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Review

Exploring The Potential of Microalgae-Fungi Co-Cultivation for Sustainable Bioprocessing in Microalgae Biorefinery

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ABSTRACT

Developing co-cultivation systems involving microalgae and fungi has shown promising potential for microalgae harvesting technology. As discussed in this review, the co-cultivation of microalgae and fungi has emerged as a novel approach for enhancing biomass and lipid production, wastewater treatment, biofuel production, and high-value products. However, despite being used for a few years, this technique is still in its early stages of development and has yet to be widely applied in the industry. The main challenges associated with co-cultivation include designing effective cultivation systems, managing nutrient requirements, selecting compatible strains, and implementing contamination control measures. In this study, bibliometric analysis was conducted (using the Web of Science database) to examine global trends and developments in microalgae-fungi co-cultivation research between 2014 and 2023, which aimed to identify the research progression, prominent contributors, and leading countries in the research field. The dataset comprised 682 articles, 242 reviews, 31 book chapters, and 22 conference papers. The results showed a rapid increment of publications with China as an active nation in this research area, followed by India, the USA, Italy, Spain, etc. As demonstrated in this study, the immense potential of co-cultivation techniques suggests further exploration, particularly in employing different microalgae species with exceptional characteristics in conjunction with non-pathogenic and edible fungi for profitable industrialization.

Key words: Bioflocculation, biofuels, co-cultivation, fungi, microalgae, wastewater

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INTRODUCTION

The rising implementation of microalgal biotechnology has showcased the capacity of microalgae to contribute to multiple Sustainable Development Goals (SDGs) set by the United Nations, including clean energy and zero hunger (Sutherland *et al*., 2021). This approach is especially crucial considering that the world's population is projected to reach 9 billion by 2050, leading to substantial obstacles in future food, energy, and feed supply (Fayyaz *et al*., 2020; Trovão *et al*., 2022). Without a doubt, dealing with the challenge of rapidly increasing energy demand and its impact on the environment is a task that must be addressed in the coming years (Liu, 2020). Despite huge potential, microalgae-based products are restricted to laboratory-scale production (Kumar *et al*., 2020). One of the primary factors hindering the commercialization of microalgae-related products is the issues related to the harvesting of this organism (Matter *et al*. 2019; Hadiyanto *et al*. 2022).

The successful commercialization and industrialization of microalgae biorefinery depend significantly on the ability and effectiveness of cultivating microalgae on a large scale (Wang *et al*., 2022), as well as harvesting and downstream processes (Premaratne *et al*., 2022). Harvesting and dewatering algae biomass is a crucial step in producing algae-based products, as it separates the biomass from the growth medium and concentrates it for

downstream processing. However, it is also a significant cost factor, accounting for up to 20-30% of the total production cost (Musa *et al*., 2019; Tan *et al*., 2020). The problematic features of microalgae, such as growth in a diluted suspension, small size (<30 µm in diameter), and cell-to-cell electrostatic repulsion, create difficulties in extracting the biomass from the liquid media (Singh & Patidar, 2018).

However, co-cultivation techniques have shown the potential to address these difficulties, particularly in producing microalgae-based biofuels. Filamentous fungi strains have effectively harvested microalgae biomass by immobilizing the microalgal cells through interaction with the mycelium. This technique forms pellets of fungi-microalgae, which can be easily separated from the liquid media through simple filtration (Wang *et al*. 2019; Jaiswal *et al*. 2022). Recent studies have found this approach promising (Mathushika & Gomes, 2022). Although most research on algal biofuel production has been based on mono-culture, attention is now turning toward co-culture methods for producing biodiesel (Suchitra & Karthikeyan, 2019). Co-cultivating microalgae with fungi can improve biomass yield and lipid accumulation (Gopal *et al*., 2023). The symbiotic relationship between microalgae and fungi boosts the expression of citric acid, succinic acid, and malic acid, signifying increased activity in the tricarboxylic acid (TCA) cycle. This heightened metabolic activity fosters an effective exchange of energy and substances, facilitating mutual growth. The symbiosis accelerates energy and substance metabolism in both microalgae and fungi, forming a solid foundation for material synthesis and energy, thereby ensuring the stability of the symbiotic system (Wang *et al*., 2023). This approach could be a practical option for surpassing the limitations and enhancing biomass and lipids production (Yen & Chen, 2015; Das *et al*., 2022). In addition, the co-cultivation method has been utilized for wastewater treatment, generating biomass and bioactive compounds (Khan & Kim, 2018).

Previous studies have predominantly focused on utilizing microalgae-fungi co-cultivation for wastewater treatment. This approach has proven effective in remediation efforts (Jiang *et al*., 2019; Guo *et al*., 2020; Padri *et al*., 2022). However, it is essential to couple this approach with producing other valuable products to enhance its viability for large-scale production. This review holds significant importance due to its ground-breaking nature to conduct a bibliometric analysis that focuses solely on the co-cultivation of microalgae and fungi. This unique approach contributes to the existing literature by providing valuable insights into the research landscape of this specific field.

MATERIALS AND METHODS

Data extraction

The data collection approach utilized in this study adhered to the methodology outlined by (Omoregie *et al*., 2022), with adjustments tailored to meet the specific research objectives. The analysis used information retrieved from the Web of Science database from 2014 to 4th December 2023. One notable advantage of the Web of Science is its comprehensive coverage of scholarly literature, providing researchers with a reliable platform to access a wide array of high-quality, peer-reviewed publications across various disciplines (Pranckutė, 2021). The utilization of the keywords "microalgae" and "fungi" in this study served as a strategic approach to investigate various applications and research trends within the chosen topic, with a specific focus on co-cultivation. By incorporating these keywords, the research aimed to capture comprehensive information and insights related to the synergistic interactions between microalgae and fungi, shedding light on their collaborative applications and exploring emerging trends in this specific field of study. To capture relevant publications, the search string incorporated the key term ("Microalg*") AND ("Fung*") NOT (Actinomycetes). The asterisk (*) was utilized to encompass singular/plural variations and diverse word forms like "microalgal" and "microalga" AND “Fungi, Fungus or Fungal”. The search was executed on December, 04, 2023. The search outcomes were examined by considering the year of publication, citations report, and countries/regions. The "analyze results" feature offered by the WoS database was utilized for this purpose. Additionally, using citation report data from the Web of Science, we gathered information on the annual distribution of publications and citations. Microsoft Office Excel 2019 and VOSViewer (version 1.6.11) were used to conduct statistical analyses and visually process the retrieved results. Nine hundred seventy-seven (977) articles were published between 2014 and May 2023, including 682 articles, 242 reviews, 31 book chapters and 22 conference papers. The methodology for extracting the data from WoS is visually depicted in the flowchart in Figure 1.

Bibliometric analysis

Bibliometrics has been widely used to investigate trends in a particular researcher's or discipline's publications, validate the impact of a researcher, identify emerging areas of research, locate potential research collaborators, and discover appropriate outlets for publication (Chen *et al*., 2021). The comma-separated values (CSV) file obtained from the WOS database was imported into VOSviewer to streamline mapping and visualization processes. VOSviewer allows the creating of co-occurrence networks for essential terms extracted from scientific literature using text-mining functionality (Bukar *et al*., 2023). The current study used VOSviewer for bibliometric analysis, utilizing citation data from Web of Science databases. The software employs standardized weights, including the number

and total strength of links, to visually represent the nodal network, where nodes size and intertwining lines indicate the significance and strength of connections (Donthu *et al*., 2020). To identify the major research trend, co-occurrence analysis based on text data from titles and abstract fields was used. Of the total of 314 terms that were obtained, 49 met the threshold (the minimum number of occurrences of a term is 10, based on binary count). The analysis resulted in 45 terms that were used for the co-occurrence network visualization map of research topics. To identify author keywords, a total of 1073 keywords were gathered from the Web of Science. A thesaurus file was employed in a data cleaning procedure to ensure data consistency, eliminating synonymous keywords or those denoting the same concept. This process aimed to harmonize the data, acknowledging the potential variation in keywords used by different authors to articulate their research focus. Following applying a minimum threshold of 3, a final set of 106 keywords was utilized in this study. Overlay visualization was employed to explore these keywords' yearly publications, occurrences, and connections, with different colours indicating the article's year. Analyzing keywords is a crucial step in identifying contemporary trends.

