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Indole-3-acetic acid regulation of fungal–algal consortia for oxytetracycline removal: Physiological responses, extracellular polymeric substances, and biomass clarification

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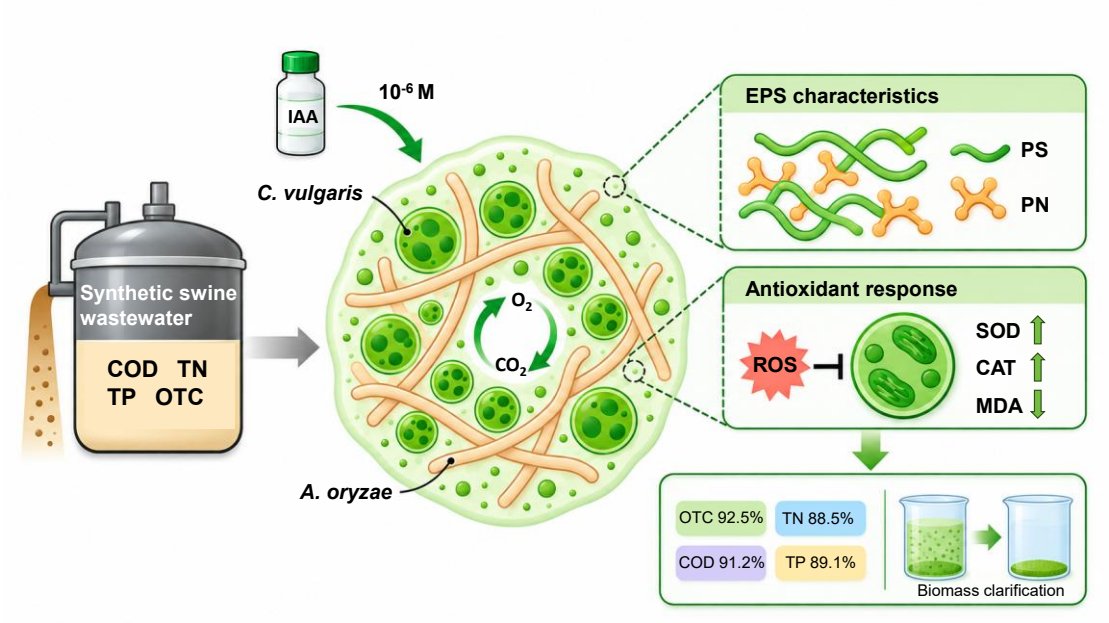
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Graphical abstract



Highlight

- Fungal cocultivation increased OTC removal from 48.2% to 74.6%.
- IAA at 10^{-6} M achieved 92.5% OTC removal after 14 days.
- IAA enhanced antioxidant responses and extracellular polymer accumulation.
- The IAA-treated coculture achieved 88.2% clarification within 30 min.

Abstract: Microalgal treatment of antibiotic-containing wastewater is often constrained by physiological stress and the difficulty of biomass separation. This study evaluated the effects of exogenous indole-3-acetic acid (IAA) on a *Chlorella vulgaris–Aspergillus oryzae* coculture treating synthetic swine wastewater containing oxytetracycline (OTC). An algal monoculture, an IAA-free coculture, and cocultures supplemented with different IAA concentrations were compared over 14 days. Fungal cocultivation increased OTC removal from 48.2% to 74.6%. Supplementation with 10^{-6} M IAA yielded the highest overall performance, with removal efficiencies of 92.5% for OTC, 91.2% for chemical oxygen demand, 88.5% for total nitrogen, and 89.1% for total phosphorus. This treatment also increased biomass concentration to 3.47 g L^{-1} and chlorophyll a content to 4.26 mg L^{-1}, accompanied by higher superoxide dismutase and catalase activities and a 48.6% reduction in malondialdehyde content relative to the IAA-free coculture. Extracellular polymeric substances (EPS) content reached 132.04 mg g^{-1} dry weight, representing a 36.7% increase, while the zeta potential of extracted EPS solutions shifted from −17.45 to −11.36 mV. Clarification efficiency reached 88.2% after 30 min and 93.6% after 60 min. These findings demonstrate that appropriate IAA supplementation can improve wastewater treatment and early-stage biomass clarification, accompanied by coordinated physiological and extracellular responses in fungal–algal consortia.

Keywords: Indole-3-acetic acid; Oxytetracycline; Fungal – algal coculture; Extracellular polymeric substances; Antioxidant response; Biomass clarification

1. Introduction

Microalgae-based wastewater treatment has attracted increasing interest as an approach that integrates pollutant removal, nutrient recovery, and biomass production. Through photosynthetic and metabolic activities, microalgae can assimilate nitrogen and phosphorus while contributing to the removal of organic contaminants, offering opportunities for the development of resource-oriented treatment systems (Kuß et al. 2025; Xenaki et al. 2026). The presence of antibiotics in livestock and aquaculture wastewater, however, introduces additional complexity because these compounds can interact with algal metabolism and extracellular constituents. Oxytetracycline (OTC), a widely used tetracycline antibiotic, has received particular attention owing to its occurrence in agricultural wastewater and its concentration-dependent effects on photosynthetic microorganisms (Luo et al. 2023; Fekry et al. 2026). Previous investigations have demonstrated that microalgal systems can contribute to OTC removal through a combination of biological and physicochemical processes. For example, Luo et al. (2023) documented changes in nutrient uptake, growth, and extracellular polymeric substances (EPS) in Chlorella vulgaris exposed to OTC-containing swine wastewater, while Li et al. (2025) reported enhanced OTC removal and adaptive microbial responses in microalgal–bacterial granular sludge. These findings have encouraged the development of cultivation strategies that integrate antibiotic removal with the maintenance of biological activity and efficient biomass recovery.

