209.945
C20:0	140.367	67.065	75.677	50.484	39.747	74.184
SFA	5000.481	3505.033	2825.025	3090.983	1692.114	2537.420
C18:1	201.017	-	-	243.053	39.205	119.911
C20 :1	16.189	-	-	-	-	-
MUFA	217.207	-	-	243.053	39.205	119.911
C18 :2	-	429.794	312.135	-	201.143	85.442
C16:3	-	37.065	-	-	-	-
C18:3	-	1003.487	652.646	-	514.508	-
PUFA	-	1470.346	964.782	-	715.651	85.442
C22:0	91.058	28.163	28.493	41.981	14.319	25.948
C24:0	49.653	80.856	80.211	69.472	43.953	44.673
C25:0	-	-	10.018	-	5.325	25.924
C26:0	21.405	16.171	15.496	-	10.780	23.001
VLCHA	162.117	125.191	134.219	111.453	74.379	119.547

Table 11: Effect of ACE on Alkanes, alkenes, and Sterols changes under Pb and Cd stress

Metabolites ($\mu\text{g g}^{-1}$)	Ctr	ACE	Pb	Pb +ACE	Cd	Cd + ACE
Heptadecane	-	-	-	200,463	-	-
Octadecane	125,791	17,330	107,743	15,688	7,137	85,464
Tetracosane	165,065	-	210,364	-	52,562	-
Penntacosane	-	-	59,667	-	-	-
Hexacosane	-	-	51,342	-	-	-
Heptacosane	69,850	-	51,342	-	-	-
Octacosane	27,898	-	55,779	-	-	-
Heneicosane	60,722	-	28,410	-	14,819	-
Eicosane	-	144,670	97,450	-	-	-
Nonacosane	-	-	-	29,086	-	-
Triacontane	-	53,221	59,152	120,791	41,279	25,305
Alkanes	449,325	215,222	721,249	366,027	115,797	110,770
1-Octadecene	10,771	-	-	-	-	-
Cholesta-6,22,24-triene	-	42,169	-	-	-	-
Cholest-5-ene, 3-methoxy-, (3. beta)	-	28,962	-	-	-	-
Alkenes	10,771	71,131	-	-	-	-
Stigmasta-3,5-diene	-	92,666	25,241	-	-	-
y-sitosterol	-	12,230	-	-	8,068	-
Stigmastan-6,22-dien, 3,5-dedihydro-	-	-	26,061	-	12,663	-
Stigmastan-3,5,22-trien	-	-	-	-	12,669	-
3,7,11,15-tetramethyl-2-Hexadecen-1-ol	-	-	40,493	-	-	-
Sterols	-	104,896	91,795	0,000	33,400	0,000
Other metabolites						
Furanone, 5 dodecyldihydro	148,663	-	-	-	-	-
Aalpha.-Tocopherol	40,581	-	-	19,575	8,765	24,727
Alpha.-Tocospiro A	-	-	12,956	-	-	-
Tris(2,4-di-tert-butylphenyl) phosphate	67,859	-	33,285	-	13,968	-
1-(4-Fluorophenyl)-2- [(4-hydroxy-6-methylpyrimidin-2-yl)thio]ethan-1	-	-	65,229	-	-	-

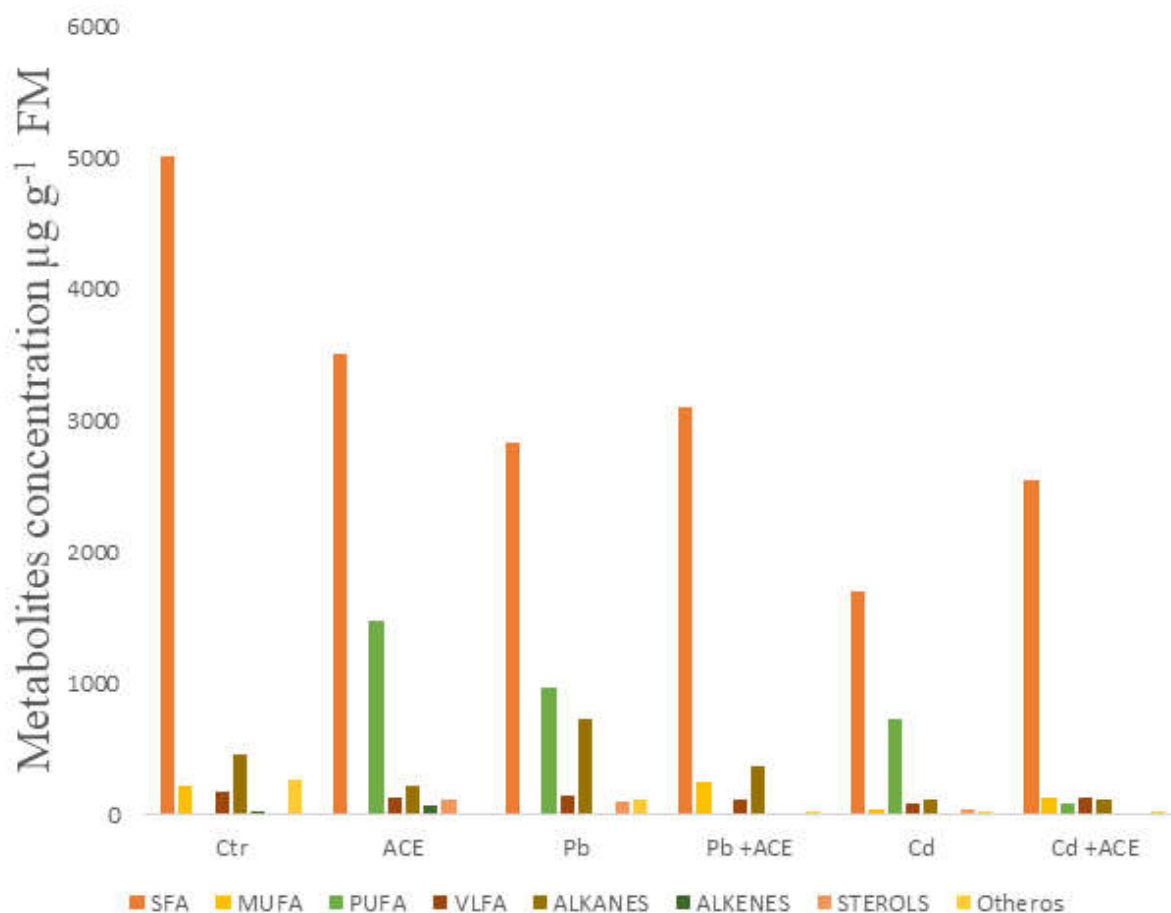


Fig. 21: Effect of ACE on metabolites changes under Pb and Cd stress.

5. Effect of ACE on heavy metals accumulation and distribution in tomato plant

Tomato plant showed significant accumulation of Pb and Cd in root by $10.7 \mu\text{g g}^{-1}$ and $13.4 \mu\text{g g}^{-1}$, respectively. As well as a significant accumulation in shoot also with $32.7 \mu\text{g g}^{-1}$ and $3.4 \mu\text{g g}^{-1}$ for Pb and Cd, respectively (Figure 22 (a) and (b)). Moreover, ACE supplementation under Pb significantly enhanced the accumulation of Pb in root by 88.78% compared to 2 mM Pb. In shoot, ACE significantly reduced the accumulation of Pb by 30.18% compared to 2 mM Pb. Furthermore, ACE significantly reduced Cd concentration in root and shoot by 75.77% and 44.69% compared to 2mM Cd. Indeed, ACE application showed reduction of metal accumulation in root or/and shoot. Our results showed translocation inhibition from root to shoot, which tomato plants exposed to 2 mM Pb showed a TF exceed 1, which was 3.01 ± 0.037 . Moreover, ACE application to plant stressed by 2 mM Pb showed reduction in TF, which was 1.11 ± 0.028 . For Cd, ACE showed in inhibition of Cd uptake and accumulation and both BCF and TF doesn't exceed 1 (Table 12).

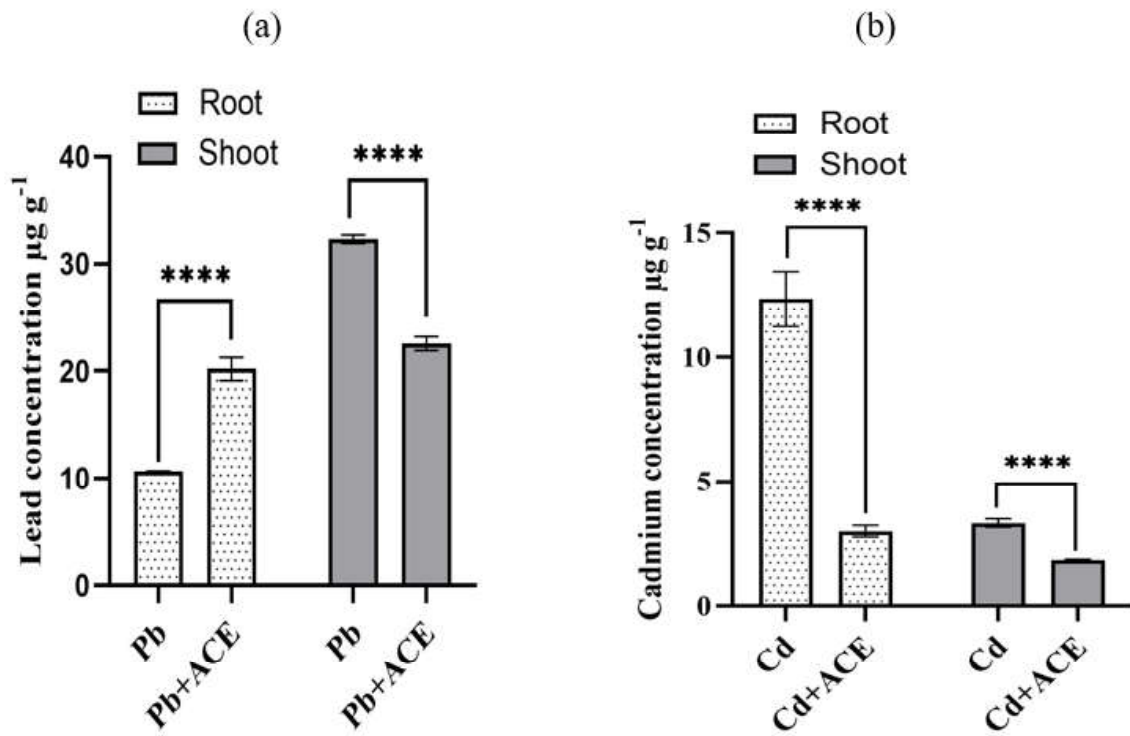


Figure 22: Effect of ACE on Pb (a) and Cd (b) uptake and translocation in tomato plant.