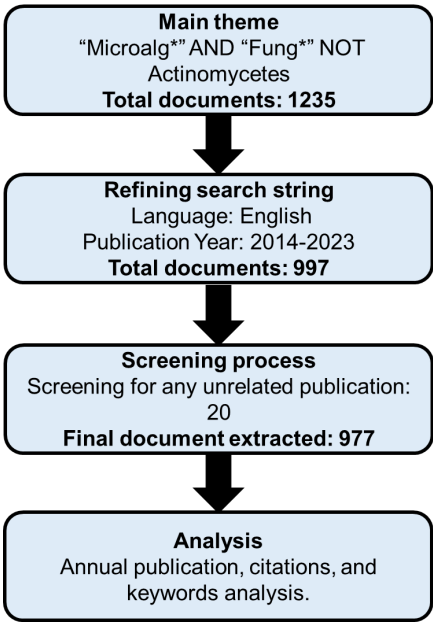

Fig. 1. Flowchart depicting the comprehensive research methodology utilized in data extraction

RESULTS AND DISCUSSIONS

Annual publications and citations

For ten years, 977 articles were published (shown in Figure 2a). From the figure, the yearly distribution of articles was obtained, and only 225 articles from 2014 – 2018 were published, totaling 1,789 citations. Since then, the overall number of publications has fluctuated and grown yearly to 2023 (total articles published were 752 & citations of 18,772), having accelerated growth during 2022. The increasing number of publications may be due to technology's increasing attention and development in recent years. The heightened research interest observed from 2020 to date reflects a compelling emphasis on the imperative to expand microalgae biomass and enhance lipid productivity. This surge in scientific curiosity is grounded in recognizing microalgae's potential as a crucial player in the sustainable production of biofuels. Microalgae known for their rapid growth and high lipid content, are increasingly considered promising third-generation biofuel feedstock (Khoo *et al*., 2020; Feng *et al*., 2022). This recognition stems from the urgency to transition towards renewable and environmentally friendly energy sources, with microalgae standing out as a viable candidate to address the growing demand for sustainable alternatives. However, despite COVID-19 that prevailed during 2019 and 2020, which resulted in the temporary closure of the Universities, there was an increasing number of publications and citations. Similarly, as depicted in Figure 2b, China has emerged as a leading country in publications and citations in microalgae-fungi research. The high number of publications from China indicates the depth and breadth of research in this field. Likewise, the increased number of citations reflects the recognition and importance of this particular study within the scientific community.

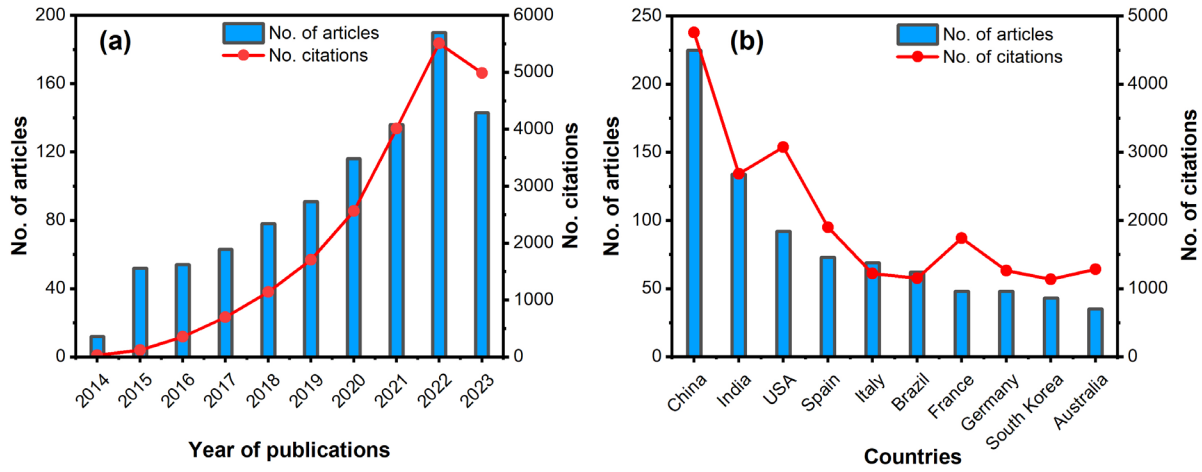

Fig. 2. (a) Number of publications and citations by year from 2014 – 2023. (**b)** Most productive countries and their respective number of published articles and citations

Keywords analysis

The author's co-occurrence network map is shown in Figure 3. Three distinct clusters were identified in the network visualization map represented by different colors. The clusters were categorized based on their colors and size, with larger nodes indicating the importance and frequency of keywords. Out of a minimum of 314 occurrences, only 49 keywords met the threshold and were considered relevant for the co-occurrence network. The red cluster is the cluster I. This cluster focuses on the beneficial interactions between filamentous fungi and algae in co-cultivation systems. Co-culture is the cultivation strategy where filamentous fungi and algae are grown together; wastewater can serve as a nutrient source in co-cultivation systems, allowing for the removal of nutrients and contaminants. The keywords within this cluster shed light on aspects of the co-cultivation process, leading to higher biomass and lipid production, harvesting, and other potential applications. Extraction techniques are employed to recover specific compounds from the biomass produced in co-cultivation systems, such as lipids, pigments, and bioactive molecules, which can be extracted using various methods, e.g., solvent extraction, supercritical fluid extraction, and enzymatic extraction. These compounds hold potential for various applications in industries such as biodiesel production, nutraceuticals, and pharmaceuticals. Cluster II is in blue. The keywords within this cluster highlight the co-cultivation of microalgae and fungi for biofuel production, wastewater treatment, biogas upgrading, nutrient reduction, photoperiod manipulation, CO_2 removal, and utilization. This integrated approach holds great promise in addressing environmental challenges and promoting sustainable development. Furthermore, using CO_2 removal and utilization technologies contributes to greenhouse gas mitigation and sustainability. By embracing this integrated approach, renewable energy can be promoted, and pressing environmental issues can be effectively tackled. Cluster III is in green, encompasses keywords such as performance, nutrient removal, growth, *C. vulgaris* (a type of microalgae), activated sludge, bioremediation, removal efficiency, endophytic bacteria, dilution ratio, photosynthetic performance, nitrogen, CO_2, and intensity. This cluster suggests a research focus on evaluating and optimizing the performance of systems involving *C. vulgaris* and activated sludge for nutrient removal and bioremediation, particularly considering photosynthetic performance, nitrogen utilization, CO_2 dynamics, and light intensity. The research within this cluster likely involves assessing the performance of *C. vulgaris* and activated sludge systems in terms of their efficiency in removing nutrients from various sources, such as wastewater or nutrient-rich environments. The emphasis may be placed on evaluating removal efficiency, growth rates, and overall system performance.

The overlay visualization mapping of the co-occurrence of author keywords is shown in Figure 4. Table 1 presents the frequent recurring keywords and their total link strength. Following applying a minimum threshold of 3, a final set of 106 keywords was utilized in this study. From the figure, it was observed that the term “Microalgae” was the most used author keyword with 84 occurrences, a total link strength of 494, and 91 links to other author keywords. This keyword (Microalgae) also had an average publication year of 2019.93, and average citations of 27.38. Moreover, the term "co-cultivation," which has recently garnered attention from researchers, is notable for its 23 occurrences, a total link strength of 170, and connections to 53 other authors' keywords. Notably, this keyword, "co-cultivation," exhibits an average publication year of 2020.65. Its significance is further emphasized by its strong associations with various topics, including fungi, microalgae, biodiesel production, biomass production, *Chlorella vulgaris*, wastewater treatment, nutrient removal, microalgae harvesting, accumulation, bioflocculation,

and CO_2 removal with the following link strength 12, 11, 10, 10, 10, 7, 7, 6, 5, 4 respectively. Additionally, keywords like proteins, feasibility, anaerobic digestion, wastewater, extraction, optimization, extracellular polymeric substances, flocculation mechanisms, phosphorus, photosynthetic performance, and inhibition were identified as linked to "co-cultivation" with link strengths ranging from 2 to 3. This interconnected network underscores the broad scope of "co-cultivation" within the research landscape, revealing its associations with diverse topics and contributing to a comprehensive understanding of its implications. The variation in the color of the keywords corresponds to shifts in the time frame. In the figure, nodes appearing more yellow signify recent usage of the keywords, whereas purple nodes indicate their appearance before 2014. This color differentiation reveals a dynamic temporal trend, suggesting that the field has experienced growth and development over the past 10 years.