Cocultivation with filamentous fungi provides a practical approach to integrating wastewater treatment and microalgal biomass separation. Fungal hyphae can serve as attachment surfaces for algal cells, promoting the development of larger biological aggregates that can be recovered through gravitational settling. The complementary metabolic activities of algae and fungi may also support organic matter conversion and nutrient removal, making fungal-assisted cultivation attractive for wastewater applications (Leng et al. 2021). Recent investigations have expanded the application of this strategy across different fungal–algal combinations. Tölle and Kuenz (2025) demonstrated the potential of *Aspergillus oryzae* for the bioflocculation of *Tetraselmis subcordiformis*, while Mohamed et al. (2025) examined *C. vulgaris* harvesting using *Aspergillus niger* pellets. In antibiotic-containing systems, Wang et al. (2025) identified relationships among extracellular constituents, cellular metabolic responses, tetracycline removal, and biomass harvesting in microalgae–fungi consortia. An important link among these processes is the extracellular matrix, particularly its protein and polysaccharide components, which participate in cell attachment, surface interactions, and aggregate formation (Zhou et al. 2024). Recent work on microalgae–bacteria flocs has also demonstrated the importance of EPS for aggregate integrity and settling behavior (Hering-Peter et al. 2026). Together, these studies establish extracellular regulation as a relevant consideration in the design of fungal–algal treatment systems.

Indole-3-acetic acid (IAA), a naturally occurring auxin, offers an additional means of regulating microalgal physiological and extracellular responses. Auxin-mediated effects on *C. vulgaris* growth, metabolite accumulation, and antioxidant activity were documented by Piotrowska-Niczyporuk and Bajguz (2014), providing an early basis for investigating IAA as a microalgal growth regulator. More recent studies have extended this approach to environmentally relevant cultivation systems. Li et al. (2023) reported that IAA supplementation improved *Tetradesmus obliquus* biomass production and extracellular protein and polysaccharide accumulation during the treatment of toxic sludge extracts. Xie et al. (2024) further demonstrated that IAA could influence the initial adhesion of microalgae through changes in EPS-associated proteins and interfacial characteristics. Phytohormone-assisted cultivation has also been investigated for antibiotic-containing wastewater. Xu et al. (2024) examined gibberellin regulation in microalgae – bacteria – fungi consortia, whereas Chai et al. (2024) evaluated mixed phytohormone supplementation for nutrient and antibiotic removal in microalgae-based systems. More recently, Wang et al. (2026) reported IAA-associated improvements in antibiotic removal, antioxidant responses, and sedimentation performance in *C. vulgaris*. These findings highlight the potential of phytohormone regulation to influence both intracellular physiological processes and extracellular properties relevant to wastewater treatment and biomass separation.

However, the concurrent effects of IAA supplementation on physiological responses, extracellular characteristics, and biomass clarification in OTC-exposed fungal–algal consortia remain insufficiently characterized. In these systems, antioxidant enzyme activities and lipid peroxidation indicators reflect cellular physiological responses, while EPS production and composition provide information on the extracellular environment surrounding algal and fungal cells. Surface electrokinetic properties offer an additional perspective on interparticle interactions, and time-resolved clarification measurements indicate how readily suspended biomass can be separated from the treated medium. Studies of phytohormone-assisted cultivation have established relationships between growth regulation and extracellular responses (Li et al. 2023; Xie et al. 2024), while investigations of microbial aggregates have connected EPS characteristics with flocculation and biomass recovery (Wang et al. 2025; Hering-Peter et al. 2026). Integrating physiological, extracellular, and clarification measurements within an OTC-exposed fungal–algal system provides a basis for evaluating the coordinated responses to IAA supplementation.

In this study, a *C. vulgaris–A. oryzae* coculture was investigated for the treatment of OTC-containing synthetic wastewater. An algal monoculture and an IAA-free coculture were compared to evaluate the effects of fungal cocultivation, followed by the examination of three IAA concentrations to assess phytohormone-associated changes in treatment performance. OTC, organic matter, and nutrient removal were evaluated together with biomass accumulation, chlorophyll a production, and antioxidant responses. EPS production and composition, the zeta

potential of extracted EPS solutions, and static biomass clarification were further investigated to characterize extracellular and separation-related properties. Particular emphasis was placed on the relationships among IAA supplementation, OTC removal, EPS-associated surface characteristics, and early-stage clarification behavior. The study provides an integrated assessment of phytohormone-assisted fungal – algal cultivation for antibiotic-containing wastewater treatment and biomass recovery.

2. Materials and Methods

2.1 Microorganisms and inoculum preparation

The green microalga *Chlorella vulgaris* FACHB-2723 was obtained from the Freshwater Algae Culture Collection at the Institute of Hydrobiology, Chinese Academy of Sciences (FACHB, Wuhan, China). The microalga was cultivated in sterile BG-11 medium (Hajri et al. 2024) at 25 ± 1 °C under an illumination intensity of approximately 60 μmol photons m^{-2} s^{-1} with a 12 h:12 h light/dark cycle. Exponentially growing cells were harvested by centrifugation, washed twice with sterile deionized water, and resuspended for subsequent inoculation.

The filamentous fungus *Aspergillus oryzae* CICC 40214 was obtained from the China Center of Industrial Culture Collection (Beijing, China). The fungus was maintained on potato dextrose agar (PDA) at 28 ± 1 °C for 7 days. Mature spores were collected using sterile 0.1% (v/v) Tween-80 solution, filtered through sterile gauze to remove mycelial fragments, and counted using a hemocytometer. The spore suspension was adjusted to 1×10^6 spores mL^{-1} and inoculated into potato dextrose broth (PDB). After cultivation at 28 °C and 150 rpm for 48 h, the resulting fungal pellets were harvested and washed three times with sterile deionized water before use.

Oxytetracycline hydrochloride (OTC·HCl, purity ⩾ 98%) and indole-3-acetic acid (IAA, purity ⩾ 98%) were obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Stock and working solutions were prepared separately, sterilized by filtration through 0.22 μm membranes, and protected from light before use. All other chemicals were of analytical grade.