Table 12: Bioaccumulation and translocation factors of H.M

	BCF	TF
Pb	0.008 ± 0.00	3.018 ± 0.037
Pb + ACE	0.008 ± 0.00	1.117 ± 0.028
Cd	0.006 ± 0.0003	0.270 ± 0.035
Cd + ACE	0.0018 ± 0.00	0.615 ± 0.037

6. Effect of ACE on nutrients uptake and distribution under Pb and Cd stress

In the present study we investigate the effect of ACE on nutrients uptake and redistribution under Pb and Cd stress (Figure 23 and 24). 2 mM Cd significantly increased Nitrogen (N) concentration in root by 17.83% compared to control. Moreover, ACE supplementation under 2mM Cd further enhanced this accumulation to 27.60%. N concentration in shoot was increased by 2 mM Pb and Cd to 49.23% and 73.86%, respectively in comparison to control. Furthermore, ACE treatment enhanced N concentration in shoot to 54.64% and 133.01% under Pb + ACE and Cd + ACE, respectively. Phosphorus concentration showed no significant difference in root and shoot under metal stress and even after ACE supplementation. For potassium (K), 2 mM Pb and Cd showed significant increase of K in root accumulation by 14.38% and 7.22%, respectively. The treatment by ACE significantly reduced K concentration by 11.38% and 14.19% under Pb + ACE and Cd + ACE, respectively compared to Pb and Cd. Moreover, K exhibited a significant increase in shoot by 19.71% and 19.51% under Pb and Cd, respectively compared to control. The effect of metal and treatment by ACE on other nutrients concentration was showed in Figure 24. 2 mM Pb significantly reduced the concentration of Ca, Zn, Mg, Cu and Cr in root by 84.10%, 30.70%, 14.88%, 22.31% and 50%, respectively compared to control. ACE supplementation significantly increased the concentration of Zn (92.31%), Mg (31.78%), Cu (34.39%) and Cr (42.85%) compared to 2 mM Pb. 2 mM Cd significantly reduced the concentration of Ca (81.76%), Cu (0.82%) and Cr (39.28%) in root compared to control. Furthermore, ACE supplementation significantly enhanced concentration of Ca and Cu by 644.29% and 26.39%, respectively compared to 2 mM Cd. In shoot 2 mM Cd significantly reduce Mn, Mg, Cu and Cr concentrations by 15.10%, 4.91%, 44.88% and 77.08%, respectively compared to control. The concentration of these element was increased significantly after treatment by ACE with Mn (27.91), Mg (9.67%), Cu (79.63%) and Cr (327.43%) compared to 2 mM Cd. 2 mM Pb significantly reduced Mg concentration by 12.49% compared to control, while enhanced after treatment by ACE with 12.71% compared to 2 mM Pb.

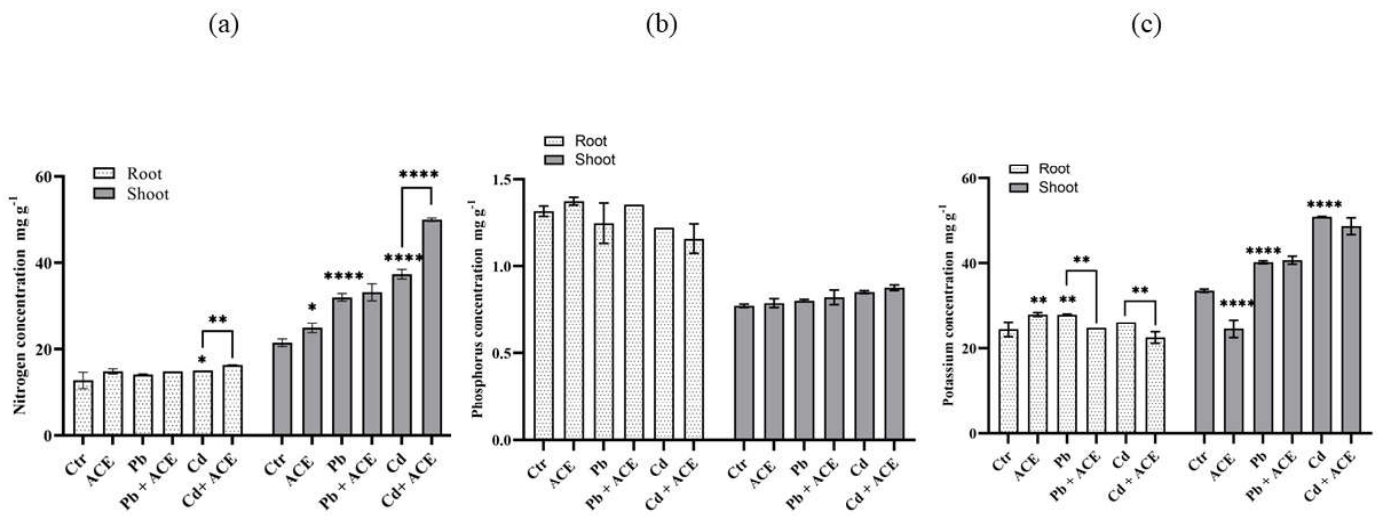


Fig. 23: Effect of ACE on macro element N (a), P (b), K (c) uptake and translocation under Pb and Cd stress.

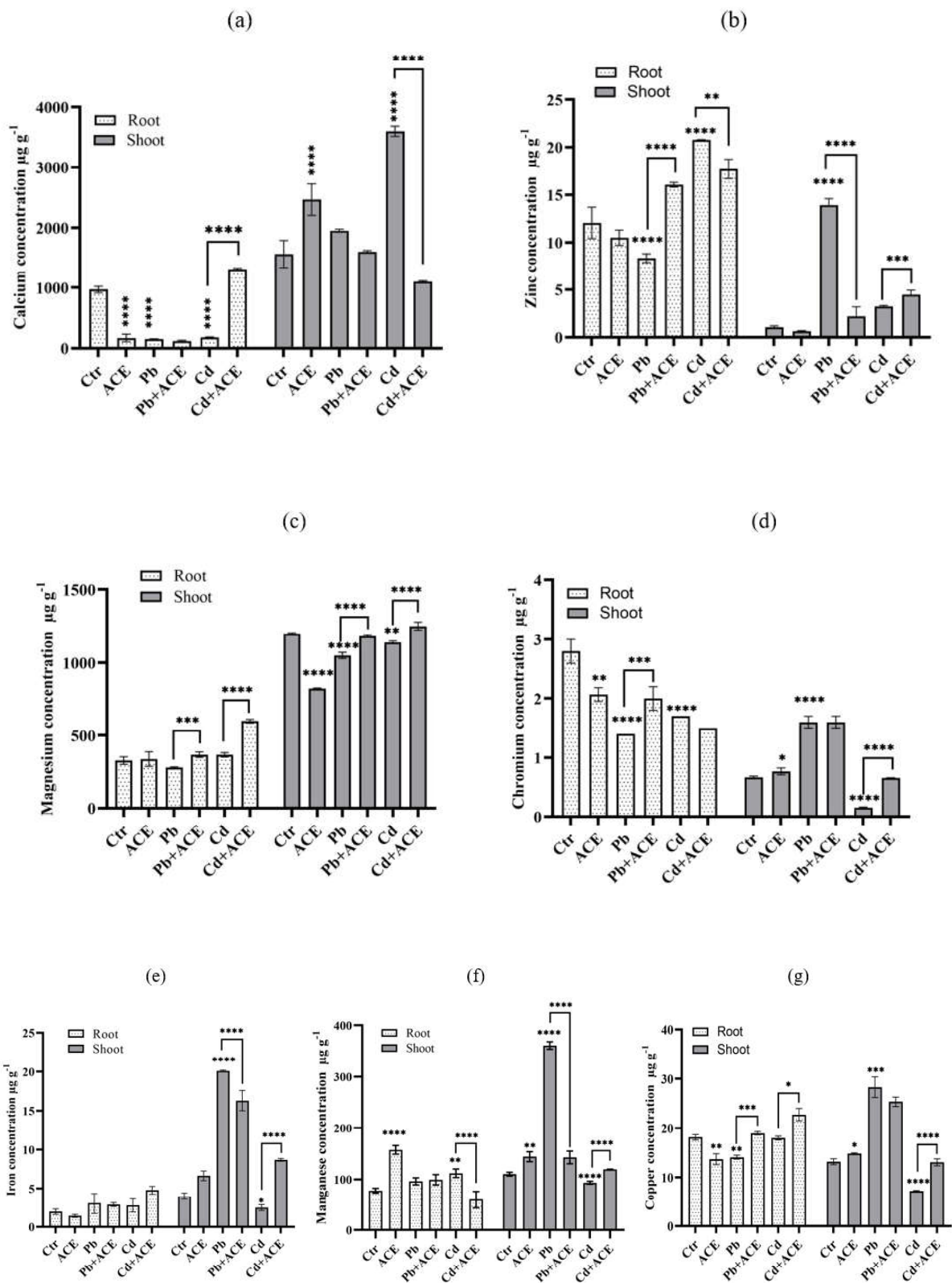


Fig 24: Effect of ACE on minerals element (a) Ca, (b) Zn, (c) Mg, (d) Cr, (e) Fe, (f) Mn, (g) Cu uptake and translocation under Pb and Cd.

DISCUSSION

In the present study, the effect of ACE on physiological, biochemical, and metabolic responses under metallic stress has been investigated. The results showed no significant difference in tomato dry weights for root and shoot under 2 mM Pb (Figure 18). In accordance, spinach, parsley and cilantro exposed to 0.3 mM Pb and 0.005 mM Cd showed no significant difference in dry weight of shoot and root compared to control (Sharifan et al., 2020). Conversely to our results for Pb stress, Galal et al., (2021) study found that elevated concentration of lead stunted growth parameters and 300 mg L⁻¹ of Pb as the high concentration decreased root and shoot dry weight by 0.58 g and 1.48 g compared to control 0.89 g and 2.25 g, respectively. However, a significant reduction was noted in tomato dry weight of shoot and root under 2mM Cd by 29.25% and 39.48%, respectively compared to control. In accordance, Naciri et al.(Naciri et al., 2021) found that tomato plant in hydroponic culture exposed to 0.025 mM of Cd and 232 ppm of K induced significant reduction of shoot and root dry weights by 1.6 g and 0.31 g compared to control by 2.5 g and 0.47 g, respectively. Nogueira et al., (2021) reported that tomato biomass (shoot and root) produced by Cd treated plants was reduced when grown in contaminated soil with Cd at 0.02 mM. Generally, plants under HM stress reduce the number of lateral roots, root elongation and root hair density in the order to limit H.M uptake including Pb and Cd (Feng et al., 2021). The growth reduction caused by Pb and Cd in plants may be due to size reduction of intracellular space and the strong binding between pectin molecules in cell wall. Moreover, Pb and Cd as stress may incorporate the lignin to cell-wall, which increase cell-wall rigidity and reduce expansion. Moreover, Pb and Cd as stress caused microtubule damage, in results caused cell division inhibition (Pál et al., 2006). The treatment of tomato under 2 mM Cd by ACE showed a significant recovery of dry weights shoot and root by 26.11% and 40.35%, respectively compared to plants under 2 mM Cd. These results are in agreements with the finding of Abd El-All et al., (2021) which showed that the treatment by seaweed extract (SWE) has a recovering to deleterious effect of heavy metals on tomato plant growth in comparison with control. The interaction with 6 mM of Pb and SWE enhanced the dry weight of root, stem, leaves and whole of tomato plant with 0.61g, 1.13g, 2.62g and 4.36g, respectively compared to control with 0.57g, 1.03g, 2.30g and 3.90g, respectively. The application of SWE with 1.6 mM Cd enhanced the dry weight of root, stem, leaves and whole of tomato plant with 0.57g, 0.95g, 2.16g, 3.68g, respectively compared to control with 0.43g, 0.84g, 1.52g and 2.79g, respectively (Abd El-All et al., 2021). In the light of this point, microalgae based biostimulant showed positive effect on plant growth and yield by reducing stress and restoring previous damages

(Ronga et al., 2019). The biostimulant effect of microalgae extract was reported in numerous studies. Li et al., (2021), showed that 1% of microalgae extract enhanced significantly bean seedling growth. Tomato plants treatment by microalgae and cyanobacteria crude extract (CBEs) showed significant growth enhancement, particularly *Aphanothece* sp. which improved significantly root and shoot DW (34.81% and 58.69%), respectively (Mutale-joan et al., 2020). The stimulation effect of microalgae extract may be due to the presence of plant growth promoting substances such as: macro-and micro nutrients, amino acids, fatty acids, polysaccharides, phytohormones etc., which can affect cellular physiology of plants enhancing plant growth and productivity (Behera et al., 2021; Ferreira et al., 2021; Li et al., 2021; Ronga et al., 2019).