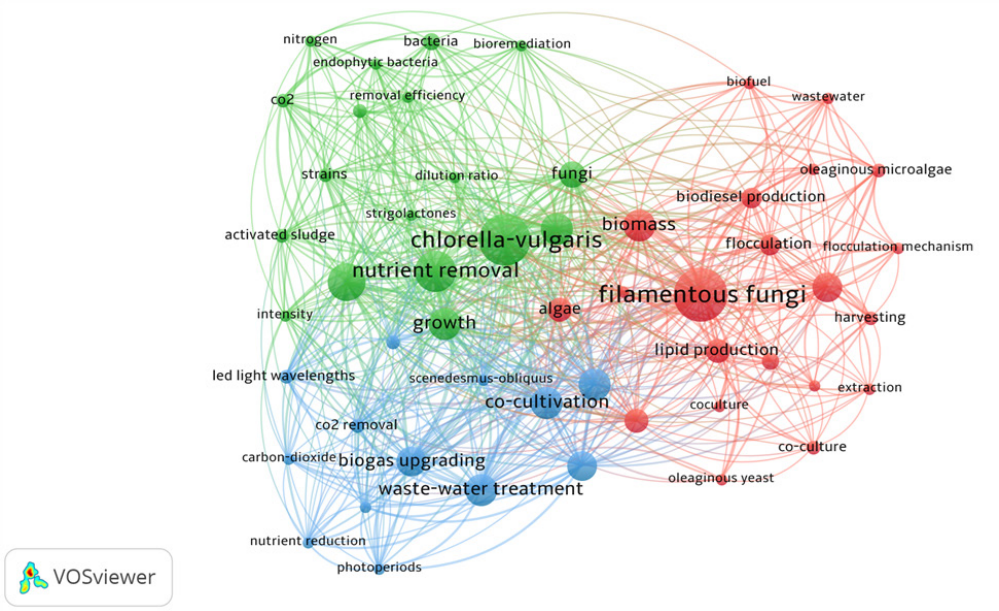

Fig. 3. Co-occurrence networks map of keywords

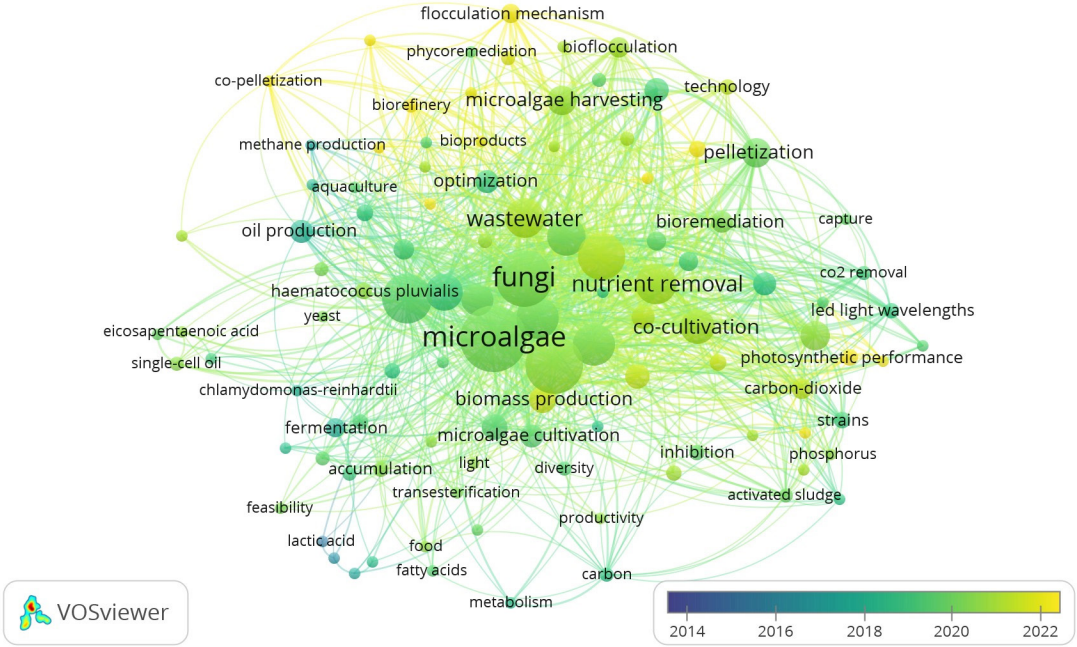

Fig. 4. Overlay visualization based on keywords from 2014 – 2023

Table 1. Frequent recurring keywords and their total link strength

S/no.	Keywords	Occurrences	Total links strength
1	Microalgae	84	494
2	Fungi	62	439
3	Cultivation	64	387
4	Biodiesel production	48	313
5	*Chlorella vulgaris*	43	311
6	Nutrient removal	32	246
7	Growth	35	227
8	Wastewater treatment	29	210
9	Biofuel production	26	189
10	Wastewater	30	186
11	Co-cultivation	23	170
12	Palletization	17	134
13	Microalgae harvesting	17	134
14	Lipid accumulation	20	121
15	Flocculation	12	94

Co-cultivation of microalgae and fungi

The symbiotic relationship between fungi and microalgae allows the formation of pellets that can be separated from the liquid medium. Depending on the pellet's size and shape, they can transport and store microalgae biomass. Besides their mechanical properties, the spherical morphology of these materials also offers various advantages, such as a larger surface area and enhanced mass transfer rate (Wang *et al*., 2021; Jaiswal *et al*., 2022). However, to be considered for harvesting, the fungi should not be environmentally harmful and safe for downstream applications, such as feeds (Ummalyma *et al*., 2017). Interestingly, the co-existence of fungi with microalgae protects cultures from external microbial contaminations (Laezza *et al*., 2022). Similarly, it was reported that the presence of fungi in microalgae biomass did not change its bio-oil or carbon, hydrogen, or nitrogen contents and overall biomass composition (Li *et al*., 2017). The following subsections of this review paper emphasize the application of microalgae-fungi co-cultivation in five key areas: improving microalgae biomass harvesting, improving biomass and lipid production, wastewater treatment, biofuels production, and high-value compound production.

Harvesting microalgae biomass

The co-cultivation of microalgae and fungi has been acknowledged as a cost-effective and environmentally friendly technique for microalgae biomass harvesting. The method is classified as chemical-free and not toxic to algal biomass, enabling the culture medium to be recycled and potentially reducing overall costs (Ummalyma *et al*., 2017). Several studies have explored the combination of filamentous fungi and microalgae cultivation to achieve efficient harvesting and recover valuable biomass. These studies have provided novel insights and focused on obtaining fungal-algal pellets to improve harvesting. The pellets formed after flocculation can be separated from liquid media by simple filtration due to their apparent size ranging from 1-2 cm, facilitating their easy retrieval (Leng *et al*., 2021).

Generally, there are two approaches to co-cultivation: adding pre-cultured fungal pellets to the culture broth of microalgae or inoculating fungal spores together with algal cells to form pellets as depicted in Figure 5. The fungal pellets-assisted bioflocculation (FPA) is usually performed in two steps: preparing fungal spores from the agar plate and adding them to the culture broth of microalgae. Though it can be performed quickly, it requires an extra cultivation medium and a longer cultivation time (at least 48 to 72 hr) for fungal pellet formation (Miranda *et al*., 2015; Muradov *et al*., 2015). Secondly, the flocculation-assisted fungal technique could reduce the processing steps and increase the flocculation efficiency (Srinuanpan *et al*., 2018a). Chen *et al*. (2018) compared the fungal spore-assisted (FSA) and fungal pellet-assisted (FPA) flocculation for harvesting microalgae. Under optimized conditions (40 ºC, 1.1 × 10^4 cells mL^{-1}, 160 r.p.m., and 5 g glucose L^{-1}), fungal pellet formation harvested 99.26%. In contrast, the latter harvested 98.26% at 160 r.p.m. 34 ºC with fungus: microalga 1:2. This technology is expected to be used in industrial microalgae biomass production.

The primary challenge fungi and microalgae co-cultivation faces are the requirements of different growth conditions, such as carbon sources and pH (Lal *et al*., 2021). In addition, when the fungal spores concentration is high, the development of the microalgae-fungal pellet production process can be affected (Leng *et al*., 2021). However, non-edible and toxic fungi commonly used in bioflocculation processes are unsuitable for applications involving microalgae products, such as food and feed production. Although edible fungi can be used in the flocculation process of microalgae, careful attention should be paid to their growth rates and potentially harmful effects (Luo *et al*., 2019).

The toxins produced by fungi can potentially be harmful to the environment. Therefore, using edible fungi in flocculation is also essential to properly harvest biomass.

However, it is vital to identify the properties of the target fungal strains to develop effective strategies to improve the microalgae harvest. These include their surface activity, mycelial pellet morphology, and their secretions. Table 2 summarises the harvesting of microalgae biomass using different fungal species. The table provides an overview of the specific combinations of microalgae and fungi used in co-cultivation, highlighting the effectiveness of their bioflocculation capabilities. The table includes information on the optimal conditions required for bioflocculation, such as pH range and temperature, which promote the formation of flocculates or aggregates. Additionally, the table presents the harvesting efficiencies achieved through fungal bioflocculation for each species combination.

Harvesting mechanisms

The precise mechanisms by which fungal spores or pellets aid in harvesting algal cells still lack a definitive conclusion. The specific processes and interactions involved in successfully harvesting algal cells with the assistance of fungal spores or pellets remain the subject of ongoing research and investigation. The mechanisms of fungal bioflocculation for harvesting microalgae are complex and vary depending on the microalgae species (Bansfield *et al*., 2021). Fungi are promising bioflocculants due to their higher harvesting efficiency for improving biomass yields owing to their biochemical contents, such as nutrients and enzymes (Li *et al*., 2020; Nazari *et al*., 2021). The flocculation mechanism between microalgae and fungi depends on the contact between their cell surfaces having varying electrostatic charges. The negative charge on the surface of microalgae cells might contain a carboxylic group, phosphodiester, phosphoric, hydroxyl group, and amines functional groups with a zeta potential value ranging from −10 to −35 mV (Muradov *et al*., 2015).

A few theories explain the mechanism by which fungi trap microalgal cells. Leng *et al*. (2021) proposed the mechanism of microalgal flocculation to be either chemical adsorption, physical adsorption, or electrostatic attraction. Either way, the fungal pellets with positively charged functional groups interact with negative charges on the surface of microalgae, leading to microalgal cell aggregation via charge neutralization. Microalgal cells are adsorbed and twined by mycelium, which brings about an intricate network structure between the interactions on the cell surface that could also lead to flocculation. However, no significant changes in the surface morphology of microalgae are observed during the flocculation, which shows that microalgae cells are not damaged after flocculation (Chu *et al*., 2021).

The fungal mycelial surface area and network structure provide a wider surface area for attaching microalgae. The fungi flocculant could also contain high molecular weight polymers, including exopolysaccharides and extracellular proteins. These polymers can help initiate the attachment of microalgae to fungal mycelial pellets through chemical combination. It was reported that exopolysaccharide (EPS) produced during interaction with microalgal cells established the attachment via hydrogen bond formation (Muradov *et al*., 2015; He *et al*., 2021). Bhattacharya *et al*. (2017) reported that N-acetyl-D-glucosamine released by *A. fumigatus* can cause flocculation of *C. pyrenoidosa* via a bridging mechanism.