2.2 Synthetic wastewater and experimental design

Synthetic swine wastewater was prepared using glucose and sodium acetate as carbon sources, NH_4Cl and urea as nitrogen sources, and KH_2PO_4 and sodium β-glycerophosphate as phosphorus sources. Mineral constituents were adapted from BG-11 medium (Hajri et al. 2024), with nitrate omitted and the modified composition provided in Table 1. The nominal initial concentrations of chemical oxygen demand (COD), total nitrogen (TN), ammonium nitrogen

158 (NH_4^+-N), total phosphorus (TP), and orthophosphate phosphorus (PO_4^{3-}-P) were 800, 80, 65,
159 15, and 12 mg L^{-1}, respectively. OTC was added at a nominal concentration of 10 mg L^{-1}, and
160 the initial pH was adjusted to 6.8 using 0.1 M HCl or NaOH. The composition of the synthetic
161 wastewater is presented in Table 1. The basal wastewater was sterilized at 121 °C for 20 min
162 and cooled to room temperature before the addition of filter-sterilized OTC and IAA solutions.

163 Table 1. Composition of the synthetic swine wastewater

Component	Concentration	Unit
Glucose	562	mg L^{-1}
Sodium acetate	254	mg L^{-1}
NH_4Cl	248	mg L^{-1}
Urea	32	mg L^{-1}
KH_2PO_4	53	mg L^{-1}
Sodium β-glycerophosphate	21	mg L^{-1}
$MgSO_4 \cdot 7H_2O$	75	mg L^{-1}
$CaCl_2 \cdot 2H_2O$	36	mg L^{-1}
Citric acid	6	mg L^{-1}
Ferric ammonium citrate	6	mg L^{-1}
Na_2EDTA	1	mg L^{-1}
A5 trace element solution	1	mL L^{-1}
OTC	10	mg L^{-1}

164 Note: Nominal initial concentrations were COD 800, TN 80, NH_4^+-N 65, TP 15 and PO_4^{3-}-P 12
165 mg L^{-1}. Initial pH was 6.8.

166 Five biological treatments (T1–T5) were established to evaluate the effects of fungal
167 cocultivation and exogenous IAA supplementation under OTC exposure. Two uninoculated
168 controls (AB0 and AB10) were included to assess abiotic OTC loss. The experimental design
169 and treatment compositions are summarized in Table 2.

170

171 Table 2. Experimental treatments and initial IAA concentrations

Group	Microbial composition	IAA (M)	IAA (mg L^{-1})
T1	*C. vulgaris*	0	0
T2	*C. vulgaris* + *A. oryzae*	0	0
T3	*C. vulgaris* + *A. oryzae*	10^{-8}	0.00175
T4	*C. vulgaris* + *A. oryzae*	10^{-6}	0.17518
T5	*C. vulgaris* + *A. oryzae*	10^{-4}	17.518

AB0	Uninoculated	0	0
AB10	Uninoculated	10^{-6}	0.17518

Note: All treatments contained a nominal initial OTC concentration of 10 mg L^{-1}, added as oxytetracycline hydrochloride. AB10 is the existing control-group identifier and does not represent an IAA concentration of 10 mg L^{-1}.

Batch experiments were conducted in 500 mL Erlenmeyer flasks with a final working volume of 250 mL. The initial algal biomass concentration was adjusted to approximately 0.30 g dry weight (DW) L^{-1}, while the fungal inoculum was introduced at an initial biomass concentration of 0.15 g DW L^{-1} in the coculture treatments. The microalgal monoculture received the same initial algal biomass without fungal inoculation.

IAA was added once at the beginning of cultivation using appropriately diluted working solutions, with consistent carrier volumes across treatments. All cultures were incubated at 25 ± 1 °C and 120 rpm under an illumination intensity of 60 μmol photons m^{-2} s^{-1} with a 12 h:12 h light/dark cycle for 14 days. Samples were collected on Days 3, 7, and 14 for water quality, biomass, antioxidant, and extracellular polymeric substance (EPS) analyses. Parallel cultures were allocated to endpoint morphological observations, zeta potential measurements, and static clarification assays. All treatments were conducted in triplicate using independent experimental flasks (n = 3).

2.3 Water quality and OTC determination

Culture samples were centrifuged at 6000 rpm for 10 min, and the supernatants were filtered through 0.45 μm membrane filters before aqueous-phase water quality analysis. COD was determined using the potassium dichromate method (HJ 828-2017). TN was measured by alkaline potassium persulfate digestion followed by ultraviolet spectrophotometry (HJ 636-2012), whereas NH_4^+-N was quantified using Nessler's reagent colorimetry (HJ 535-2009). Phosphorus concentrations were determined using the ammonium molybdate spectrophotometric method (GB 11893-89), with digestion applied to determine the digestible phosphorus content of the aqueous fraction.

Residual OTC concentrations were determined using high-performance liquid chromatography (HPLC; Agilent 1260 Infinity, Agilent Technologies, Santa Clara, CA, USA) equipped with a diode-array detector and a ZORBAX Eclipse Plus C18 column (4.6 × 250 mm, 5 μm). Culture supernatants were filtered through 0.22 μm membranes before analysis. The mobile phase consisted of 0.01 M aqueous oxalic acid and acetonitrile (80:20, v/v), delivered isocratically at a flow rate of 1.0 mL min^{-1}. The column temperature was maintained at 30 °C, the injection volume was 20 μL, and the detection wavelength was set at 355 nm. OTC concentrations were quantified using external calibration standards, with initial and residual concentrations expressed on the same analytical basis.

The removal efficiency of each pollutant was calculated using Eq. (1):

$$\text{Removal efficiency (\%)} = \frac{C_0 - C_t}{C_0} \times 100 \qquad (1)$$

where C_0 and C_t represent the initial and residual aqueous concentrations of the corresponding pollutant at sampling time t, respectively.