To investigate the effect of metallic stress on the photosynthesis as one of the most fundamental metabolic processes in plants, we exposed tomato plant to Pb and Cd stress. The result presented in Figure 19 (a), (b), (c), and (d) demonstrates the effect of Pb and Cd stress alone and in combination with ACE on Chl a, Chl b, total Chl and carotenoids in the leaves of tomato plants. 2 mM Pb and Cd decreased significantly Chl a (49.97 % and 41.92 %, respectively), Chl b (44.73 % and 41.37 %), total Chl (48.73 % and 41.79 %) and significantly increased carotenoids (39.26 % and 24.15 %) content in tomato leaves. However, ACE supplementation normalized the content of chlorophyll pigments increasing significantly Chl a (23.70 % and 24.15 %), Chl b (14.70 % and 30.74 %), total Chl (21.57 and 25.62 %) and significantly decreased carotenoids content (3.15 % and 4.18 %) to meet control values. The both harmful heavy metals Pb (3 and 6 mM of soil) and Cd (0.5 and 1.6 mM of soil) concentrations decreased photosynthetic pigments (Chl a, Chl b, Total Chl and carotenoids in tomato leaves. However, foliar application of seaweed extract resulted in a significant increase and recovery in chlorophyll content and carotenoids after harmful effect of H.M (Abd El-All et al., 2021). Moreover, Cd stress reduced significantly Chl a, b, total chl and carotenoids, which were 132.7%, 152.6%, 143%, and 322%, respectively ($P < 0.05$) (Rahmatizadeh et al., 2019). Indeed, Chl stability index in tomato leaves reduced from 95% in control to 55 % at Pb 0.6 mM and 25 % under Cd 1.2 mM (Baruah et al., 2019). In accordance with our result, Siddiqui et al., (2022) found that 0.025 mM of Cd reduced Chl a and Chl b content in tomato leaves. Generally, Chl destruction may be due to the high H_2O_2 generation or chlorophyllase activity (Faizan et al., 2021). Moreover, it may be due also to low nutrients absorption required for chlorophyll production, which reduced by metal stress (Jimenez et al., 2020). (Naciri et al., 2021)Naciri et al., (2021) showed that potassium has an important role in photosynthetic pigments formation

and prevents formed photosynthetic pigments degradation (Tanaka and Tsuji, 1980). Moreover, dos Santos Sarah et al., (2021) related the reduction of total chl content and carotenoids to deficiency on K. H.M can also substitute Mg^{2+} ion in chlorophyll molecule (Blanco et al., 2021). Furthermore, microalgae biomass used as fertilizer showed a positive effect in photosynthetic pigments contents (Jimenez et al., 2020).

In the present study, tomato leaves showed significant elevation of H_2O_2 level with 63.45% and 110.15% under 2 mM Pb and Cd stress, respectively. Moreover, 2mM Pb and Cd showed an increase of MDA level with 98.67 % and 66.24 %, respectively (Figure 20 (a), (b)). Which, indicates that metals disrupt cellular metabolic by increasing lipid peroxidation via free radical molecules liberation (Faizan et al., 2021). In accordance, an increase in MDA was recorded in soybean from $185.09 \pm 16.53 \mu\text{mol g}^{-1}$ in control to $261.15 \pm 36.02 \mu\text{mol g}^{-1}$ at 2.5 mM Pb (Blanco et al., 2021). Moreover, 200 μM Cd caused oxidative stress in tomato leaves by increasing MDA and H_2O_2 level by 421.4% and 202.2%, respectively (Rahmatizadeh et al., 2019). Cd stress at 25 μM significantly increased tomato leaves in H_2O_2 and MDA content by 175% and 75%, respectively (Siddiqui et al., 2022). In the present study, 1% ACE supplementation reduced significantly H_2O_2 and MDA level by 15.41% and 64.79 % for Pb and 56.34% and 45.11% for Cd, respectively after elevation by 63.45% and 98.67% for Pb and 110.15% and 66.24% for Cd, respectively. Tomato plants treatment with K^+ (5 mM) or Melatonin (50 μM) alone or combined showed significant reduction of H_2O_2 and MDA content under Cd stress with K (45%; 25%), Melatonin (36.36%; 17.85%) and K^+ +Melatonin (49.09%; 42.85%) (Siddiqui et al., 2022). Moreover, microalgae polysaccharides showed improvement in H_2O_2 content, which indicate that represent a good biost

imulant for plant defense (Rachidi et al., 2021). Microalgae extract showed reduction of H.M stress toxicity by lowering lipid peroxidation. In addition, ACE application alone showed increase of H_2O_2 level by 68.37% (Figure 20 (a)), which indicate that ACE stimulate plant oxidative system to reduce H_2O_2 and lipid peroxidation under H.M stress. In this regard, microalgae extract can modulate oxidative stress in plants (Mutale-joan et al., 2021; Ronga et al., 2019). Furthermore, microalgae contain antioxidant molecules such ascorbic acid, carotenoids, tocopherols, phycocyanin, and phenolic compounds. Which has an important role in redox homeostasis (El-Baky and El-Baroty, 2013; Chokshi et al., 2017).

In the present study, the exposure to Pb and Cd showed an accumulation of proline and polyphenols by 43.01% and 8.20% under 2 mM Pb, respectively and 58.21% and 9.26% under 2 mM Cd, respectively (Figure 20 (c), (d)). Furthermore, ACE supplementation showed

reduction in proline and polyphenols accumulation under stress by 31.46% and 7.56% under Pb +ACE in comparison to 2 mM Pb. For Cd, ACE application showed reduction of 35.98% and 15.16% in proline and polyphenols, respectively. Rahmatizadeh et al., (2019) reported the increase in proline content by 89.9% in shoot under 200 μM Cd. Furthermore, selenium application as biostimulant at 1 μM under Cd stress reduced proline accumulation in tomato leaves from 105 $\mu\text{mol g}^{-1}$ FW to 67 $\mu\text{mol g}^{-1}$ FW at 0.5 μM CdCl_2 (Alves et al., 2020).

The exposure to 2 mM Pb and Cd exhibited significant increase of soluble sugar and proteins content in tomato leaves (Figure 19 (e), (f)). This elevation may be due to the enzymatic activities under metal stress (Fig 20 (e), (f) and (g)). However, ACE treatment normalized proteins and carbohydrates content in tomato leaves. Blanco et al., (2021) reported the increase in starch and proteins under 2 mM Pb, which were $195.5 \pm 41.49 \text{ mg g}^{-1}$ and $92.97 \pm 26.51 \text{ mg g}^{-1}$ compared to $95.05 \pm 31.11 \text{ mg g}^{-1}$ and $11.10 \pm 5.62 \text{ mg g}^{-1}$ in control, respectively. In line of our results Rahmatizadeh et al., (2019) reported that proteins and soluble sugars were increased in tomato shoot under 0.2 mM Cd, which were 228.1% and 121%, respectively. Furthermore, plants used soluble and reduced sugar from starch as source of energy under abiotic stress (Blanco et al., 2021). That can explain the elevation of soluble sugar under pb and cd stress. Moreover, H.M stress increase sugar content and proteins synthesis for preserving osmotic balance and water absorption ability. Moreover, proteins content under Cd stress synthesis might be due to antioxidative activities especially scavenging enzymes (Baruah et al., 2019).

Scavenging enzymes are another barrier of antioxidative system played to reduce free radicals (Kumar et al., 2020). In the present study, we noted a significant increase of scavenging enzymes SOD (68.23%; 33.28%), POD (759.25%; 150.63%), and CAT (93.14; 1.34%) under 2 mM Pb and Cd, respectively except CAT under Cd (Figure 20 (e), (f) and (g)). The highest elevation recorded in POD under 2 mM Pb with 759,25%. Siddiqui et al., (2022) showed that tomato leaves have an increase in antioxidant enzymes (SOD, GR, APX, DHA, MDHAR) under Cd stress in the range of 1.7–2.6 fold. Moreover, metal stress significantly increased CAT activity in tomato leaves from 17 U mg^{-1} of protein to 46 U mg^{-1} of protein under Pb at 175 ppm as the highest activity recorded in different concentration of Pb and 57 U mg^{-1} of protein in Cd at 1.2 mM (Baruah et al., 2019). Pb and Cd in different concentrations caused increase in POD activity in tomato leaves with 70% as the max concentration in Cd at 1.6 mM of soil compared to control (Abd El-All et al., 2021). This elevation in scavenging enzymes is a natural response of antioxidant system to dismutase H_2O_2 and maintain ROS homeostasis (Baruah et al., 2019). First line of defense is SOD, which dismutase H_2O_2 to H_2O and O (Zhang et al.,

2020). Any excess of H₂O₂ result from SOD or other molecule is detoxified by other enzymes CAT, POD, G.R and APX (Alves et al., 2020). Furthermore, ACE supplementation showed neutralization of all enzymes after their elevation under H.M stress (Figure 20 (e), (f) and (g)). The lowest reduction recorded in SOD under 2 mM Cd + ACE with 25.65%. In accordance, seaweed extract (SWE) application by foliar spray mode reduced significantly POD activity with 11 % and in result recovered the deleterious effect of H.M (Abd El-All et al., 2021). Microalgae biostimulant contains bioactive molecules such as proline, betaines, phytohormones which can regulate plant redox homeostasis leading to stress resistance and tolerance (Ferreira et al., 2021). Zinc oxide nanoparticles (ZnONPs) prepared from *Ulva lactuca* biomass at 25 mg⁻¹ showed reduction of scavenging enzymes after elevation in *Leucaena leucocephala* leaves under Pb and Cd stress. It showed significant reduction of SOD activity which were 95.7% and 97.4% under 50 mg L⁻¹ Cd and 100 mg L⁻¹ Pb, respectively compared to control. While 25 mg⁻¹ ZnONPs decreased CAT activity to 85.3% and 90.9% for Cd (50 mg L⁻¹) and Pb (100 mg L⁻¹), respectively. Moreover, POD activity found increased to 110.2% and 127.1% under 50 mg L⁻¹ Cd and 100 mg L⁻¹ Pb, respectively after 25 mg⁻¹ ZnONPs supplementation to medium (Venkatachalam et al., 2017). Moreover, tomato plants treatment by Ca (10 mmol L⁻¹), salicylic acid (100 µmol L⁻¹) and epi-brassinolide (1 mol L⁻¹) in combination showed reduction of SOD (137.64%), CAT (62.91%) and POD (74.08%) after their elevation under 5 mg L⁻¹ of Cd by 151.89%, 155.18% and 99.49%, respectively (Guo et al., 2018). Our results showed that ACE can improve plant tolerance to H.M stress by stimulating plant oxidative system at early stage. ACE treatment alone showed elevation of H₂O₂ by 68.37% (Figure 20 (a)). However, ACE supplementation with Pb or Cd stress showed reduction of H₂O₂, MDA and scavenging enzymes activities after their elevation under Pb and Cd stress. Which indicate that plant early immunized for any stress by the first elevation of H₂O₂ from ACE. Furthermore, microalgae biostimulant act as elicitor contain bioactive molecules such as proline, betaines, phytohormones which can regulate plant redox homeostasis molecules leading to stress resistance and tolerance (Oancea et al., 2013; Sharma et al., 2014).