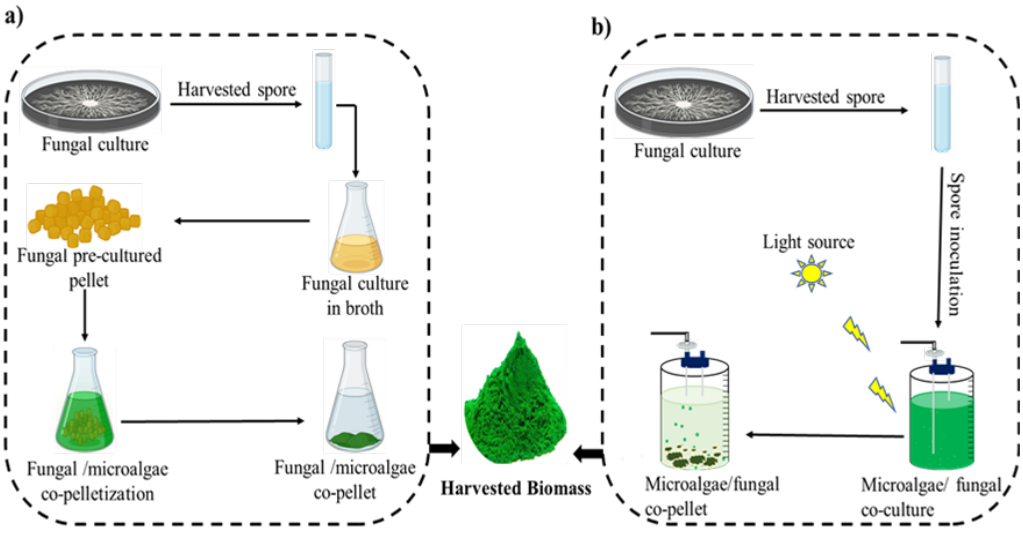

Fig. 5. Co-cultivation modes a) Fungal pellets assisted (FPA) b) Fungal spores assisted (FSA)

Table 2. Harvesting microalgae biomass using different fungal species

Microalgae species	Fungal species	Cultivation Types	Optimum Flocculation parameters	Harvesting Efficiencies (%)	Time (hr)	References
Scenedesmus quadricauda	*Aspergillus fumigatus*	Pellets	150 r.p.m., 25 ºC	>95	48	(Wrede *et al*., 2014)
Nannochloropsis sp.	*A. nomius*	Pellets	Microalgae/fungi ratio 4:1, 1 L/min. air flow rate, 23 ºC, pH 6.0	97.2	3	(Talukder *et al*., 2014)
Chlorella sp.	*Pleurotus ostreatus*	-	28 ºC, 100 r.p.m.	64.86	2.5	(Luo *et al*., 2019)
Scenedesmus sp.	*Aspergillus niger*		160 r.p.m., pH 8.0, 30 ºC	99.4	48	(Pei *et al*., 2021)
Chlorella sp.	*Aspergillus niger*	Pellets	125 r.p.m.	99	72	(Mohd *et al*., 2019)
C. vulgaris	*Aspergillus oryzae*	Pellets	Microalgae/fungi ration 1:1, 130 r.p.m., 30 ºC, pH 9.68	99.23	5	(Chu *et al*., 2021)
Chlorella sp.	*Penicillium* sp.	Spores	1.10×10^4 (spores/mL), 160 r.p.m., 40 ºC	99	28	(Chen *et al*., 2018)
		Pellets	Microalgae/fungi ratio 1:2, 160 r.p.m., pH 4.0, 34 ºC	98.26	2.5	
Chlorella sp.	*Aspergillus niger*	Spores	$2\times10^6 – 3\times10^6$ cell/mL, 100–150 r.p.m., pH 3.0- 9.0	>90		(Mohd Nasir *et al*., 2019)
Scenedesmus obliquus	*Cunninghamella echinulate*	spores	120 r.p.m., pH 5.5, 30 ºC	92.7	24	(Srinuanpan *et al*., 2018a)
C. vulgaris	*Aspergillus niger*	Pallets	150 r.p.m., pH 7	96	72	(Zamalloa *et al*., 2017)
Chroococcus sp.	*A. lentulus*	Pallets	150 r.p.m., pH 7.82	100	24	(Prajapati *et al*., 2014)
Synechocystis sp. PCC6803	*Aspergillus oryzae*	Pallets	Microalgae/fungi ratio 1:4.26, 130 r.p.m., pH 7.4, 30 ºC	90	48	(Choi *et al*., 2016)
C. pyrenoidosa	*A. fumigatus*	Pallets	Fungi: algae ratio of 1:5, 100 r.p.m., 38 ºC	99	3	(Bhattacharya *et al*., 2017)
C. vulgaris	*Aspergillus* sp.	Spore	Spores: algae 1:100, 80 r.p.m., 35 ºC	>97	4	(Yang *et al*., 2019)
C. sorokiniana	*Isaria fumosorosea*	Spore	Spores/microalgae 1:10, 125 r.p.m., pH 7.0–8.0	94–97	72	(Suparmaniam *et al*., 2020)

Similarly, *A. niger* has demonstrated bridging as a flocculation mechanism by adding 5 mM calcium chloride, improving flocculation efficiency (Li *et al*., 2017). The attachment of fungi to different surfaces of microalgae was reported to be mediated by a mechanism known as hydrophobicity, generated by the surface-active proteins called hydrophobin (Epstein & Nicholson, 2016; Ogawa *et al*., 2019). The hydrophobins are associated with fungi secretions that contain amino acids ranging from 100-150 with a low molecular weight of approximately 10 kDa (Kulkarni *et al*., 2017). It was discovered that the hydrophobic components (e.g. proteins) of fungi surfaces could adhere to solid substrates (e.g. microalgae cells), resulting in the development of fungal-algal pellets (Zhou *et al*., 2013; Gao *et al*., 2020).

The surface characteristics of mycelial pellets play an essential role in the flocculation mechanism, as shown in Figure 6. Although there is no specific mechanism of fungal-mediated microalgae flocculation, immense efforts have been devoted to comprehending the mechanisms of fungal-mediated flocculation of microalgae cells. Due to differences in surface charges between the fungal and microalgae, many findings reported that charge neutralization is the primary mechanism of flocculation. Other researchers believed that exopolysaccharides (EPSs) and hydrophobins produced by fungi during interaction could cause attachments of microalgae to the fungal pellet leading to flocculation. Thus, providing a comprehensive mechanism to understand fungal-mediated bioflocculation is necessary.

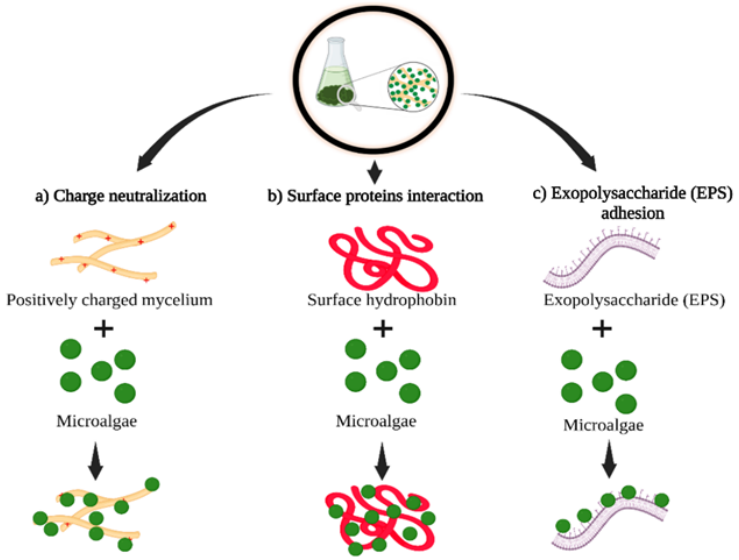

Fig. 6. Mechanisms of fungal-mediated bioflocculation of microalgae cells

Microalgae/fungi co-culture improves biomass and lipid production

Numerous strategies were employed to increase the productivity of lipids and biomass, which are essential components of microalgal biofuel production. These include manipulating environmental parameters, plant hormones, and genetic modifications (Das *et al*., 2022). The microorganisms utilized in the co-culture of algae and fungi are known to be beneficial for wastewater treatment and lower the cost of harvesting and biodiesel production (Zhou *et al*., 2012). The increase in biomass and lipid accumulation within the microbial co-cultivation system has been attributed to the development of a mutualistic relationship that could improve the metabolic activities of the organisms involved (Bader *et al*., 2010). Wang *et al*. (2022) noted that microbial co-culture approaches might improve the characteristics of biofuels and the productivity of biomass and lipids.

Few fungi are known for self-palletisation ability which includes *Isaria fumosorosea* (Suparmaniam *et al*., 2020), *Aspergillus* sp. (Yang *et al*., 2019), *Penicillium* sp. (Chen *et al*., 2018), *Cunninghamella* sp. (Xie *et al*., 2013), *Pleurotus ostreatus* (Luo *et al*., 2019) and these fungi when cultivated with microalgae has been demonstrated to cause flocculation of the microalgal cells. Besides increasing the productivity of the lipids by microalgae, the bioflocculation process can also help decrease the energy input required in the harvesting process. Previous studies have shown that certain strains of fungi could enhance biomass and lipid productivity when co-culture with microalgae, as summarised in Table 3**.** The table noted that many researchers observed a significant increase in biomass and lipids in the co-cultivation system. Wang *et al*. (2022) cultivated *A. oryzae* and *Chlorella pyrenoidosa* in wastewater and reported that the biomass concentration was higher in the co-culture and the individual culture yielding 1.79 g/L biomass and 0.99 g/L lipid concentration.