2.4 Biomass, chlorophyll a, and morphological characterization

Biomass dry weight (DW) was determined gravimetrically. A 10 mL aliquot of homogeneous culture suspension was filtered through a pre-dried and pre-weighed Whatman GF/C glass microfiber filter (nominal particle retention: 1.2 μm). The retained biomass was washed three times with deionized water to remove soluble salts and dried at 105 °C to constant weight. Biomass concentration was calculated from the difference between the final and initial filter masses and expressed as g L^{-1}.

Chlorophyll a was determined by ethanol extraction followed by spectrophotometric analysis. Briefly, 5 mL of culture suspension was centrifuged, and the collected biomass was resuspended in 5 mL of 95% (v/v) ethanol. Extraction was conducted at 4 °C in the dark for 24 h. After centrifugation, the absorbance of the extract was measured at 664 and 649 nm using a UV–Vis spectrophotometer. Chlorophyll a concentration was calculated using the spectrophotometric equations applied by Dritsas and Aggelis (2025):

$$\text{Chl } a \text{ (mg L}^{-1}) = (13.36A_{664} - 5.19A_{649}) \times \frac{V_e}{V_s} \qquad (2)$$

where A_{664} and A_{649} are the absorbance values of the ethanol extract, and V_e and V_s represent the extraction volume and original culture sample volume, respectively. The calculation assumes a 1 cm optical path length.

The morphology of *C. vulgaris* cells and fungal–algal aggregates was examined using field-emission scanning electron microscopy (FE-SEM, TESCAN, Czech Republic). Biomass samples collected on Day 14 were fixed with 2.5% (v/v) glutaraldehyde, dehydrated through a graded ethanol series, dried, and sputter-coated with gold before observation. Culture pH was monitored using a calibrated digital pH meter.

2.5 Antioxidant enzyme activities and lipid peroxidation

Antioxidant responses were evaluated by measuring superoxide dismutase (SOD) and catalase (CAT) activities and malondialdehyde (MDA) content. Biomass samples were harvested by centrifugation at 4 °C, washed with ice-cold phosphate-buffered saline (PBS, pH 7.4), and

homogenized under refrigerated conditions. The homogenates were centrifuged at 10,000 rpm for 10 min at 4 °C, and the resulting supernatants were collected for biochemical analysis.

SOD, CAT, and MDA were quantified using commercial assay kits supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China), according to the manufacturer's instructions. SOD activity was determined using the water-soluble tetrazolium salt-1 (WST-1) method at 450 nm. CAT activity was measured using the ammonium molybdate method at 405 nm, whereas MDA content was determined using the thiobarbituric acid (TBA) reaction at 532 nm.

SOD and CAT activities were expressed as U mg^{-1} fresh weight (FW), while MDA content was expressed as μmol g^{-1} FW.

2.6 EPS extraction and compositional analysis

EPS were extracted from harvested biomass using a thermal extraction method. The biomass was washed twice with deionized water, resuspended in 0.05% (w/v) NaCl solution, and heated at 75 °C for 50 min. The suspension was subsequently centrifuged at 18,000 rpm for 15 min at 4 °C, and the supernatant was filtered through a 0.45 μm membrane to obtain the thermally extractable EPS fraction.

Extracellular protein (PN) was quantified using the Coomassie Brilliant Blue G-250 method at 595 nm, with bovine serum albumin as the calibration standard. Extracellular polysaccharide (PS) was determined using the phenol–sulfuric acid method at 490 nm, with glucose as the standard. Both PN and PS contents were normalized to the corresponding biomass dry weight and expressed as mg g^{-1} DW. The measured EPS content was calculated as the sum of PN and PS, and the protein-to-polysaccharide mass ratio (PN/PS) was determined accordingly.

2.7 Zeta potential and static clarification assays

The zeta potential of the extracted EPS solutions was measured using a Zetasizer Nano ZS90 analyzer (Malvern Panalytical, Malvern, UK) at 25 °C.

Static clarification assays were conducted at the end of cultivation to evaluate gravitational settling performance. Homogeneous culture suspensions (50 mL) were transferred into graduated cylinders and allowed to settle under quiescent conditions at 25 °C. Separate settling columns were used for different sampling intervals to avoid disturbances caused by repeated sample withdrawal.

The optical density of the supernatant was measured at 680 nm (OD_{680}) at 0, 15, 30, and 60 min. Clarification efficiency was calculated using Eq. (3):

$$\text{Clarification efficiency (\%)} = \frac{OD_0 - OD_t}{OD_0} \times 100 \qquad (3)$$

where OD_o and OD_t represent the initial optical density and the optical density of the supernatant at settling time t, respectively.

2.8 Statistical analysis

All experiments were conducted in three independent replicates (n = 3), and quantitative results were expressed as mean ± standard deviation (SD). Differences among treatments at individual sampling time points were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test for multiple comparisons. Statistical significance was established at $p < 0.05$. Different lowercase letters were used to indicate statistically significant differences among treatments in the corresponding figures. Statistical calculations were performed using Python, and figures were prepared using OriginPro 2022 (OriginLab Corporation, Northampton, MA, USA).

3. Results

3.1 Pollutant removal performance

The removal efficiencies of TP, TN, COD, and OTC increased during the 14-day cultivation period, with distinct differences among treatments (Fig. 1a–d). Fungal cocultivation improved pollutant removal compared with the *C. vulgaris* monoculture, particularly during the early cultivation stage. On Day 3, OTC removal reached 65.3% in T2, compared with 25.4% in T1. By Day 14, TP, TN, COD, and OTC removal efficiencies in T2 reached 76.2%, 75.1%, 78.3%, and 74.6%, respectively, compared with 55.6%, 68.5%, 62.4%, and 48.2% in T1. The largest improvements were observed for OTC and TP removal, with increases of 26.4 and 20.6 percentage points, respectively.