Metal stress application showed that 2 mM Pb and Cd reduced SFA and MUFA, while increased PUFA, alkanes and sterols compared to control (Figure 21 and table 10, 11). This increase in PUFA, alkanes and sterols can be considered as plant response to preserve the membrane from metal alteration. PUFA, alkanes and sterols are the major metabolites that have a role in tolerance to abiotic and biotic stress (Jump, 2002). PUFAs are the main compound of membrane, which keep plasma membrane permeability, integrity, and fluidity. They have also

a role in ROS generation via activation of NADPH oxidase ((Di Palma et al., 2020; Yaeno et al., 2004). In the present study, tomato plants exhibited synthesis of PUFA under Pb and Cd stress which were 964.78 $\mu\text{g g}^{-1}$ FW and 715.65 $\mu\text{g g}^{-1}$ FW, respectively compared to 0 in control $\mu\text{g g}^{-1}$ FW. The majors PUFA found under Pb and Cd stress were C18:2, C16:3 and C18:3, which are particularly important in plant defense as the precursors of LOX pathway resulting in oxylipins biosynthesis after their peroxidation (Lim et al., 2017). Oxylipin play the role of signaling and defense molecules (Sugimoto et al., 2022). In addition, the oxylipin induce expression of genes involved in the biosynthesis and accumulation of secondary metabolites (Mithöfer et al., 2004; Viswanath et al., 2020). Moreover, Alkanes are also a compound of membrane and wax cuticle, which play a vital role as a defense barrier against abiotic and biotic attacks (Bourdenx et al., 2011; Wang et al., 2015). Tomato plant showed an increase in alkanes concentration under 2 mM Pb by 60% compared to control. Sterols have a role in membrane formation and preservation, against abiotic and biotic shocks (Rogowska and Szakiel, 2020). In the present study, Pb and Cd showed accumulation of sterols which were 91.79 $\mu\text{g g}^{-1}$ FW and 33.40 $\mu\text{g g}^{-1}$ FW compared to 0 $\mu\text{g g}^{-1}$ FW in control. Plants sterols can act as precursors of BRs (Kour et al., 2021). Moreover, BRs improve plant tolerance to metal, thereby increasing crop yield and quality (Vriet et al., 2012). They can eliminate toxic metal via assisted phytoremediation system by plant growth regulators. BRs reduce H.M uptake via membrane permeability alteration, improve soluble proteins and increasing ATPase activity (Barbafieri and Tassi, 2011). Moreover, they help in metal detoxification by enhancement of antioxidative system via scavenging enzymes and proline accumulation (Vriet et al., 2012). However, ACE supplementation under Pb and Cd showed increase of SFA and MUFA, while decrease PUFA, alkanes and sterols (Figure 21 and table 10, 11). Moreover, ACE application alone showed increase of PUFA, alkanes and sterols, which have a protective role against stress. The accumulation of these metabolites can explain the role of ACE to alleviate H.M stress regarding to metabolites accumulated in comparison with plants under metal stress alone. In accordance, Rachidi et al., (2021) reported that microalgae polysaccharides showed redistribution of metabolites with improvement of lipids, alkanes, and sterols in tomato plants. Moreover, the treatment of tomato plant by liquid microalgae extract showed enhancement of SFA especially palmitic and stearic acid which are the first stage of de novo lipid synthesis (Mutale-joan et al., 2020).

To investigate the effect of ACE on Pb and Cd accumulation, tomato plants exposed to 2 mM of Pb and Cd alone and combined with ACE. Tomato plant showed significant accumulation of Pb and Cd in root by $10.7 \mu\text{g g}^{-1}$ and $13.4 \mu\text{g g}^{-1}$, respectively. As well as a significant accumulation in shoot also with $32.7 \mu\text{g g}^{-1}$ and $3.4 \mu\text{g g}^{-1}$ for Pb and Cd, respectively (Figure 22 (a) and (b)). In line of our results, Ishaq et al., (2020) showed the accumulation of Pb (80 mg Kg^{-1}) and Cd (1.3 mg Kg^{-1}) in tomato leaves irrigated with sewage water compared to 0 mg Kg^{-1} in Pb and Cd for plants treated with tube well. The treatment of tomato plant by 5 mg L^{-1} showed accumulation of Cd in root, stem and leaf by 1800 mg Kg^{-1} , 700 mg Kg^{-1} and 800 mg Kg^{-1} , respectively (Guo et al., 2018). Tomato plant showed accumulation of Cd by 650.9% and 524.1% in shoot and root, respectively at 0.2 mM CdCaCl₂ (Rahmatizadeh et al., 2019). Furthermore, tomato root showed high concentration of Pb and Cd with BCF value of 3.03 and 15.1 at 20 ppm and a TF of 74% and 33.9% (Baruah et al., 2019). This high accumulation of metal in root due to the fact that root is the first part of plant exposed to metal (Eid et al., 2021). The root system ensures the metal uptake and translocation to the above part. It acts also as barrier for their absorption or reducing their passage to the above part of plants. Moreover, plants deploy some physiological process to reduce metal transportation such as cell wall binding, apoplastic barriers, chelating by photoheating and vacuole sequestration (Clemens et al., 2013; Eid et al., 2021). Moreover, ACE supplementation under Pb significantly enhanced the accumulation of Pb in root by 88.78% compared to 2 mM Pb. Moreover, in shoot ACE significantly reduced the accumulation of Pb by 30.18% compared to 2 mM Pb. In accordance, spent mushroom compost (SMC) used as biostimulant of *Megathyrus maximus* in contaminated soil improved significantly H.M uptake and remediation including Pb and Cd from $0.333 \pm 0.00 \text{ mg/100 g}$ of soil and $0.020 \pm 0.01 \text{ mg/100 g}$ of soil, respectively to $0 \pm 0.00 \text{ mg/100 g}$ of soil after treatment at 20% (Asemoloye et al., 2020). Furthermore, ACE significantly reduced Cd concentration in root and shoot by 75.77% and 44.69% compared to 2mM Cd. In line of our results, the treatment by Ca (10 mmol L^{-1}), salicylic acid ($100 \mu\text{mol L}^{-1}$) and epi-brassinolide (1 mol L^{-1}) in combination showed reduction of Cd concentration in root (66.66%), stem (87.14%) and leaf (86.25%) (Guo et al., 2018). ACE application showed reduction of metal accumulation in root or/and shoot, which make ACE to be used for phytostabilisation strategy. Indeed, the use of biostimulant directly in contaminated soil may have metal chelator effect, which enhance metal solubility and uptake by plants (Asemoloye et al., 2020). It may be due also to nutrients competition which ACE enhance nutrients uptake. In this regard, Gharaibeh et al., (2016) showed that zinc combination with Cd may reduce metal concentration in different part of tomato plants. In fruit Cd concentration was 2.38 ± 0.29

growth in irrigated soil by 2.56 mg L^{-1} and 2.03 ± 0.32 under Cd 2.56 mg L^{-1} + Zn 256 mg L^{-1} . Moreover, potassium supplementation at 310 ppm significantly reduced Cd translocation from root to shoot in tomato (Naciri et al., 2021). Calcium also reported as competitor ion of metals. The application of Ca at 2.5 mM in combination with Pb at 2.5 mM in soybean significantly reduced Pb accumulation in roots. Pb concentration in root was 4.5 mg g^{-1} without Ca and 1.7 mg g^{-1} after Ca supplementation to Pb stress (Blanco et al., 2021). Moreover, exogenous application of Ca at 10 mmol L^{-1} reduce Cd uptake and translocation to the above-part of tomato because Cd and Ca competes for calcium channel. K supplementation confers plant exposed to Cd a positive response (Guo et al., 2018). K can efficiently reduce Cd-toxicity and improve health of plant by enhancing photosynthesis activity and the biosynthesis of photosynthetic pigments (Naciri et al., 2021). Ishaq et al., (2020) reported that tomato fruits collected from the land irrigated with sewage water has concentrations of Pb (40 mg Kg^{-1}) and Cd (2 mg Kg^{-1}) exceeded limit imposed by WHO-FAO (FAO/WHO, 2019). Tomato plant showed a significant increase in Cd concentration in root (from 1.41 ± 0.37 to 27.01 ± 9.60 , shoot (from 0.95 ± 0.25 to $33.44 \pm 1.14 \text{ mg kg}^{-1}$) and fruits (from 0.31 ± 0.08 to $2.38 \pm 0.29 \text{ mg kg}^{-1}$) with increasing Cd level in soil (from 2.51 ± 0.26 to $5.03 \pm 0.20 \text{ mg kg}^{-1}$) (Gharaibeh et al., 2016).

Our results showed translocation inhibition from root to shoot, which tomato plants exposed to 2 mM Pb showed a TF exceed 1, which was 3.01 ± 0.037 . Moreover, ACE application to plant stressed by 2 mM Pb showed reduction in TF, which was 1.11 ± 0.028 . For Cd, ACE showed in inhibition of Cd uptake and accumulation and both BCF and TF doesn't exceed 1 (Table 12). Even that the concentration found in tomato plants parts doesn't exceed the limit imposed by the FAO/WHO for both Pb and Cd (FAO/WHO, 2019). But tomato showed the capability of metal uptake and translocation to the above part of plant, which represent a health risk to animal and humans. For this reason, an inhibition of metal uptake and translocation to the above part is necessary. Indeed, our study showed the potential of ACE as biostimulant of plant tolerance to heavy metals, which can minimize Pb and Cd uptake and translocation to the above part. In accordance, Gharaibeh et al., (2016) reported that fruits showed low Cd accumulation than shoot and root. Moreover, Eid et al., (2021) exhibited that TFs of H.M < 1.0 in *L. esculentum*, were while BCFs of H.M exceed 1 except for Al, Fe and Mn. Therefore, Metal accumulation may perturbate other nutrients uptake and distribution because of the competition in the transporters channel.