Microalgae/fungi co-culture use for wastewater treatment

The traditional methods of controlling the pollution in wastewater include techniques such as filtration, aeration, and anaerobic-anoxic systems. However, these technologies cannot fully utilize and recycle the nutrients in wastewater (Abyar *et al*., 2018; Han *et al*., 2019). Inadequate and poor

Table 3. Co-culture of microalgae and fungi for biomass and lipid production

Fungi species/ Microalgae species	Cultivation conditions	Time (hr)	Mono-culture yield		Co-culture yield		References
			Biomass (g/L)	Lipid (mg/L)	Biomass (g/L)	Lipid (mg/L)	
A. niger / C. vulgaris	27 ºC, 200 r.p.m., pH 5.0	72	0.215 ± 0.02/ 0.142 ± 0.01	38.03 ± 10.05/ 53.11 ± 1.51	0.968 ± 0.129	275.88 ± 52.40	(Zhang & Hu, 2012)
Freshwater microalgae	25 ºC, 150 r.p.m., 200 mmol $m^{-2}s^{-1}$	48					(Wrede *et al.*, 2014)
A. fumigatus/ C. vulgaris			NR/ 0.20± 0.02	NR/45.74 ± 5.5	0.47± 0.0	46.2 ± 5.5	
A. fumigatus/ P. subcapitata			NR/0.29 ± 0.04	NR/72.42 ± 8.7	0.56± 0.0	30.76 ± 4.1	
A. fumigatus/ C. reinhardtii			NR/0.27 ± 0.03	NR/15.35 ± 1.8	0.54 ± 0.1	25.99 ± 3.1	
Marine microalgae							
A. fumigatus/ Thraustochytrium sp			NR/0.28 ± 0.08	NR/36.20 ± 8.8	0.71 ± 0.2	125.34 ± 36.3	
A. fumigatus / T. chuii			NR/0.20 ± 0.04	NR/11.80 ± 3.5	0.31 ± 0.7	20.69 ± 8.2	
A. fumigatus / D. salina			NR/0.12 ± 0.01	NR/12.58 ± 1.5	0.39 ± 0.1	25.02 ± 3.0	
A. fumigatus/ C. protothecoides	25 ºC, 150 r.p.m., 200 µmol m^{-2}	48	2.21 ± 0.5/2.25 ± 0.4	240.20 ± 41.9/ 699.7 ± 120.4	8.96 ± 2.1 4.49	2041.9 ± 440.6	(Muradov *et al.*, 2015)
A. fumigatus/ Synechocystis PCC 6803	25 ºC, 150 r.p.m., 200 µmol $m^{-2}s^{-1}$	48	1.50 ± 0.21/ 1.70 ± 0.24	60 ± 9/1.85	4.50 ± 0.6 1700	250 ± 35.7	(Miranda *et al.*, 2015)
A. awamori/ Chlorella minutissima	NR	NR	NR/1.14 ± 0.1	NR/250 ± 19.0	2.80 ± 0.1	510.40	(Dash & Banerjee, 2017)
Cunninghamella sp./ *Scenedesmus obliquus*	30 ºC, 120 r.p.m.	24	1.75 ± 0.1/1.99 ± 0.12	NR/810 ± 60	4.45 ± 0.06	1210 ± 80.00	(Srinuanpan *et al.*, 2018a)
Trichoderma reesei/ Scenedesmus sp.	30 ºC, 3500 lux	7	5.50 ± 0.5/1.00	NR/1200 ± 500	6.64 ± 0.7	1700 ± 90	(Srinuanpan *et al.*, 2018b)
Penicillium sp./ *Chlorella* sp.	35 ºC, 120 r.p.m., 60 µmol $m^{-2}s^{-1}$	36	NR	20.7 ± 4.01/12.9 ± 3.09	0.63	13.01 ± 2.36	(Chen *et al.*, 2018)
Ganoderma lucidum/ C. vulgaris	10-9 M of GR24,	240	0.162 ± 0.015	NR	0.299 ± 0.027	NR	(Zhang *et al.*, 2021)
Aspergillus sp./ *Chlorella* sp. MJ11/11	Optimisation	45	3.75 ± 0.19/1.21 ± 0.06	0.064 ± 0.03/0.21 ± 0.01	2.37 ± 0.12	0.14 ± 0.01	(Lal *et al*., 2021)
A. oryzae/ C. pyrenoidosa	25 ºC, 150 r.p.m.	72	2.23 ± 0.05/ 1.73 ± 0.04	31.20 ± 3.68/23.90 ± 2.40	3.53 ± 0.03	28.10 ± 0.99	(Wang *et al.*, 2022)
M. circinelloides/ S. obliquus	26 ºC, 250 r.p.m., 150 µmol $m^{-2}s^{-1}$	108	120 ± 0.4/ 69.8 ± 0.4	8.6 ± 0.4/ 19.9 ± 0.4	133.3 ± 0.4	30.7 ± 0.4	(Zorn *et al.*, 2022)

NR Not reported

wastewater treatment facilities to remove nutrients and other contaminants can lead to eutrophication, which can cause detrimental effects on the environment. To effectively remove these contaminants, biological approaches using microalgae and fungi have been shown to remove the contaminant from wastewater (Dhangar & Kumar, 2020).

The wastewater characteristics must be conducive to growing the microalgae and fungi co-culture. The process is being carried out synergistically in which fungi serve as absorbents, while nutrient removal is achieved by microalgae (Padri *et al*., 2022). According to previous studies, microalgae and fungi co-cultivation can remove contaminants from wastewater which is more effective than mono-culture (Han *et al*., 2019; Lin *et al*., 2022). Muradov *et al*. (2015) reported that the filamentous fungus co-culture with microalgae in swine wastewater could increase biomass production, bioremediation efficiency, and lipid yield. They also noted that this method could address the shortcomings of mono-culture biotechnology.

Filamentous fungi and microalgae cooperate to remove nutrients and organics from the waste solution (Lau *et al*., 2022). The fungi do not have chlorophyll to carry out photosynthesis; therefore, they require heterotrophic conditions for their growth (Chu *et al*., 2021). Despite differences in metabolisms between the organisms (microalgae & fungi), they are known to play a significant role during wastewater treatment. Microalgae can utilize the CO_2 generated heterotrophically by fungi to release O_2, nutrients, and sugars, which can then be consumed by fungi and can also protect the microalgae by retaining the water and necessary nutrients for growth (Trivedi *et al*., 2015; Lin *et al*., 2022). The overall process is mutual, where microalgae promote fungal growth and nutrient removal by establishing an aerobic condition, while fungi supply inorganic carbon to microalgal cells (Yang *et al*., 2019).

Nitrogen is essential in microalgae metabolism for protein and nucleic acid synthesis. The nitrogen source must also be sufficient for microalgae to grow effectively (Leng *et al*., 2020). Nitrogen sources in the form of ammonia (NH_3), nitrate (NO_3^-), and nitrite (NO_2^-), are utilized by microalgae after it has been transported. Thus, in an aquatic environment, nitrate is the most stable and oxidizing form of nitrogen. This means that the nitrate uptake does not begin until the ammonium nitrogen (NH_4 – N) is completely used (Gonçalves *et al*., 2017), which is then reduced into ammonium ion (NH_4^+) before assimilation (Rahimi *et al*., 2020). The process is carried out through a two-step reaction catalyzed by cytosolic nitrate reductase and chloroplastic nitrite reductase as shown in an equation; $NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O$. Then, $NO_2^- + 8H^+ + 6e^- \rightarrow NH_4^+ + 2H_2O$. Figure 7 describes the mechanisms of nitrogen utilization by microalgae and fungal consortia. However, depending on the ammonium level, the release of NH_4^+can join the amino acids by glutamate dehydrogenase or the glutamine synthetase/glutamate synthase cycle (Gonçalves *et al*., 2017; Rahimi *et al*., 2020).

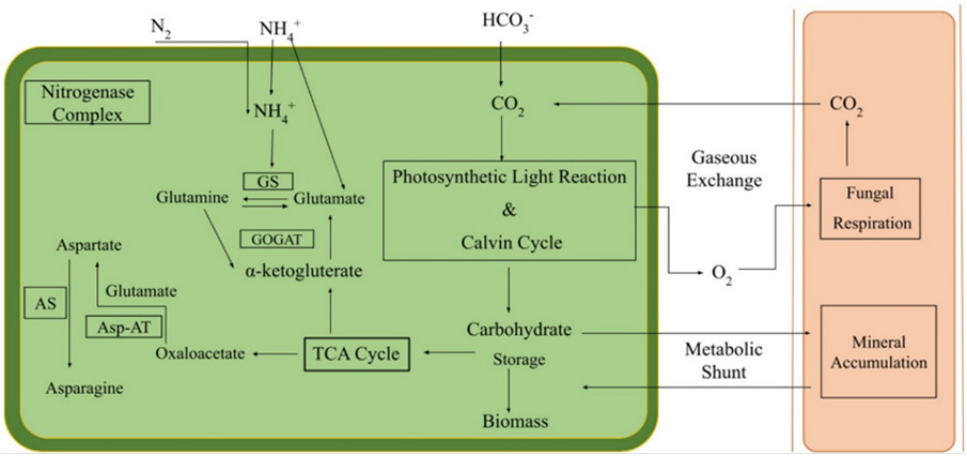

Fig. 7. Mechanisms of nitrogen utilization for microalgae and fungal consortia. Reproduced from with permission from Gopal *et al*. (2023).