IAA supplementation at 10^{-6} M (T4) further enhanced pollutant removal, yielding the highest removal efficiencies among the tested treatments. OTC removal in T4 reached 75.6% on Day 3 and 88.4% on Day 7, increasing to 92.5% by Day 14 (Fig. 1d). At the end of cultivation, T4 achieved TP, TN, COD, and OTC removal efficiencies of 89.1%, 88.5%, 91.2%, and 92.5%, respectively, representing increases of 12.9, 13.4, 12.9, and 17.9 percentage points relative to the IAA-free coculture (T2). Significant improvements were observed in TP, TN, and OTC removal. Among the IAA concentrations examined, 10^{-6} M consistently provided the highest overall treatment performance.

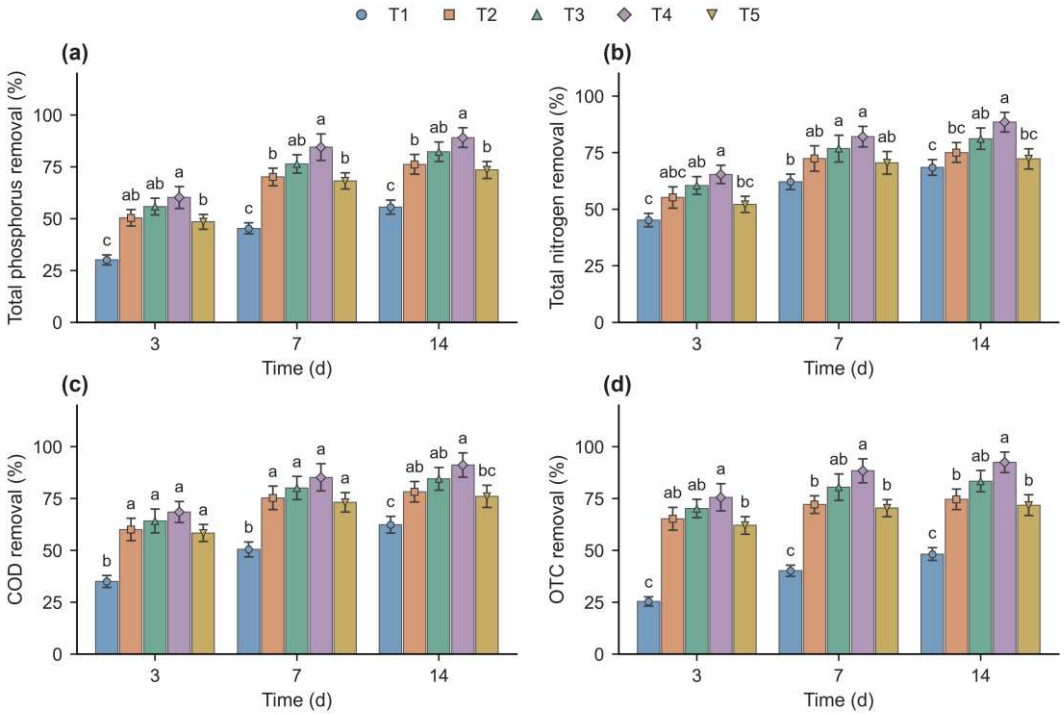


Fig. 1 Effects of indole-3-acetic acid (IAA) supplementation on pollutant removal performance in microalgal monoculture and fungal–algal coculture systems: (a) TP, (b) TN, (c) COD, and (d) OTC removal efficiencies. Data are presented as mean ± SD (n = 3 independent biological replicates). Different lowercase letters indicate significant differences among treatments at the same sampling time (one-way ANOVA followed by Tukey's HSD test, $p < 0.05$)

Abiotic OTC loss remained substantially lower than that observed in the biological treatments (Table 3). By Day 14, OTC removal reached 11.6% and 11.9% in AB0 and AB10, respectively, compared with 48.2% in T1 and 92.5% in T4.

Table 3. OTC removal efficiencies in abiotic controls and selected biological treatments during cultivation.

Treatment	Day 3 (%)	Day 7 (%)	Day 14 (%)
AB0	4.8 ± 0.39	8.4 ± 0.51	11.6 ± 0.76
AB10	5.0 ± 0.40	8.6 ± 0.51	11.9 ± 0.74
T1	25.4 ± 2.25	40.2 ± 2.68	48.2 ± 3.07
T4	75.6 ± 6.63	88.4 ± 5.77	92.5 ± 4.96

Note: Values are presented as mean ± standard deviation (n = 3). AB0 and AB10 represent uninoculated controls without and with IAA supplementation, respectively. T1 represents the microalgal monoculture, whereas T4 represents the fungal–algal coculture supplemented with

314 10^{-6} M IAA.

315 3.2 Biomass growth and physiological responses

316 Biomass accumulation increased throughout the cultivation period, with pronounced
317 differences among the treatments (Fig. 2a). The fungal–algal coculture exhibited greater total
318 biomass accumulation, with DW reaching 2.61 g L^{-1} in T2 on Day 14, compared with 1.82 g L^{-1}
319 in T1. IAA supplementation further enhanced biomass accumulation, with T4 exhibiting the
320 highest DW throughout cultivation. Biomass concentration in T4 increased from 1.27 g L^{-1} on
321 Day 3 to 2.64 g L^{-1} on Day 7, reaching 3.47 g L^{-1} on Day 14, representing a 33.0% increase
322 compared with T2. A similar pattern was observed for Chl a accumulation (Fig. 2b). By Day 7,
323 Chl a concentration in T4 had reached 3.68 mg L^{-1}, increasing to 4.26 mg L^{-1} on Day 14,
324 significantly exceeding the corresponding values in T2 (3.12 mg L^{-1}) and T1 (2.15 mg L^{-1}).

325 SEM observations revealed an interconnected network of fungal hyphae with algal cells
326 distributed along the filamentous structures and within the aggregates (Fig. 2c, d). Changes in
327 culture pH were also observed throughout cultivation (Fig. 2e). Following a slight decrease
328 during the initial 3 days, the pH increased progressively in the subsequent cultivation period.
329 T4 exhibited the highest pH, increasing from an initial value of 6.80 to 7.18 on Day 7 and 7.42
330 on Day 14, compared with final values of 7.12 and 6.85 in T2 and T1, respectively.