In this regard, we investigate the effect of ACE on nutrients uptake and redistribution under Pb and Cd stress (Figure 23 and 24). 2 mM Cd significantly increased N concentration in root by 17.83% compared to control. Moreover, ACE supplementation under 2mM Cd enhanced more this accumulation to 27.60%. N concentration in shoot was increased by 2 mM Pb and Cd to 49.23% and 73.86%, respectively in comparison to control. Furthermore, ACE treatment enhanced N concentration in shoot to 54.64% and 133.01% under Pb + ACE and Cd + ACE, respectively. P concentration showed no significant difference in root and shoot under metal stress and even after ACE supplementation. For K, 2 mM Pb and Cd showed significant increase of K in root accumulation by 14.38% and 7.22%, respectively. The treatment by ACE significantly reduced K concentration by 11.38% and 14.19% under Pb + ACE and Cd + ACE, respectively compared to Pb and Cd. Moreover, K exhibited a significant increase in shoot by 19.71% and 19.51% under Pb and Cd, respectively compared to control. NPK are the most important nutrient for plant growth and development (Fontana et al., 2020). N is an important mineral element for plant productivity, which found mostly in nitrate, ammonium and organic molecules such as amino acids (Hussain et al., 2018). In the present study metal stress induced increase in N content in root and shoot even when added ACE. Moreover, our results showed increase of soluble proteins and enzymes activity which indicate the functional use of N assimilated by plant under each treatment. In accordance, Schreiber et al., (2018) showed the potential of *Chlorella vulgaris* as physiostimulators to enhance N and P accumulation in wheat plant. Generally, K deficiency decreased Chl a and b biosynthesis, which negatively affect plant growth and development (Fontana et al., 2020). In the present study, metals stress induced increase in K level in plant. However, we found a decrease in Chl a and b content in plant. This finding indicates that Pb and Cd affect the function of nutrients in photosynthetic pigments not the essential nutrients uptake. In accordance, Li et al., (2016) reported significant increase of K concentrations in welsh onion roots, pseudostems and leaves by 21-24%, 111-116%, and 66-74%, respectively under 2.5 mg Kg⁻¹ Cd. Contrary, H.M stress showed a significant reduction of NPK concentration in soil irrigated with wastewater by 124.38%, 40.39%, 65.80%, respectively (Ahmed and Slima, 2018). Furthermore, the increase of some mineral concentrations under H.M stress might be attributed to their incorporation for Cd detoxification (Li et al., 2016). In conclusion, ACE may enhance nutrients uptake and translocation to minimize the uptake of Pb and Cd and detoxify these metals when they are present in plants parts, in turn enhance plant tolerance to metal stress.

The effect of metal and treatment by ACE on other nutrients concentration was showed in Figure 24, 2 mM Pb significantly reduced the concentration of Ca, Zn, Mg, Cu and Cr in root by 84.10%, 30.70%, 14.88%, 22.31% and 50%, respectively compared to control. ACE supplementation significantly increased the concentration of Zn (92.31%), Mg (31.78%), Cu (34.39%) and Cr (42.85%) compared to 2 mM Pb. 2 mM Cd significantly reduced the concentration of Ca (81.76%), Cu (0.82%) and Cr (39.28%) in root compared to control. In line of our results, 200 μ M Cd application in tomato plant showed significant decrease in Fe content by 123.3% and 110.5%, K content by 132.2% and 217.5%, Mg content by 182.3% and 518.7%, Ca content by 246.5% and 252.3% in both shoot and root ($P < 0.05$) (Rahmatizadeh et al., 2019). Moreover, a significant reduction of macroelement (N, P and K) and micro elements was recoded in *Pisum sativum* cultivated in polluted site which contains important concentrations in Pb and Cd (Galal et al., 2021). Both Pb and Cd reduced Mg content in shoot, which represent the main element of chlorophyll molecule. That's can explain the reduction in chlorophyll pigments content in our study (Fig 19 (a), (b) and (c). Moreover, Mg deficiency showed chloroplast destruction, reduction in chlorophyll synthesis and photosynthetic carbon fixation (Ge et al., 2022). Furthermore, ACE supplementation significantly enhanced concentration of Ca and Cu by 644.29% and 26.39%, respectively compared to 2 mM Cd. In shoot 2 mM Cd significantly reduce Mn, Mg, Cu and Cr concentrations by 15.10%, 4.91%, 44.88% and 77.08%, respectively compared to control. The concentration of these element was increased significantly after treatment by ACE with Mn (27.91), Mg (9.67%), Cu (79.63%) and Cr (327.43%) compared to 2 mM Cd. 2 mM Pb significantly reduced Mg concentration by 12.49% compared to control, while enhanced after treatment by ACE with 12.71% compared to 2 mM Pb. In this regard, Asemoloye et al., (2020) reported that the use of SMC at 20% as biostimulant of *Megathyrus maximus* enhanced nutrients status in contaminated soil with P (88.49%), K (63,44), Ca (84.83%), Mg (3036,36%), Mn (351,68%). The use of *C. reinhardtii* and *C. sorokiniana* as biostimulant improved Mn (9.58%; 14.96%) and Cu (28.75%; 3.56%), respectively in maize seedling (Martini et al., 2021). However, microalgae extract application improved nutrients uptake and concentration in broccoli by (7%–182%) (Kusvuran, 2021). Kusvuran, (2021) showed that plant leaves treatment by *C. vulgaris* extract may affect positively plant nutrients content. The use of biostimulants may enhance nutrients uptake, which improve plant growth and productivity. Moreover, limiting the use of chemical fertilizers and protecting environment (Wozniak et al., 2020). Numerous studies showed that biostimulant may protect plants from the excess and deficiency of nutrients (Kapoor et al., 2021; Kusvuran, 2021; Wozniak et al., 2020). Vernieri et al., (2006) reported that the application of biostimulant

reduced the nitrate content in leaves. Moreover, microalgae may act as physioactivators, which can stimulate nitrate reductase and other enzymes incorporated in minerals absorption and transformation in plants (Kusvuran, 2021). Microalgae have bioactive molecules which has a major role in agriculture such improving nutrient uptake, physiological status, crop performance and abiotic stress tolerance (Kholssi et al., 2019; Renuka et al., 2018).

CONCLUSION

Pb and Cd treatment reduced tomato growth and photosynthetic pigments through the generation of ROS and nutrients uptake disruption. However, ACE application showed corrective effect which increased tomato growth under Pb and Cd and photosynthetic pigments. Tomato exposure to Pb and Cd showed increase in H₂O₂, which in results increase lipids peroxidation (MDA). In response, tomato accumulated proline, polyphenols and increased scavenging enzymes as SOD, CAT, and POD against heavy metal stress. Furthermore, ACE can improve plant tolerance to H.M stress by stimulation of plant oxidative system at early stage. ACE treatment alone showed elevation of H₂O₂. However, ACE supplementation showed reduction of H₂O₂, MDA and scavenging enzymes activities after their elevation under Pb and Cd stress. Metal stress application showed that 2 mM Pb and Cd reduced SFA and MUFA, while increased PUFA, alkanes and sterols compared. However, ACE supplementation under Pb and Cd showed increase of SFA and MUFA, while decrease PUFA, alkanes and sterols. Tomato plant showed significant accumulation of Pb and Cd in root. As well as a significant accumulation in shoot. Whereas ACE application favored heavy metals accumulation in root and inhibited their translocation to shoot. Thus, ACE can be used as biostimulant of plant tolerance to heavy metals by minimizing H.M translocation to the aboveground part and to be used in phytostabilisation strategy. Pb and Cd inhibited nutrients uptake and their distribution in plants. ACE application showed a positive effect in nutrients uptake and translocation under Pb and Cd stress. ACE can alleviate metal stress by enhancing antioxidative system and nutrients status from the uptake to redistribution in different plant parts.

IV. Synthèse

Le traitement des plantes de la tomate sous 2 mM de Pb et Cd par ACE a montré une amélioration de la croissance et des pigments photosynthétiques des plantes de la tomate en réduisant le stress métallique et en restaurant leurs dommages. Ces résultats sont en cohérence avec nombreuses études qui ont permis de révéler aussi un effet positif des biostimulants à base des microalgues sur la croissance, la photosynthèse et le rendement des plantes en récupérant les effets délétères des métaux lourds (Ronga *et al.*, 2019; Abd El-All *et al.*, 2021; Li *et al.*, 2021). L'effet stimulant de l'extrait des microalgues peut être dû à la présence des substances favorisant la croissance des plantes telles que : macro et micronutriments, acides aminés, acides gras, polysaccharides, phytohormones, etc., qui peuvent affecter la physiologie cellulaire des plantes en améliorant la croissance et la productivité des plantes (Behera *et al.*, 2021; Ferreira *et al.*, 2021; Li *et al.*, 2021).

De plus, nos résultats ont montré que l'ACE peut améliorer la tolérance des plantes au stress des métaux lourds en stimulant le système oxydatif à un stade précoce. En effet, le traitement des plantes de tomate par ACE seul a montré une élévation de H₂O₂. Par la suite, le traitement des plantes sous Pb et Cd par ACE a montré une réduction de la concentration des H₂O₂, MDA, enzymes et molécules antioxydantes après leur élévation sous Pb et Cd stress. Ce qui indique que les plantes ont été immunisé contre le stress par la première élévation de H₂O₂ de l'ACE. En accord, des études antérieures ont montré que l'utilisation des biostimulants a réduit les enzymes et molécules du système oxydatif après leur élévation sous stress des métaux lourds (Alves *et al.*, 2020; Abd El-All *et al.*, 2021). En effet, les biostimulants à base des microalgues contiennent des molécules bioactives telles que la proline, les bêtaïnes, polyphénols, caroténoïdes et ect. les phytohormones qui peuvent réguler l'homéostasie des redox de la plante conduisant à une tolérance/résistance au stress (Sharma *et al.*, 2014; Ferreira *et al.*, 2021).

L'application de l'ACE aux plantes sous Pb et Cd a montré une augmentation des SFA et MUFAs, tout en diminuant les PUFAs, les alcanes et les stérols. En revanche, le traitement des plantes de tomate par l'ACE seule, a montré une augmentation des PUFAs, des alcanes et des stérols. Ces derniers métabolites jouent un rôle primordial dans la tolérance aux stress abiotiques et biotiques (Jump, 2002). Les PUFAs sont les composés principaux de la membrane, qui maintiennent leur perméabilité, intégrité et fluidité. En plus, ce sont les précurseurs de la voie LOX entraînant une biosynthèse des oxylipines après leur peroxydation (Lim *et al.*, 2017; Sugimoto *et al.*, 2022). Les alcanes et alcènes sont également des composés de la cuticule de la

membrane, qui jouent un rôle indispensable en tant que barrière de défense contre les attaques abiotiques et biotiques (Bourdenx *et al.*, 2011; Wang *et al.*, 2015). Par ailleurs, Les stérols des plantes peuvent agir comme des précurseurs des Brassinostéroïdes (BRS) (Kour *et al.*, 2021). L'accumulation de ces métabolites peut expliquer le rôle de l'ACE dans l'amélioration de la tolérance et la résistance des plantes aux métaux lourds. Ces résultats sont en cohérence avec des études antérieures qui montrent que les microalgues favorisent l'accumulation des métabolites (PUFAs, alcanes, alcènes, stérols etc.) incorporés dans la résistance et la tolérance aux stress abiotiques et biotiques (Mutale-joan *et al.*, 2020; Rachidi *et al.*, 2021).