Many enzymes produced by fungal mycelia make them capable of efficiently decomposing organic matter. These enzymes are essential for decomposition because they allow the fungus to extract energy and nutrients from organic matter efficiently (Zhang *et al*., 2018). Due to the metabolic capabilities of fungi, they are known to use both organic and inorganic nitrogen but are less effective than microalgae. Although fungi have only a minimal capacity for total nitrogen (TN) and ammonium nitrogen (NH_3^+ - N) removal (usually less than 20%) (Leng *et al*., 2021). Therefore, simultaneous cultivation of fungi and microalgae in wastewater treatment can improve the efficiency of removing TN, (NH_3^+ - N), and NO_3^- (Yang *et al*., 2019). Researchers have demonstrated that combining algae and fungi in co-cultivation can greatly enhance nutrient removal efficiency (Wrede *et al*., 2014; Muradov *et al*., 2015; Guo *et al*., 2017; Zhao *et al*., 2019; Zhang & Liu 2021; Liu *et al*. 2022).

Fungi-assisted microalgae palletization could remove nutrients from wastewater more efficiently than mono-cultures (Yang *et al*., 2019). Wrede *et al*. (2014) used co-culture of *A. fumigatus* and *Thraustochytrid* pellets and reported that major nutrients were effectively assimilated in swine wastewaters, with removing efficiencies of 86% (NH_4^+ - N) and 69% PO_4^{3-} after 48 hr. According to Guo *et al*. (2017), a corporation between the fungi *G. lucidum* and *C. vulgaris* was able to remove 84.61% (COD), 80.41% (TN), and 92.21% (TP) from swine wastewater treated anaerobically. Another finding by Mujtaba and Lee (2017) found that the co-culture of *C. vulgaris* and activated sludge considerably eliminated 98–100% nitrogen, 92–100% phosphorus, and 94–96% COD from artificial municipal wastewater. Surprisingly, the nitrogen removal efficiency of a co-culture of *A. niger* and *C. vulgaris* was lower than that of a mono-culture, as reported by Padri *et al*. (2022). The researchers noted that the low nitrogen removal by fungi and microalgae might be due to the inhibition of nitrogen uptake during the co-culture. This suggests that further studies on the various operational parameters can help improve nitrogen removal efficiency.

Another essential element is phosphorus which is required in microalgae culture and is a crucial component of adenosine triphosphate (ATP) and cell membrane phospholipid (Wang *et al*., 2016). However, microalgae can only make use of soluble forms of phosphorus, such as hydrogen phosphate (HPO_4^{2-}) and dihydrogen phosphate ($H_2PO_4^-$) (Martínez *et al*., 1999). Microalgae mono-culture cannot adsorb the nutrients efficiently when added during wastewater treatment. Interestingly, fungi and microalgae consortia in the treatment process of wastewater can yield significant biomass. Miranda *et al*. (2015) reported that industrial wastewater is a low-cost medium for producing microalgae-fungal pellets. They noted that the immobilized cells could reduce the wastewater's total nitrogen and phosphorus content by 44% and 93%, respectively. In addition to harvesting microalgae cells, the concurrent cultivation of microalgae and fungi possesses huge potential in wastewater treatment. This technique may also be easier and less expensive to harvest the microalgal cells used in biofuel production, making it an extra benefit of using a co-culture system. Table 4 summarises the nutrient-removing efficiencies of microalgae and fungi co-culture systems.

Microalgae/fungi co-culture improves biofuel production

Microalgae is a desirable renewable source for oil production compared to other oil crops since lipids constitute about 20-70% of the total biomass (Bibi *et al*., 2022). Although the yield and composition of lipids are not always linked to the biomass produced after co-culture, they are closely related to the conditions under which fungi and microalgae grow (Leng *et al*., 2021). Studies have shown that certain environmental conditions, such as high salt levels, pH, and nutrient starvation, can stimulate the synthesis of triacylglyceride (TAG) (Li *et al*. 2017; Bibi *et al*. 2022; Zhao *et al*. 2022). Although TAG has no definite structural function, it can still function efficiently as a cellular storage device for both energy and carbon (Morales *et al*., 2021).

The older method to promote the accumulation of lipids by microalgae is by exposing them to different abiotic stress environments, including high salinity levels, nutrient starvation, and solid light intensity (Suparmaniam *et al*., 2020). Interestingly, microalgae and fungi co-cultivation enhances the total lipid and other chemical products suitable for biofuel production (Ogbonna & Nwoba, 2021). It has been proven to increase lipid production by microalgae, and fungi co-cultivation has the potential for biodiesel production, as depicted in Figure 8. The added advantage of using fungi co-culturing with algae is that the fungi can store lipids around 20 – 30% made up of primarily saturated and monounsaturated fatty acids, which are desirable feedstock for biodiesel production (Yen *et al*., 2015; Das *et al*., 2022; Muhammad *et al*., 2022). Generating viable biodiesel from algal and fungi needs the implementation of both lipid and biomass simultaneous harvest.

The microalgae biomass harvested through fungi-aided flocculation can concurrently contribute to lipid and fatty acid methyl esters (FAMEs) composition, making the technique suitable for biodiesel generation (Muradov *et al*., 2015). There are many reports of microalgae-fungi pellets where lipid yield has been significantly improved due to symbiotic interaction between the two organisms. For instance, when the *C. echinulata* and *C. vulgaris* were co-cultivated, the total lipid yield increased to 99% in 48 hr, with an increase in biomass harvesting efficiency (Xie *et al*., 2013). Chu *et al*. (2021) found that the harvested biomass of *C. vulgaris* with the pallets of *A. niger* promoted lipid yield to 33.97%. The study results indicated that pellet-assisted algae harvesting could be a viable alternative to conventional methods of increasing biofuel production.

Microalgal and fungal interactions may influence lipid production (Dash & Banerjee, 2017). The gas exchange and nutrient sharing between fungi and microalgae can help maintain the metabolic activity of the two organisms (Piercey-Normore & Athukorala, 2017). It was noted that due to differences in the nutritional requirement, therefore the consortia of microalgae and fungi required mixotrophic conditions. Muradov *et al*. (2015) reported that a higher lipid yield of 2041.96 g/L was produced when the *Chlorella protothecoides* co-cultivated with *A. fumigatus* which was higher than the mono-culture. In addition to the content of the lipids, the degree of unsaturation and length of lipids are essential factors that can be considered for selecting suitable microalgae species for biodiesel production. The

Table 4. Co-culture of microalgae and fungi for nutrient removal in wastewater

Microalgae species/ Fungal species	M/F ratio	Nature of Media	Nutrient removal			Duration (Days)	Biomass (g/L)	References
			TN %	TP %	COD %			
C. vulgaris/ Ganoderma lucidum	10%	Swine wastewater	74.28	85.37	79.74	7	4.77	(Guo *et al.*, 2017)
Chlorella variabilis/ G. lucidum	1:3	Synthetic wastewater	76.7	74.7	75.5	6	0.89	(Jiang *et al.*, 2019)
Chlorella sp./ *Aspergillus* sp.	1:1	Molasses wastewater	67.09	88.39	70.68	5	4.215	(Yang *et al.*, 2019)
Scenedesmus obliquus / G. lucidum	100:5	Biogas slurry	83.31± 4.72	84.26± 5.58	85.82± 5.37	10	NR	(Wang *et al.*, 2017)
S. obliquus/ G. lucidum	NR	Piggery wastewater	87.26	90.17	87.29	10	0.129 ± 0.009	(Guo *et al.*, 2020)
Thraustochytrid sp./ *A. fumigatus*	NR	Swine wastewater	96	84	NR	2	1.06 ± 0.2	(Wrede *et al.*, 2014)
C. vulgaris/ G.lucidum	100 mL: 5 mL	Biogas slurry (55% CO_2 Conc.)	61.75	64.21	68.29	10	0.174	(Zhou *et al.*, 2018)
C. vulgaris/ G. lucidum	100 mL: 5 mL	Biogas slurry with the synthetic analog of strigolactone (GR24) concentration of 10^9	78.86 ± 7.51	78.12 ± 7.57	77.33 ± 7.25	7	0.342 ± 0.029	(Zhang *et al.*, 2021)
C. vulgaris/ G. lucidum	5:1	Piggery wastewater (COD of 1200 mg/L)	80.23 ± 6.31	89.37 ± 6.58	79.86 ± 6.11	10	0.102±0.008	(Gao *et al.*, 2018)
C. vulgaris/ G. lucidum	10:1	Biogas slurry	89.83 ± 4.36	90.31 ± 4.69	92.17 ± 5.28	10	0.168 ± 0.008	(Zhao *et al.*, 2019)
C. vulgaris/ A. niger	NR	Cassava biogas effluent wastewater	73	82	72	10	1273 mg L	(Padri *et al.*, 2022)
Haematococcus pluvialis, Simplicillium lanosoniveum	30: 1	Local wastewater with the addition of $NaHCO_3$	83.3	88.2	100	12	1.95	(Liu *et al.*, 2022)
Scenedesmus sp./ *Trichoderma reesei*	1:2	Secondary effluent from seafood processing plants	>44	>93	>74	7	NR	(Srinuanpan *et al.*, 2018b)
Chlorella vulgaris/ Aspergillus sp.	NR	Diluted swine manure wastewater	44.68	84.70	70.34	3–5	NR	(Zhou *et al.*, 2012)

M/F ration Microalgae/fungi ration
TN Total nitrogen
TP Total phosphorus
COD Chemical oxygen demand
NR Not reported

most common fatty acids found in the majority of algae species, according to the literature, are myristic (14:0), palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids. Generally, microalgal oils often have significant levels of PUFAs which are excellent properties of biodiesel requirements (Bibi *et al*., 2022). The *A. fumigatus* has been known to enhance the unsaturated fatty acids when co-cultivated with the microalgae system, yielding 60% of oleic (C18:1) and linoleic (C18:2) acids (Miranda *et al*., 2015). Table 5 presents the lipid yield and composition of the mono-culture of microalgae and fungi and their respective co-culture. Based on the table, It was indicated that the fungi can contribute to the production of fatty acids; their complete profile is mainly composed of oleaginous species.