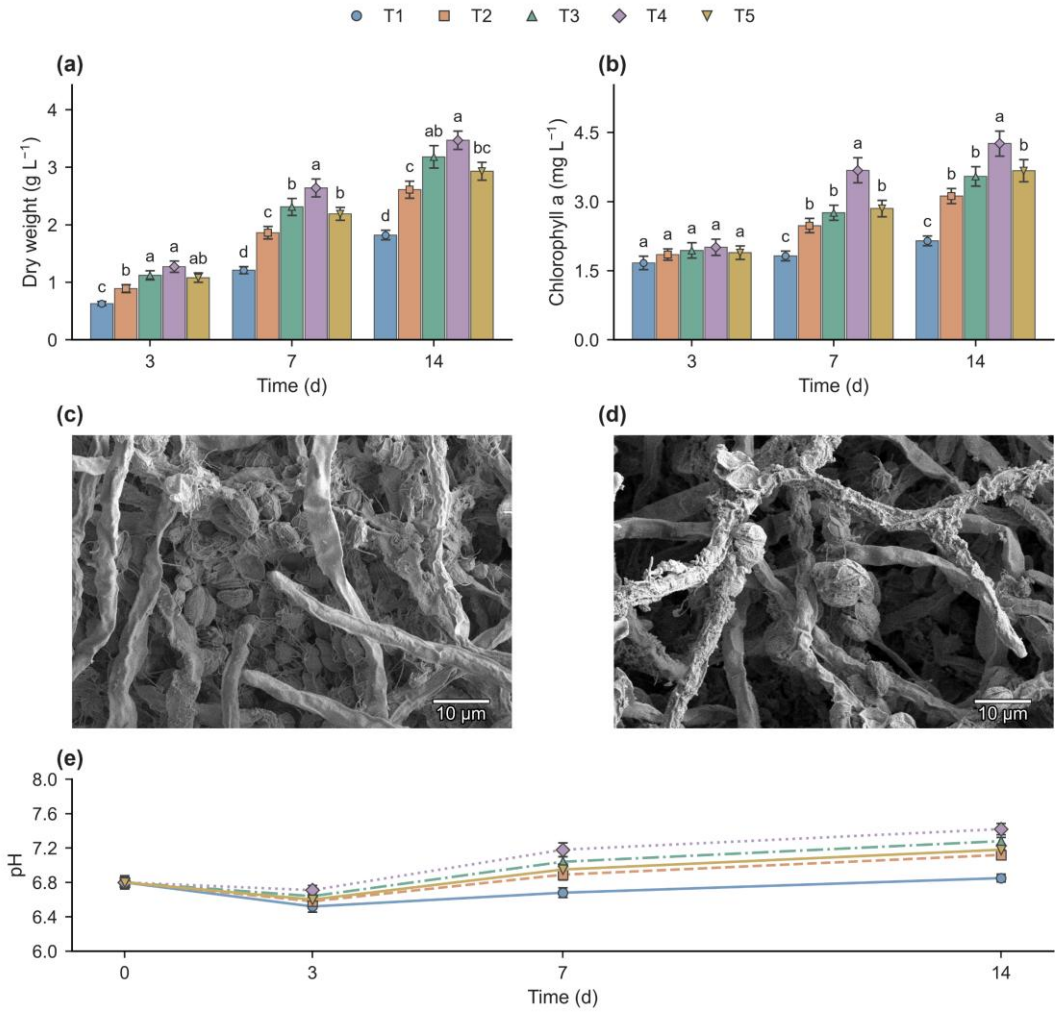


Fig. 2 Effects of indole-3-acetic acid (IAA) supplementation on biomass growth, morphological characteristics, and culture pH in microalgal monoculture and fungal–algal coculture systems. (a) Biomass dry weight (DW); (b) chlorophyll a (Chl a) concentration; (c, d) representative scanning electron microscopy (SEM) images showing fungal hyphae and associated microalgal cells within fungal–algal aggregates; and (e) changes in culture pH during cultivation. Quantitative data are presented as mean ± SD (n = 3 independent biological replicates). Different lowercase letters in (a) and (b) indicate significant differences among treatments at the same sampling time (one-way ANOVA followed by Tukey's HSD test, $p < 0.05$). Scale bars in (c) and (d) represent 10 μm

Antioxidant responses varied considerably among treatments during cultivation (Fig. 3a–c). Both SOD and CAT activities were enhanced by fungal cocultivation and further increased following IAA supplementation, with the highest activities consistently observed in T4. SOD and CAT activities in T4 peaked on Day 7, reaching 24.68 and 0.41 U mg^{-1} FW, respectively, and remained at 22.53 and 0.37 U mg^{-1} FW on Day 14 (Fig. 3a, b). These values were significantly higher than those in T2, which exhibited corresponding activities of 14.15 and 0.22 U mg^{-1} FW on Day 14. In contrast, MDA content exhibited an opposite pattern (Fig. 3c).

MDA in T4 decreased from 1.52 μmol g^{-1} FW on Day 3 to 0.75 μmol g^{-1} FW on Day 14, representing a 48.6% reduction compared with T2 (1.46 μmol g^{-1} FW). The simultaneous enhancement of antioxidant enzyme activities and reduction in MDA accumulation characterized the physiological response of the IAA-supplemented fungal–algal consortium.