La présente étude a montré l'accumulation de Pb et Cd dans les racines et la pousse de la tomate. En accord, plusieurs études ont rapporté l'accumulation des métaux lourds dans les différentes parties de la tomate (Guo *et al.*, 2018; Ishaq *et al.*, 2020). Le traitement par l'ACE a montré l'augmentation de l'accumulation de Pb et Cd dans les racines. En revanche, l'application de l'ACE a minimisé la translocation de ces métaux vers la pousse ce qui ouvre la possibilité d'utilisation de l'ACE dans la stratégie de phytostabilisation. En accord, l'utilisation des biostimulants a montré une amélioration considérable de l'absorption des métaux lourds et la remédiation des sols contaminés (Guo *et al.*, 2018; Asemoloye *et al.*, 2020). En effet, l'utilisation directe du biostimulant dans un sol contaminé, peut causer un effet de chélateur métallique, ce qui améliore la solubilité des métaux et leurs absorption par les plantes (Asemoloye *et al.*, 2020). Cela peut également être dû à la compétition en absorption avec autres nutriments, en tant que les biostimulants améliorent l'absorption des nutriments (Gharaibeh *et al.*, 2016; Blanco *et al.*, 2021; Naciri *et al.*, 2021). À cet égard, l'absorption des métaux lourds par la plante peut perturber l'absorption des autres nutriments et leur distribution en raison de la compétition dans les canaux des transporteurs. En revanche, le traitement par ACE peut améliorer l'absorption et la translocation des nutriments pour minimiser l'absorption de Pb et Cd et détoxifier ces métaux lorsqu'ils sont présents dans les parties des plantes, améliorent à leur tour la tolérance des plantes au stress métallique.

V. Conclusion

L'exposition des plantes de tomate aux Pb et Cd a réduit leur croissance et pigments photosynthétiques en générant des ROS et en perturbant l'absorption des nutriments. Cependant, le traitement de ces plantes par l'ACE a montré un effet correctif qui a augmenté la croissance de la tomate et des pigments photosynthétiques sous Pb et Cd. Par ailleurs, l'ACE peut améliorer la tolérance des plantes au stress métallique en stimulant le système oxydatif des plantes à un stade précoce. En effet, le traitement par l'ACE seul a montré une élévation de H_2O_2 . En revanche, le traitement de la tomate exposé aux Pb et Cd par ACE a montré une réduction de H_2O_2 , MDA et des enzymes du système antioxydant après leur élévation sous 2 mM de Pb et Cd. De plus, 2 mM de Pb et Cd réduisaient les SFA et les MUFAs, tandis que les PUFAs, les alcanes et les stérols augmentaient par rapport au témoin. Cependant, le traitement par ACE sous Pb et Cd a montré une augmentation des SFA et des MUFAs, tandis qu'une diminution des PUFAs, des alcanes et des stérols. Par ailleurs, la tomate a montré une accumulation significative de Pb et Cd dans les racines et la partie aérienne. Le traitement de ces plantes par ACE favorisait l'accumulation de Pb et Cd dans les racines et inhibait leur translocation vers la partie aérienne de la plante. Par conséquent, l'ACE peut être utilisé comme biostimulant de la tolérance des plantes au stress métallique en minimisant la translocation des métaux lourds vers la partie aérienne des plantes et ouvre l'utilisation de l'ACE dans la stratégie de phytostabilisation. Le Pb et le Cd ont inhibé l'absorption des nutriments et leur distribution dans la plante. Par la suite le traitement par l'ACE a montré un effet positif sur l'absorption et la translocation des nutriments sous 2 mM Pb et Cd. En conclusion, l'ACE peut atténuer le stress métallique en améliorant le système antioxydant, l'absorption et redistribution des nutriments dans différentes parties de la plante, l'accumulation des métabolites qui aident à la résistance au stress et l'inhibition de la translocation des métaux vers la partie aérienne.

DISCUSSION GENERALE

Cette thèse s'insère dans le cadre de la mise en place d'un projet de bioraffinerie intégrée sous forme d'un bioprocédé à base des microalgues pour le traitement des eaux usées et la valorisation de la biomasse issue en chaîne pour la production des bioproduits de haute valeur ajoutée. En effet, cette biomasse sera l'objet d'une étude approfondie pour sa valorisation en biodiesel principalement la partie lipidique. Bien que, la biomasse résiduelle de ce processus sera orientée vers la production des biostimulants de la tolérance des plantes au stress métallique. Ce travail va permettre d'une part de sélectionner des souches de microalgues candidates pour le développement des bioprocédés intégrés. Et d'autre part, comprendre l'effet des biostimulants sur les voies physiologiques, biochimiques et métabolomiques impliquées dans la tolérance des plantes au stress métallique.

Les microalgues ont été cultivées dans des eaux usées domestiques afin d'avoir leur potentiel d'épuration des eaux usées. Notre étude a montré la performance de croissance des microalgues dans les eaux usées et leur aptitude à traiter les polluants chimiques des eaux usées, principalement l'azote (N) et le phosphore (P) avec une efficacité qui dépasse 99%. Cette efficacité s'accorde avec les standards d'épuration des eaux usées imposés par Directive 98/15/CE avec pouvoir épuratoire de 80% et 70–80% pour un total de P et N, respectivement. Ces résultats sont en accord avec des études précédentes où l'utilisation des microalgues dans le traitement des eaux usées a montré un pouvoir épuratoire entre 90% et 100% surtout pour N et P (Daneshvar *et al.*, 2019; Li *et al.*, 2020).

En outre, les microalgues ont marqué par leurs aptitudes à croître dans les eaux usées avec une production de biomasse élevée par rapport au milieu conventionnel (BG11). Nous avons noté une production maximale de 0,82 g. L⁻¹ chez *C. pyrenoidosa* et *C. ellipsoidea* au niveau des eaux usées. L'augmentation de la production de biomasse dans les eaux usées par rapport au milieu synthétique (BG11) peut être attribuée au mode de culture mixotrophique dans les eaux usées riche en matière organique (Fuentes-Grünwald *et al.*, 2021; Manzoor *et al.*, 2022). Cette approche est mentionnée par d'autres études dont *C. vulgaris* and *S. obliquus* ont révélé une haute production de biomasse avec 1,81 et 1,86 g. L⁻¹ respectivement, sous la mixotrophie dans les eaux usées (Trivedi *et al.*, 2019; Kabir *et al.*, 2020).

De plus, l'analyse biochimique de la biomasse a montré une accumulation des lipides dans les cellules des microalgues par rapport aux protéines et sucres. *C. sorokiniana* a exhibé le taux le plus élevé des lipides dans les eaux usées avec 37% contre 15% au niveau du milieu conventionnel (BG11) suivi par *Aphanothece* sp. et *S. obliquus* avec 35,16% et 33,7% dans les eaux usées. Généralement les microalgues sous stress tend vers l'accumulation des lipides comme forme de réserve pour assurer

leur survie (Yadav *et al.*, 2019; Li *et al.*, 2020). En conséquence, l'utilisation des eaux usées pour la culture des microalgues favorise l'accumulation des lipides qui peuvent se transformer en biodiesel.

La production du biodiesel à partir des microalgues au niveau industriel est conditionnée par le coût de production et la rentabilité de l'approche adopter. À cet égard nous avons proposé une stratégie intégrée de production des microalgues en utilisant les eaux usées comme milieu de culture à faible coût avec un impact environnemental positif. De plus, la valorisation de la biomasse issue de ce processus pour la production des bioproduits en bioraffinerie. Dans ce projet, les souches de microalgues cultivées en eaux usées ont présenté un taux de lipides très important qui peut atteindre, jusqu'à 38-40% chez *C. sorokiniana*, *Aphanothece* sp. et *S. obliquus*. La transestérification de ces lipides résulte en Acides Gras Méthyle Ester (AGME) qui sont eux-mêmes le biodiesel ont l'objet d'analyse par GC-MS.

En terme de qualité du biodiesel nous avons suivi les exigences internationales des Standards Européennes EN 14214 (European Standard EN 14214, 2009). L'abondance en acides gras saturés par rapport au acides gras polyinsaturés est le 1^{er} facteur d'évaluation de la qualité du biodiesel. En effet, toutes les souches utilisées dans cette étude ont montré un pourcentage élevé en SFA par rapport au PUFAs. Le 2^{ème} facteur c'est de pouvoir vérifier le pourcentage de l'acide linoléique (C18 :3) qui ne devait pas dépasser 12%. Nos résultats présentent un pourcentage inférieur à 12 % pour les souches des microalgues cultivées en eaux usées. Le dernier facteur biologique à vérifier pour la qualité du biodiesel est le pourcentage des PUFAs qui ne doivent pas dépasser 1%. Selon ces critères de sélection, la souche *C. reihardtii* a été retenue comme souche candidate pour la mise en place d'un bioprocédé pour le traitement des eaux usées et la valorisation de la biomasse en chaine pour la production des bioproduits à hautes valeur ajoutée.

En accordance, les acides gras les plus couramment trouvés dans les microalgues sont l'acide palmitique, l'acide stéarique, l'acide oléique et l'acide linoléique. En outre, ces FA sont les plus appropriés pour produire un biodiesel de bonne qualité y compris sa viscosité, sa qualité d'allumage, son pouvoir lubrifiant et sa chaleur de combustion (Chisti, 2007; Ji *et al.*, 2015; Ge *et al.*, 2018; Manzoor *et al.*, 2022). Par ailleurs, la richesse de la biomasse des microalgues par plusieurs métabolites que ça soit primaires ou secondaires présente un atout de leur valorisation en bioraffinerie. Dans la présente thèse après valorisation des lipides en biodiesel les résidus qui restent ont l'objet par valorisation en agriculture en tant que biostimulant face au stress métallique.

Afin de compléter la valorisation de la biomasse en bioraffinerie pour achever notre stratégie intégrée, les résidus de production du biodiesel ont l'objet de développement d'un biostimulant des plantes contre le stress métallique. Les réponses physiologiques, biochimiques et métaboliques ont été étudiés pour évaluer l'effet de ces biostimulants sur la tolérance des plantes de la tomate au stress métallique. Dans cette étude, nous avons déterminé la concentration de métal qui cause des altérations aux plantes. Ensuite, nous avons effectué un criblage de l'effet des extraits de microalgues sur la tolérance des plantes au stress et l'élimination de leurs altérations. La concentration 2 mM de Pb et Cd a été prouvée comme concentration de stress chez les plantes de la tomate. Du coup, le traitement des plantes de tomate sous ces concentrations toxiques de deux métaux par les extraits de trois souches *Aphanothece* sp., *C. vulgaris* et *C. Pyrenoidosa* à 1% ont amélioré significativement la croissance des plantes en comparaison avec les plantes non traitées. Nos résultats sont en accord avec Abd El-All *et al.*, (2021) qui a montré l'effet d'extrait d'algues (SWE) sur la croissance, la productivité et l'élévation de stress métallique chez la tomate. Aussi que, les biostimulants issus des microalgues ont un effet positive sur la croissance et la productivité des plantes par la réduction de stress et la réparation des dommages antérieurs (Ronga *et al.*, 2019).