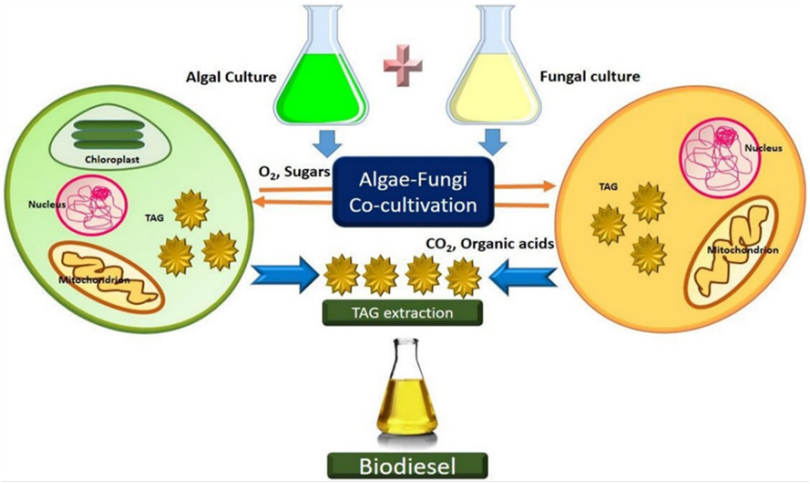

Fig. 8. Utilization of co-cultivation of microalgae and fungi for biofuel production. Reproduced from with permission from Gopal *et al*. (2023).

Table 5. Lipid yield and composition of mono-culture of microalgae, fungi, and their respective co-culture

Organisms	Lipid yield %			Lipid composition	References
	Fungi	Algae	Co-culture	Fungi; Microalgae; Coculture	
A. awamori/ C. minutissima MCC 27	7.50	40.50	35.02	C16:0, C16:1, C18:0, C18:1, C18:2, C18:3; C16:0, C18:0, C18:1, C18:2; C16:0, C16:1, C18:0, C18:1, C18:2, C18:3	(Dash & Banerjee, 2017)
Aspergillus sp./ *Chlorella* sp.	37.70	22.20	35.20	C18:1 (26), C18:2 (28); C16:0 (19), C18:2 (25); C16:0 (18), C18:2 (26)	(Yang *et al*., 2019)
A. fumigatus /*Synechocystis* PCC 6803	10.63	0.10	5.55	C18:1(30), C18:2 (30); C16:0 (49), C18:0 (18); C16:0 (37), C18:1 (22)	(Miranda *et al*., 2015)
Cunninghamella sp./ *Scenedesmus obliquus*	NR	40.86	NR	NA; C16:0 (62), C18:0 (30); C16:0 (52), C18:0 (35)	(Srinuanpan *et al*., 2018a)
Thraustochytrid sp./ *A. fumigatus*	12.10	36.20	23.35	C18:1 (30), C18:2 (29); C16:0 (28), C18:1 (5); C18:1 (18), C18:2 (18)	(Wrede *et al*., 2014)
Mortierella elongate/ *Nannochloropsis Oceanica*	23.88	11.87	16.85	C16:0 (19), C18:1 (22); C16:0 (37), C16:1 (32); C16:0 (31), C16:1 (15)	(Du *et al*., 2018)
Tribonema sp/ *Chlorella zofingiensis*	46.68	48.0	54.19	NR; NR; C20:5, C22:6	(Cheng *et al*., 2020)

[NA] Not reported

Microalgae/fungi co-culture use for high-value compound production

The protein and nutritional content of microalgae has attracted the interest of scientists since the early 1950s, especially with the increase in the earth's population. Several reports in the literature have highlighted the various biomolecules that can be produced by the co-culturing of certain microorganisms,

such as beta-carotene, astaxanthin, phycocyanin, exopolysaccharides (EPS), and organic acids. According to Shahid *et al*. (2020), integrating waste treatment with microalgae co-cultivation systems can generate substantial bioproducts while reducing the natural resources required to preserve and enhance the environment. One of the critical factors to consider when using co-cultivation to produce high-value compounds is separating the biomass, which is very challenging because the microalgae cell wall becomes hydrolyzed during the interaction. This is inappropriate in a situation where the biomass of microalgae and fungus needs to be recovered separately (Leng *et al*., 2021).

It is crucial to broaden the range of fungal-assisted microalgae collection since microalgae and fungi cells contain valuable substances that can be utilized to create valuable products. Studies have shown that the high-value ingredients in animal feed, such as biomass from mono-culture microalgae or fungi, can help improve the health performance of the animals. It can also stimulate the development of immune responses and disease resistance (Mohan *et al*., 2016; Dawood *et al*., 2020). It was reported that *A. oryzae* could be used as a beneficial probiotic for poultry, swine, and fish (Dossou *et al*., 2018; Dawood *et al*., 2019). Astaxanthin can improve the immunity of aquaculture animals, reducing their mortality rate and minimizing antibiotic abuse (Alishahi & Mesbah, 2015).

The synthesis of carotenoids by microalgae is associated with a response to nitrogen deficiency, excessive salinity, or intense light (Pereira *et al*., 2021). It was also proved that the synthesis of carotenoids by microalgae and fungi favors a decrease in nitrogen supply (Szotkowski *et al*., 2019; Coulombier *et al*., 2020). Sun *et al*. (2020) exposed *C. zofingiensis* to nitrogen stress in excess light, which enhanced astaxanthin production throughout cultivation time by enhancing intracellular carbon uptake and metabolic efficiency. The productivity of astaxanthin was 2.0 mg/L/d, while the nitrogen and light sources used in the production of carotenoids *D. salina* are crucial factors that affect its synthesis. It was shown that after the nitrogen source has been exhausted, the biomass production doubles, which caused the accumulation of carotene with a yield of 22.7 mg^{-1} L^{-1} d^{-1} (Sui *et al*., 2019).

In the past, significant attempts have been made to employ microalgal axenic cultures to boost biomass production. However, developing co-culture systems may present an innovative solution to the contamination problem in axenic cultures, thereby enhancing productivity and bioactive compound production (Lian *et al*., 2018). However, taking advantage of the synergistic relationship between microalgae and fungi can affect the co-production of high-value products to increase income and reduce production costs (Padmaperuma *et al*. 2018; Rösch & Weickert, 2019). Three different algae strains have been identified to potentially produce bioactive active compounds in their mono-culture system, including *Haematococcus pluvialis*; *Chlorella zofingiensis*; and *Scenedesmus obliquaus* as shown in Table 6. However, the use of co-culture of microalgae and fungi for high-value compound production gained little attention and has not been widely studied because it is still not used in practice. The main obstacle is the challenge of using non-edible fungi during the co-culture processes and contamination of algal biomass. There is a need to search for more edible fungi and use them for co-cultivation technology. Expanding the use of microalgae and fungal technology through these means can result in the emergence of fresh industrial prospects.

Challenges of microalgae-fungi co-cultivation

Co-cultivation of microalgae and fungi is a promising approach for producing high-value compounds. This technique can create new commercial opportunities, as it enhances the flocculation efficiency and increases the harvested biomass's nutritional content and value-added properties. Expanding the scope of fungal-mediated microalgae harvesting is essential to take full advantage of this method. Although the microalgae-fungi consortium has numerous applications, such as palletization for harvesting and biomass generation, the research is currently limited to laboratory experiments. The research has yet to be carried out on a large-scale industrial level. Most of the studies on microalgae-fungi co-cultivation are predominantly reported in small quantities, typically ranging from 50 mL to 2.0 L. The industrial challenge of microalgae and fungi co-cultivation primarily revolves around scaling up the process and optimizing its economic viability. Although co-cultivation shows potential for diverse applications, specific challenges prevent its extensive adoption in industrial settings. These challenges may include cultivation system design, nutrient management, strain selection and compatibility, contamination control, downstream processing and product extraction, and economic viability. These factors impede the implementation of co-cultivation on an industrial scale.