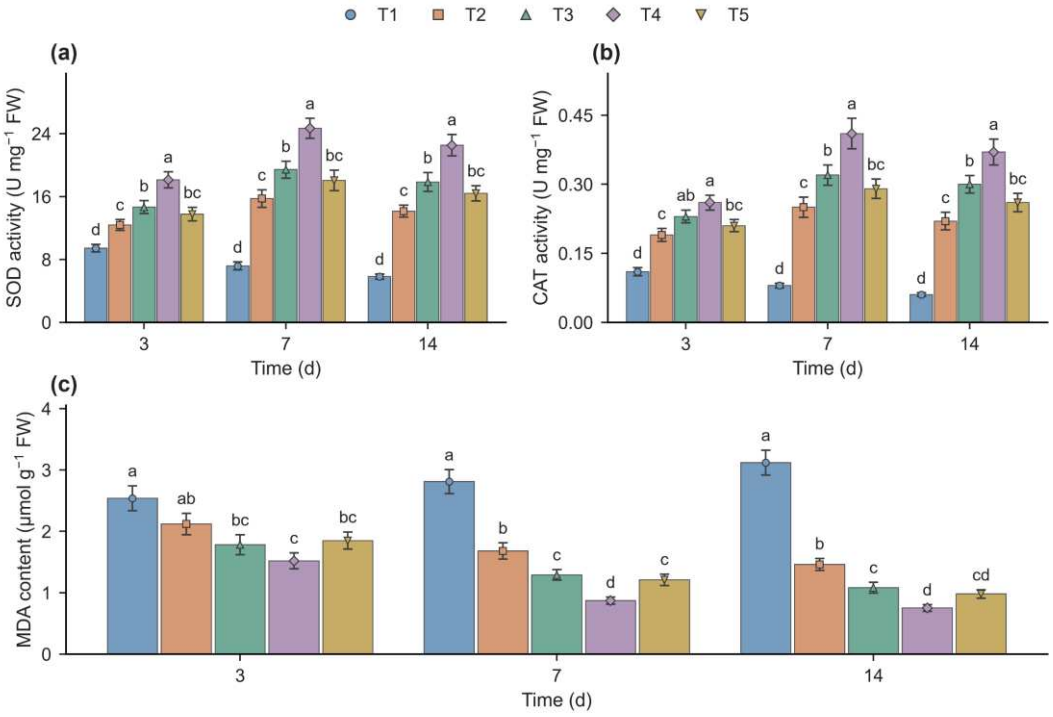


Fig. 3 Effects of IAA supplementation on antioxidant responses in microalgal monoculture and fungal–algal coculture systems under OTC exposure: (a) SOD activity, (b) CAT activity, and (c) MDA content. Data are presented as mean ± SD (n = 3 independent biological replicates). Different lowercase letters indicate significant differences among treatments at the same sampling time (one-way ANOVA followed by Tukey's HSD test, $p < 0.05$)

3.3 EPS characteristics and biomass clarification

EPS production increased throughout cultivation, with distinct differences among treatments in both PN and PS accumulation (Fig. 4a–h). The T4 treatment supplemented with 10^{-6} M IAA exhibited the highest EPS contents at all three sampling stages. In T4, PS content increased from 48.65 mg g^{-1} on Day 3 to 71.40 mg g^{-1} on Day 7 and 86.21 mg g^{-1} on Day 14, while PN content increased from 27.80 to 45.83 mg g^{-1} over the same period (Fig. 4f, g). Accordingly, the measured EPS content in T4 increased from 76.45 to 132.04 mg g^{-1} during cultivation (Fig. 4h). On Day 14, T4 exhibited significantly higher PS, PN, and total EPS contents than T2, which recorded corresponding values of 61.17, 35.42, and 96.59 mg g^{-1} (Fig. 4a–c). The total EPS content of T4 was 36.7% higher than that of T2 and 66.8% higher than that of the T1.

The EPS extracts exhibited distinct differences in zeta potential among treatments (Fig. 4e). All samples displayed negative zeta potentials, ranging from −21.84 to −11.36 mV. The zeta

potential shifted from −21.84 mV in T1 to −17.45 mV in T2 and −11.36 mV in T4, with significant differences between T4 and both T1 and T2. Meanwhile, the PN/PS ratios ranged from 0.532 to 0.605, with T4 exhibiting the lowest ratio (Fig. 4d).