Les pigments photosynthétiques ont montré une réduction significative sous une concentration de 2 mM de Pb et Cd. Le traitement par MCE a montré une normalisation des activités des pigments photosynthétiques comparés aux plantes non traitées et sous 2 mM Pb et Cd. Les résultats sont en cohérence avec d'autres études qui ont montré la réduction de Chl a et Chl b sous stress métallique. Cette réduction dépend à plusieurs causes: l'inhibition de la biosynthèse de chlorophylle, l'augmentation de l'activité de chlorophyllase, la génération des ROS et l'inhibition de l'assimilation des nutriments essentielles pour la photosynthèse comme Mg^{2+} , K^+ , P, Fe^{2+} , Mn^{2+} (Jimenez *et al.*, 2020; Faizan *et al.*, 2021; Naciri *et al.*, 2021). Par contre, le traitement des plantes par des biostimulants algaux a montré une amélioration significative des pigments photosynthétiques sous stress métallique (Jimenez *et al.*, 2020; Supraja *et al.*, 2020a; Supraja *et al.*, 2020b). L'effet stimulant de l'extrait des microalgues principalement due à la présence des substances favorisant la croissance des plantes telles que: les macro et micro nutriments, les acides aminés, les acides gras, les polysaccharides, les phytohormones, etc., qui peuvent affecter la physiologie cellulaire des plantes en améliorant la croissance, les pigments photosynthétiques et la productivité des plantes (Ronga *et al.*, 2019; Behera *et al.*, 2021; Ferreira *et al.*, 2021; Li *et al.*, 2021).

Le traitement des plantes de tomate sous 2 mM de Pb et Cd avec 1% de l'extrait brut des microalgues (ACE) a montré la réduction de H_2O_2 , MDA, l'activité des enzymes et même les molécules antioxydantes "scavenging" après leur augmentation sous stress métallique. En accord avec nos

résultats, Venkatachalam *et al.*, (2017) ont rapporté que le traitement de *Leucaena leucocephala* sous Pb et Cd par des nanoparticules d'oxyde de zinc (ZnONPs) préparées à partir de la biomasse d'*Ulva lactuca* a montré une réduction significative de SOD, POD et CAT. En outre, la pulvérisation foliaire de SWE sur tomate sous stress métallique a réduit de manière significative l'activité POD et a ainsi récupéré l'effet délétère des H.M (Abd El-All *et al.*, 2021). Des études précédentes ont montré le potentiel de l'extrait des microalgues pour moduler le stress oxydatif chez les plantes (Rachidi *et al.*, 2021; Mutale-joan *et al.*, 2021). Le biostimulant des microalgues contient des molécules bioactives telles que la proline, les bêtaïnes, les phytohormones qui peuvent réguler l'homéostasie redox des plantes conduisant à la résistance et à la tolérance au stress (Ferreira *et al.*, 2021). En effet, les EBM agissent comme des bioéliciteurs, qui améliorent la tolérance des plantes au stress métallique en activant le système antioxydant.

Pour approfondir l'étude de l'effet de l'ACE sur les plantes de la tomate sous stress des métaux lourds (Pb ou Cd) nous avons utilisé une approche métabolomique basée sur la GC-MS. L'application de stress métallique a montré que 2 mM de Pb et de Cd réduisaient les SFA et les MUFAs, tandis qu'ils augmentaient les PUFAs, les alcanes, alcènes et les stérols par rapport au témoin. Cette augmentation des PUFAs, des alcanes et des stérols peut être considérée comme une réponse de la plante pour préserver la membrane de l'altération des métaux. De plus, le traitement des plantes sous stress de Pb et Cd par l'ACE a montré une redistribution des métabolites accumulés avec diminution des PUFAs, VLCFA, alcanes/alcènes et stérols et augmentation des SFA et MUFAs. Les SFA tels que C16 et C18 sont les principaux précurseurs de la synthèse de la cuticule. De plus, l'élongation de ces AG en VLCFA, la désaturation en PUFAs ou la décarboxylation pour former des alcanes conduisant à une cuticule mature qui joue un rôle majeur dans la défense des plantes contre le stress abiotique et biotique et pour prévenir la perte d'eau (Lim *et al.*, 2017). Les PUFAs, les alcanes, les alcènes et les stérols sont les principaux métabolites qui jouent un rôle dans la tolérance aux stress abiotiques et biotiques (Jump, 2002).

Les PUFAs et VLCFA sont les principaux composés de la membrane, qui maintient la perméabilité, l'intégrité et la fluidité de la membrane plasmique (Kumar *et al.*, 2012; Kazaz *et al.*, 2022). Ils jouent également un rôle dans la génération de ROS via l'activation de la NADPH oxydase (Yaeno *et al.*, 2004; Di Palma *et al.*, 2020). En effet, l'augmentation des PUFAs est en cohérence avec l'augmentation des ROS dans les feuilles de tomate sous 2 mM de Pb et Cd dans ce travail présenté par H₂O₂. Les principaux PUFAs trouvés sous stress de Pb et Cd étaient C18:2, C16:3 et C18:3, qui sont particulièrement importants dans la défense des plantes en tant que précurseurs de la voie LOX entraînant la biosynthèse des oxylipines après leur peroxydation (Lim *et al.*, 2017).

Les alcanes sont également parmi les composés de la membrane et de la cuticule, qui jouent un rôle vital en tant que barrière de défense contre les attaques abiotiques et biotiques (Bourdenx *et al.*, 2011; Wang *et al.*, 2015). Les stérols jouent un rôle pilote dans la formation et la préservation des membranes, contre les stress abiotiques et biotiques (Rogowska and Szakiel, 2020). Les phytostérols peuvent agir comme précurseurs des Brassinostéroïdes (BRs) (Kour *et al.*, 2021). En effet, les BRs améliorent la tolérance des plantes au stress métalliques, augmentant ainsi le rendement et la qualité des cultures (Vriet *et al.*, 2012). Ils peuvent éliminer les métaux toxiques via un système de phytoremédiation assistée par des régulateurs de croissance des plantes. Les BRs réduisent l'absorption des H.M via une altération de la perméabilité membranaire, améliorent les protéines solubles et augmentent l'activité ATPase (Barbafieri and Tassi, 2011). De plus, ils aident à la détoxification des métaux en améliorant le système antioxydant via des enzymes "scavenging" et l'accumulation de la proline (Vriet *et al.*, 2012).

Pour étudier l'effet des ACE sur l'accumulation de Pb et de Cd, des plantes de tomates ont été exposés à 2 mM de Pb et de Cd seuls et combinées avec ACE. Les plantes de la tomate ont montré une accumulation significative de Pb et de Cd dans la racine par rapport au témoin. Ainsi qu'une translocation significative à la partie aérienne de la plante avec un FBC et FT qui dépassent 1. Nos résultats sont en accord avec Baruah *et al.*, (2019) qui rapportent que la tomate a montré une forte concentration de Pb et Cd au niveau des racines et la partie aérienne de la plante avec FBC et FT supérieur à 1. De plus, d'autres études ont rapporté l'accumulation de Pb et Cd dans les racines, tiges et feuilles de la tomate (Guo *et al.*, 2018; Rahmatizadeh *et al.*, 2019; Ishaq *et al.*, 2020). Cette forte accumulation de métal dans la racine est due au fait que la racine est la première partie de la plante exposée au métal (Eid *et al.*, 2021). Le système racinaire assure l'absorption et la translocation du métal vers la partie aérienne. Il agit également comme barrière pour leur absorption ou en réduisant leur passage vers la partie aérienne des plantes. De plus, les plantes déploient certains processus physiologiques pour réduire le transport des métaux tels que la liaison des parois cellulaires, les barrières apoplastiques, la chélation par photochauffage et la séquestration dans les vacuoles (Clemens *et al.*, 2013; Eid *et al.*, 2021).

Le traitement des plantes de tomate sous stress métallique par l'ACE a montré une augmentation d'accumulation de Pb dans les racines et une inhibition de la translocation à la partie aériennes de la plante. Par ailleurs, la supplémentation des ACE a montré une réduction de l'accumulation de Cd dans les racines et/ou les pousses, ce qui ouvre la possibilité d'utilisation d'ACE dans la stratégie de phytostabilisation. Conformément, le compost de champignons utilisé (CCU) utilisé comme biostimulant de *Megathyrus maximus* dans un sol contaminé a permis l'amélioration d'une manière significative

l'absorption et la remédiation des H.M, y compris le Pb et le Cd (Asemoloye *et al.*, 2020). En effet, l'utilisation des biostimulants directement dans un sol contaminé peut avoir un effet chélateur des métaux, ce qui améliore la solubilité et l'absorption des métaux par les plantes (Asemoloye *et al.*, 2020). Cela peut également être dû à la compétition avec des autres nutriments en tant que l'ACE améliore l'absorption des nutriments. Des études antérieures ont montré que la combinaison de Pb et/ou Cd avec des autres nutriments réduit la concentration de métal dans les différentes parties de la plante. La combinaison de Zinc avec Cd a réduit la concentration de métal dans les différentes parties de tomate (Gharaibeh *et al.*, 2016). De plus, K supplémentation a réduit l'absorption et la translocation de Cd chez la tomate (Naciri *et al.*, 2021). Le traitement avec le K confère aux plantes exposées au Cd, une réponse positive (Guo *et al.*, 2018). Le calcium est aussi considéré comme un compétiteur des H.M, qui réduit l'accumulation et la translocation de Pb et Cd chez la tomate (Sarah *et al.*, 2021). En effet, notre étude a montré le potentiel de l'ACE en tant que biostimulant de la tolérance des plantes aux H.M à travers la réduction de l'absorption et la translocation de Pb et Cd vers la partie aérienne de la plante. Par ailleurs, le potentiel des ACE pour stimuler l'absorption des nutriments qui rentre en compétition avec les H.M reste la voix la plus propice. A cet égard nous avons choisie d'étudier en plus l'effet d'ACE sur les nutriments sous stress métallique.

Sur le plan nutritionnel de la plante, le traitement algal a amélioré l'absorption des nutriments NPK chez les plantes cultivées dans les conditions de stress métallique. NPK est les nutriments les plus important pour la croissance et le développement des plantes (Fontana *et al.*, 2020). L'azote est un élément minéral indispensable pour la croissance et productivité des plantes. Nous le trouvons principalement sous forme de nitrate, l'ammonium et les molécules organiques telles que les acides aminés (Hussain *et al.*, 2018). Nos résultats sont en accord avec Schreiber *et al.*, (2018) qui ont rapporté le potentiel de *Chlorella vulgaris* en tant que physiostimulateurs pour améliorer l'accumulation de N et P chez le blé. De plus, une augmentation significative des concentrations de K a été signalé dans les racines, les pseudotrons et les feuilles de l'oignon sous Cd (Li *et al.*, 2016). Par ailleurs, l'augmentation de certaines concentrations de minéraux sous stress métallique pourrait être attribuée à leur incorporation pour la détoxification des H.M (Li *et al.*, 2016).