Cultivation system design

To achieve a successful co-cultivation process, it is essential to develop a reactor incorporating optimized culture parameters (Dias *et al*., 2019). Developing a scalable and efficient cultivation system for microalgae and fungi co-cultivation is complex. Designing large-scale systems that can maintain a symbiotic relationship between microalgae and fungi while maximizing productivity poses a significant challenge. The effectiveness of experimental designs for investigating co-culture systems is heavily influenced by variations in equipment types and fluctuations in operating parameters. A significant concern when operating large-scale bioreactors for co-cultivation processes is the occurrence of

oxygen or carbon-dioxide-depleted areas during the cultivation of obligate aerobic microorganisms or autotrophic microorganisms like microalgae. Forming these dead zones can lead to inadequate mass transfer, poor biomass settling, and subsequent cellular stress (da Silva & Reis, 2015). Selecting a suitable cultivation system that facilitates the growth and interaction of microalgae and fungi poses a significant challenge. Microalgae use light for photosynthesis, whereas fungi thrive in low-light or dark conditions. Achieving a system that accommodates the co-existence of both organisms while satisfying their distinct light preferences can be a complex undertaking. Another vital consideration in this co-culture system is ensuring proper access to the light source during the co-cultivation of high-cell-density symbiotic organisms such as microalgae and yeasts. The dense population can obstruct the light pathway, hindering microalgal growth and reducing lipid productivity (da Silva & Reis, 2015).

Table 6. High-value compounds produced from mono-culture and co-culture systems in different media sources

Bioactive compounds	Species involved	Nature of wastewater	Cultivation period (hr)	Biomass/Bioactive compound yield	References
Astaxanthin	*Simplicillium lanosoniveum* DT06 (DT06)/ *Haematococcus pluvialis*	Local wastewater + $NaHCO_3$	12	1.95 g L^{-1} / 27.9 mg g^{-1}	(Liu *et al*., 2022)
Astaxanthin	*Paraphysoderma sedebokerensis*/ *Haematococcus pluvialis*	NIES medium without an organic carbon source, pH 7.5	10	NA/ 4.8 mg L^{-1}	(Hwang & Sim, 2019)
Astaxanthin	*Simplicillium lanosoniveum* DT06/ *Haematococcus pluvialis*	Bold's Basal Media (BBM)	12	2.45 g L^{-1}/88.84 mg L^{-1}	(Yan *et al*., 2021)
Carotenoids	*Rhodotorula glutinis*/ *C. vulgaris*	Starch wastewater	72	5.8 g L^{-1} / 12.34 mg L^{-1}	(Zhang *et al*., 2019)
beta-carotene	*Rhodotorula glutinis*/ *Schizochytrium* sp.	Complex medium (CM)	48	22.85 ± 0.07 g L^{-1}/ 0.0671 mg L^{-1}	(Sahin & Tas, 2018)
PHA	*Rhizopus*/ *C. vulgaris*	Milk processing plant wastewater	15	440 ± 13 mg DCW $L^{-1}h^{-1}$/405 ± 12 mg $L^{-1}h^{-1}$	(Senko *et al*., 2023)
Protein	*Aspergillus oryzae*/ *Chlorella pyrenoidosa*	Starch wastewater	60	2.23 g/L 1.92 g/L	(Wang *et al*., 2021)

Nutrient management

Maintaining a balanced nutrient supply is crucial for the success of microalgae and fungi co-cultivation. Achieving this balance and managing nutrient availability at an industrial scale can be challenging, requiring the identification of cost-effective nutrient sources. Additionally, optimizing the recycling and reusing of nutrients from the system is necessary to reduce operational costs and minimize environmental impact (Goswami *et al*., 2021). Microalgae consortia exhibit varying performances depending on the species involved. However, the mechanism of keeping the consortia stable is still under investigation, with carbon, macro-, and micro-nutrients (such as vitamins, nitrogen, carbon & phytohormones) playing a central role in the interaction between the species (Yao *et al*., 2019; Alam *et al*., 2022).

Ensuring adequate nutrient availability for both microalgae and fungi poses an additional hurdle. Microalgae primarily rely on inorganic nutrients like nitrogen and phosphorus, while fungi require organic carbon sources. Balancing the nutrient supply to meet the requirements of both organisms and promote their growth represents a significant challenge in co-cultivation system design. To reduce expenses associated with microalgae and fungi co-cultivation, wastewater has been explored as a supplementary source to fulfill the nutritional needs of these organisms. This approach aims to utilize wastewater as a sustainable and cost-effective solution for nutrient provision in the co-cultivation system.

Strain selection and compatibility

Selecting suitable strains of microalgae and fungi for industrial-scale co-cultivation is critical. Compatibility between strains is determined by factors such as growth rates, nutrient requirements, and mutualistic interactions. However, identifying compatible strains that can effectively cooperate and coexist in the same cultivation system remains a significant challenge. In some cases, microalgae and fungi exhibit mutually beneficial relationships, where one organism provides essential nutrients or compounds for the other's growth. However, metabolic interactions between the two can also hinder the growth of one or both organisms. Understanding and managing these interactions are crucial for successful co-cultivation. When considering the use of filamentous fungi for microalgae harvesting, it is essential to note that the fungi primarily studied are pathogenic and lack practical applications. Therefore, it is essential to ascertain the characteristics of the targeted strains before introducing fungal spores into the algal culture broth for efficient harvesting.

The efficiency of co-pellet formation and harvesting can vary due to specific characteristics of microalgal cells, such as shape, size, hydrophobicity, and the biochemical composition of their cell walls (Wang *et al*., 2018). Furthermore, strain-dependent factors, including surface activity, intracellular structure, and secretions of pre-cultured pellets, can interact with algal cells (Chu *et al*., 2021). In summary, selecting compatible strains, understanding metabolic interactions, and considering the characteristics of microalgal cells and fungal strains are crucial for successful co-cultivation and efficient harvesting in industrial-scale systems.

Contamination control

Industrial-scale cultivation systems face a higher risk of contamination from unwanted microorganisms, leading to culture crashes, loss of bioresources, low metabolite productivity, and low-quality biomass (Ji *et al*., 2018). Therefore, it is crucial to implement robust contamination control measures to maintain the integrity and productivity of the co-cultivation process. These measures include sterile techniques, monitoring systems, and biosecurity protocols. While using an external carbon source in the co-cultivation process increases the risk of contamination compared to autotrophic mode, the advantages of heterotrophy outweigh the drawbacks. Studies have shown that heterotrophic growth offers higher algal growth rates and enhanced biomass and lipid yields compared to autotrophy (Ray *et al*., 2022). Using wastewater as a nutrient source for algal-fungal pellet production is gaining popularity. However, it is essential to consider toxic or hazardous components in wastewater. For example, wastewater from the mining and chemical industry can contain high levels of metals and chemicals that may accumulate in algal-fungal pellets and cause biomass contamination (Lin *et al*., 2022). Therefore, implementing contamination control measures, considering the advantages of heterotrophic growth, and assessing the potential risks associated with using wastewater as a nutrient source is vital for maintaining a successful and productive co-cultivation process at an industrial scale.

Downstream processing and product extraction

Extracting desired products from the co-cultivation system and separating them from the biomass can be complex and costly. Developing efficient and cost-effective downstream processing techniques to extract target compounds from biomass, such as lipids, proteins, or enzymes, represents a significant industrial challenge.

Economic viability

Scaling up microalgae and fungi co-cultivation to an industrial level requires substantial investment in infrastructure, energy supply, and operational costs. Achieving a competitive production cost for target products is crucial for the economic viability of the process. Identifying high-value markets and exploring potential applications, such as biofuels, pharmaceuticals, nutraceuticals, or bioplastics, can help commercialize microalgae and fungi co-cultivation. Extensive scale application of microalgae processing technologies seems to depend on the sustainable and continuous supply of microalgal biomass. The economic assessment of these processes implies that producing only biofuels from microalgae is not a practicable alternative without integrating various processes (Azarpour *et al*., 2022).

Future prospects

Mycelium pellets are suitable carriers for microalgae due to their various mechanisms, such as their electrical neutralization and hydrogen bond adsorption (Cai *et al*., 2019). However, factors such as insufficient mechanical strength of the fungal mycelium and low tolerance for environmental changes, the fungal mycelium must be improved to enhance its capabilities (Ding *et al*., 2018). Depending on the species, the main limitation of the fungal method is its relative time and low efficiency. Therefore, to improve the fungal mycelium's flocculation efficiency, modifying the mycelium with nanoparticles would appear as a novel approach for increasing the strength and compactness of mycelium pellets, thereby modifying the configurational characteristics of mycelium. Nanoparticles offer advantages such as a

high surface area to volume ratio, diverse functionalities, and ease of functionalization (Nguyen *et al*., 2019). However, to the best of our knowledge, no studies have been conducted on modifying mycelium with nanoparticles to bio-flocculate microalgae cells. Therefore, selecting an appropriate nanoparticle and optimizing its concentration are critical steps in developing the optimal approach for modifying fungal mycelium for harvesting microalgae biomass. Similarly, understanding the molecular interactions between co-cultivated organisms is very important to develop a sustainable and profitable process for the production of biofuels. Interestingly, in the future, introducing a desirable gene to fungi can be done through genetic modification. This process could improve the flocculation efficiency, increasing the harvested biomass's nutritional content and value-added properties.

CONCLUSION

The co-cultivation of microalgae with fungi presents a promising strategy for improving biomass harvesting, lipid production, wastewater treatment, oil quality, and the production of bioactive compounds. This interdisciplinary approach holds great potential for sustainable and efficient industrial biotechnological applications. Fungi can induce lipid biosynthesis pathways in microalgae, resulting in increased lipid production, essential for producing biofuels and other lipid-derived products. This synergistic approach harnesses the complementary capabilities of microalgae and fungi, leading to improved efficiency, sustainability, and the generation of valuable products. By leveraging the potential of co-cultivation, we can achieve advancements in various industries, paving the way for a more sustainable and resource-efficient future.

ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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