Par contre, le Pb à 2mM a réduit significativement la concentration de Ca, Zn, Mg, Cu et Cr dans la racine et la partie aérienne de la plante. 2 mM de Cd a réduit d'une manière significative la concentration de Ca, Cu et Cr dans la racine et la partie aérienne de la plante par rapport au témoin. En accordance, l'application de 200 μ M de Cd dans la tomate a montré une diminution significative de la teneur en Fe, K, Mg et Ca dans les racines et la partie aérienne (Rahmatizadeh *et al.*, 2019). De plus, une réduction significative des macroéléments (N, P et K) et des microéléments a été enregistrée

chez *Pisum sativum* cultivée en site pollué qui contient des concentrations importantes en Pb et Cd (Galal *et al.*, 2021). Le traitement des plantes de tomate sous 2 mM de Pb et Cd par l'ACE a montré une augmentation de l'absorption et la translocation des nutriments et en tourne normalisation des effets négatives des H.M. En cohérence, l'application des biostimulants issus des microalgues a permis une amélioration de la concentration des nutriments dans les plantes (Wozniak *et al.*, 2020; Kusvuran, 2021; Martini *et al.*, 2021). De nombreuses études ont montré que le biostimulant peut protéger les plantes de l'excès et de la carence en nutriments (Wozniak *et al.*, 2020; Kapoore *et al.*, 2021; Kusvuran, 2021). De plus, les microalgues peuvent agir comme physioactivateurs, qui peuvent stimuler le nitrate réductase et d'autres enzymes incorporées dans l'absorption et la transformation des minéraux dans les plantes (Kusvuran, 2021).

Les microalgues possèdent des molécules bioactives qui jouent un rôle crucial dans l'agriculture, telles que l'amélioration de l'absorption des nutriments, l'état physiologique, la performance des cultures et de la tolérance au stress abiotique (Renuka *et al.*, 2018; Kholssi *et al.*, 2019). En conclusion, l'ACE peut améliorer l'absorption et la translocation des nutriments afin de minimiser l'absorption de Pb et de Cd et de détoxifier ces métaux lorsqu'ils sont présents dans les parties des plantes, ce qui améliore à son tour la tolérance des plantes au stress métallique.

CONCLUSION GENERALE

Notre thèse révèle le potentiel d'utilisation des microalgues marocaine dans une approche intégrée de traitement des eaux usées et la valorisation de la biomasse en bioraffinerie pour la production du biodiesel et biostimulant des plantes contre le stress des métaux lourds. Nos résultats ont montré un fort potentiel d'élimination des nutriments surtout N et P conformes aux normes des directives Européennes pour la qualité d'eau. De plus, les eaux usées étaient appropriées pour la croissance de microalgues générant une productivité élevée en biomasse et lipides. Les souches de microalgues ont montré une abondance en acides gras saturés, en particulier les acides, palmitique et linoléique, avec moins des PUFA surtout dans les eaux usées. En effet, ce type de FA est le plus approprié pour la production du biodiesel. Après analyse de profile lipidique et en accordance avec les Standards Européen (EN 14214), *C. reihardtii* est qualifiée comme candidate pour la production du biodiesel à partir des eaux usées. Pour répondre à l'approche de bioraffinerie intégrée les résidus de processus de production de biodiesel ont été utilisé pour le développement des biostimulants des plantes contre le stress des métaux lourds. De fait, l'exposition des plantes de tomates aux Pb et Cd a réduit leur croissance et pigments photosynthétiques des plantes de tomate en générant des ROS et en perturbant l'absorption des nutriments. Cependant, le traitement de ces plantes par l'ACE a montré un effet correctif qui a augmenté la croissance de la tomate et des pigments photosynthétiques sous Pb et Cd. Par ailleurs, l'ACE a amélioré la tolérance des plantes au stress métallique en stimulant le système oxydatif des plantes à un stade précoce. De plus, à travers l'accumulation des métabolites comme PUFAs, Alcanes/alcènes et stérols incorporés dans la résistance au stress métallique. En outre, la tomate a montré une accumulation significative de Pb et Cd dans les racines et la partie aérienne. Pourtant, le traitement de ces plantes par l'ACE favorisait l'accumulation de Pb et Cd dans les racines et inhibait leur translocation vers la partie aérienne de la plante. Par conséquent, ACE peut être utilisé comme biostimulant de la tolérance des plantes au stress métallique en minimisant la translocation des métaux lourds vers la partie aérienne des plantes avec la possibilité de valorisation dans la stratégie de phytostabilisation. Le Pb et le Cd ont inhibé l'absorption des nutriments et leur distribution dans la plante. Par la suite, le traitement par l'ACE a montré un effet positif sur l'absorption et la translocation des nutriments sous 2 mM Pb et Cd. En conclusion, MCE peut atténuer le stress métallique en améliorant le système antioxydant, l'absorption et redistribution des nutriments dans différentes parties de la plante, l'accumulation des métabolites qui aident à la résistance au stress et l'inhibition de la translocation des métaux vers la partie aérienne. En perspectives, d'autres études seraient nécessaires pour valider ces résultats à l'échelle pilote. La combinaison avec autres types de stress paraît possible et faisable comme la culture des microalgues pour le traitement des eaux usées et la séquestration du

CO₂ avec valorisation de la biomasse dans une démarche de bioraffinerie sortiront par autres produits afin d'assurer la rentabilité. De plus, une caractérisation des extraits des microalgues paraît indispensable pour identifier les molécules responsables de la normalisation des effets délétères des métaux lourds. Leur mécanisme d'action face au stress et la régulation de production de ces métabolites est important aussi. Finalement, la validation d'utilisation de ces extraits dans la serre ou au champ jusqu'au stade fruit pour savoir le potentiel de ces extraits dans l'inhibition de la translocation des métaux lourds vers le fruit.

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ANNEXES

Annexe S1: Microalgae crude extract composition

	<i>Aphanothece</i>	<i>C. vulgaris</i>	<i>C.pyrenoidosa</i>	<i>C. ellipsoidae</i>	<i>C.sorokiniana</i>	<i>S. obliquus</i>	<i>S. dimorphus</i>	<i>C.reinhardtii</i>
Proteins	0,054	0,088	0,052	0,107	0,130	0,017	0,108	0,026
Soluble sugar	0,049	0,050	0,038	0,171	0,083	0,014	0,047	0,025
N (mg/ml)	0,146	0,059	0,043	0,077	0,095	0,083	0,003	0,145
P (mg/ml)	0,226	0,117	0,078	0,317	0,277	0,379	0,333	0,241
K (mg/ml)	0,224	0,075	0,104	0,182	0,145	0,284	0,133	0,132
Ca (mg/ml)	0,164	0,190	0,321	0,212	0,442	0,612	0,391	0,541
Zn (mg/ml)	0,005	0,001	0,002	0,001	0,002	0,001	0,002	0,001
Fe (mg/ml)	0,021	0,017	0,009	0,005	0,020	0,022	0,023	0,020
Mg (mg/ml)	0,032	0,048	0,029	0,026	0,051	0,045	0,055	0,039
Mn (mg/ml)	0,137	0,133	0,076	0,068	0,154	0,179	0,187	0,153
Cr (mg/ml)	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
Cu (mg/ml)	0,002	0,002	0,002	0,002	0,004	0,002	0,002	0,002
Pb (mg/ml)	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
Cd (mg/ml)	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000

Annexe S2: Chemical and physical characteristics of commercial peat

Major Constituents:	Mixture of weakly-moderate decomposed bog peat (H3-H5), strongly decomposed bog peat (H5-H8), with further additions like clay and perlite.	
Organic substances:	70–90% (Dry masses)	
Contents filling:	70L (EN 12580)	
Chemical properties:	pH (CaCl ₂):	5.7
	Salt g/L KCl	0.9
	N mg/L	140
	P ₂ O ₅ mg/L	100
	K ₂ O mg/L	180

Résumé

La présente thèse s'inscrit dans le cadre d'étude du potentiel des microalgues pour le développement d'un bioprocédé intégré pour le traitement des eaux usées et la valorisation de la biomasse microalgale en bioproduits tel que le biodiesel et des biostimulants contre le stress métallique dans l'agriculture. Ces microorganismes photosynthétiques ont montré un fort potentiel d'épuration des polluants majeurs dans les eaux usées, principalement l'azote et le phosphore. Ensuite la caractérisation de la biomasse issue du traitement des eaux usées a montré un taux élevé en lipides de bonne qualité pour la production du biodiesel. De plus, les résidus issus de l'extraction des lipides à partir de cette biomasse font l'objet d'une hydrolyse acide qui va servir en tant que biostimulant des plantes. L'application de cet extrait sur des plantes de tomate a exhibé une amélioration de la croissance et la tolérance des plantes au stress des deux métaux étudiés (Pb et Cd). Ces extraits ont favorisé l'accumulation de Pb et Cd dans les racines des plantes de tomate et inhibé la translocation de ces métaux vers la partie aérienne. En outre, ces extraits ont stimulé le système antioxydant des plantes de tomate face au stress métallique par réduction des enzymes et molécules antioxydantes après leurs augmentations sous ce stress. L'analyse métabolomique par GC-MS a révélé l'accumulation des AGPI, AGLC, alcanes/alcènes et stérols sous Pb et Cd stress. En effet, le traitement des plantes de tomate par les extraits des microalgues a réduit l'accumulation de ces métabolites, tout en favorisant l'accumulation des AGS principalement palmitique et stéarique. Les microalgues pourront être une base pour promouvoir une bioraffinerie efficace et durable pour le traitement des effluents liquides et gazeux et la production de la biomasse microalgale destinées pour la production des bioproduits à haute valeur ajoutée.

Mots-clefs (5) : Microalgues, Eaux Usées, Biodiesel, Métaux Lourds, *Solanum lycopersicum* L., Biostimulant.

Abstract

The present thesis is part of the study of the potential of microalgae for the development of an integrated bioprocess for the treatment of wastewater and of microalgal biomass valorisation in bioproducts such as biodiesel and biostimulants against metal stress in agriculture. These photosynthetic microorganisms have shown a strong potential for wastewater treatment, mainly nitrogen and phosphorus. Then, the biomass characterization from the wastewater showed a high rate of lipids with good quality to produce biodiesel. Moreover, the residues resulting from lipids extraction are subject to acid hydrolysis which will serve as a plant biostimulant. The application of this extract on tomato plants exhibited an improvement in growth and plant tolerance to heavy metals (Pb and Cd). These extracts promoted the Pb and Cd accumulation in the roots of tomato plants and inhibited the translocation of these metals to the aerial part. Furthermore, these extracts enhanced the antioxidant system of tomato plants against heavy metals stress by reducing scavenging enzymes and molecules after their increases under this stress. Metabolomic analysis provided by GC-MS revealed the accumulation of PUFA, UFA, alkanes/alkenes and sterols under Pb and Cd stress. Indeed, the treatment of tomato plants with microalgae extracts reduced the accumulation of these metabolites, while promoting the accumulation of SFA, mainly palmitic and stearic acids. Therefore, microalgae can be a suitable model to promote an efficient and sustainable biorefinery for liquid and gaseous effluents treatment and the production of microalgal biomass intended to produce high value-added bioproducts.

Key Words (5) : Microalgae, Wastewater, Biodiesel, Heavy Metals, *Solanum lycopersicum* L., Biostimulant.