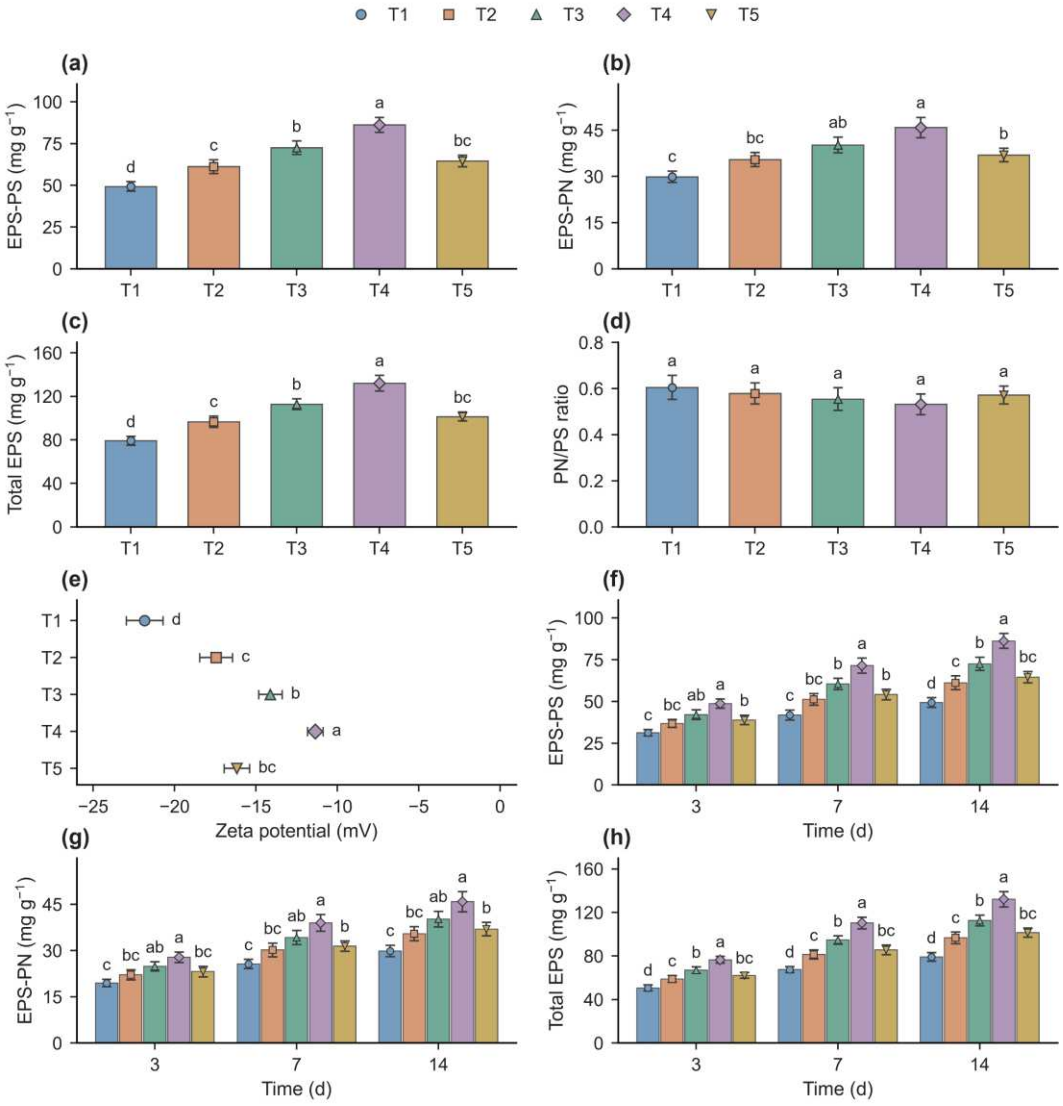


Fig. 4 Effects of IAA supplementation on EPS production, composition, and zeta potential in microalgal monoculture and fungal–algal coculture systems. (a) PS content, (b) PN content, (c) total EPS content, (d) PN/PS ratio, and (e) zeta potential of the extracted EPS solutions on Day 14; temporal variations in (f) PS, (g) PN, and (h) total EPS contents during cultivation. Data are presented as mean ± SD (n = 3 independent biological replicates). Different lowercase letters indicate significant differences among treatments within each sampling time (one-way ANOVA followed by Tukey's HSD test, $p < 0.05$)

Static clarification assays further demonstrated differences in biomass settling performance among the treatments (Fig. 5a, b). Clarification efficiency increased with settling time, with T4 consistently exhibiting the highest mean values. Within the first 15 min, T4 achieved a

clarification efficiency of 76.4%, compared with 54.2% in T2 and 38.5% in T1. After 30 min, clarification efficiency in T4 reached 88.2%, exceeding that of T2 by 16.6 percentage points. The improvement in clarification efficiency was particularly pronounced during the initial 15–30 min. By 60 min, clarification efficiency in T4 reached 93.6%, compared with 81.2% in T2 and 68.4% in T1. The results demonstrated the favorable early-stage clarification performance of the IAA-supplemented fungal–algal consortium.

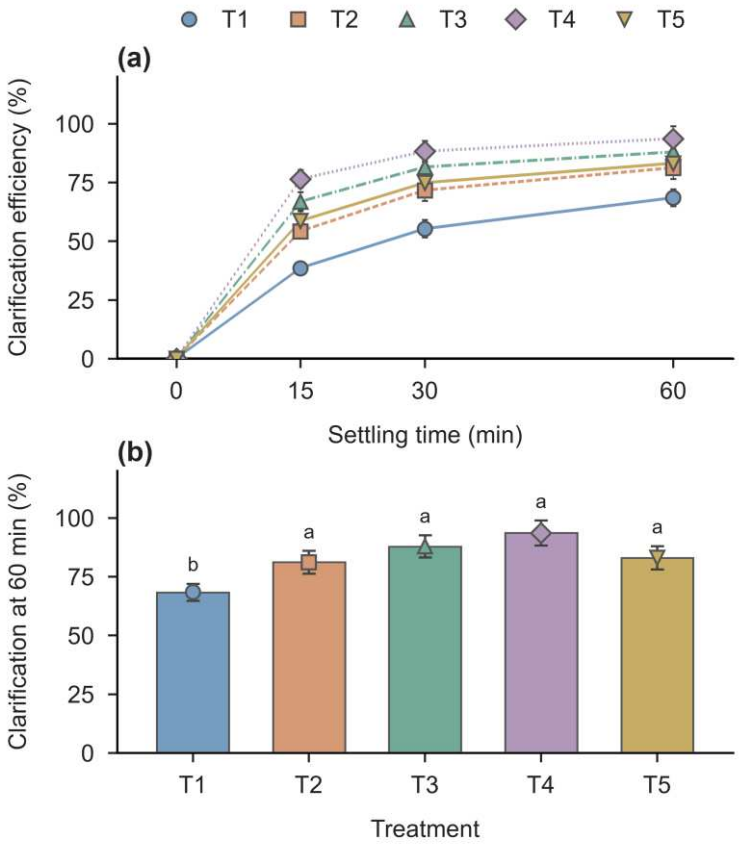


Fig. 5 Effects of IAA supplementation on static biomass clarification in microalgal monoculture and fungal–algal coculture systems. (a) Changes in clarification efficiency during 60 min of gravitational settling and (b) clarification efficiencies after 60 min. Data are presented as mean ± SD (n = 3 independent biological replicates). Different lowercase letters in (b) indicate significant differences among treatments after 60 min of settling (one-way ANOVA followed by Tukey's HSD test, $p < 0.05$)

4. Discussion

The improved pollutant removal performance following fungal cocultivation highlights the complementary functions of microalgae and filamentous fungi in antibiotic-containing wastewater treatment. The introduction of *A. oryzae* increased OTC removal from 48.2% in the algal monoculture to 74.6% in the IAA-free coculture, accompanied by enhanced COD, TN, and TP removal. Beyond increasing the biological components of the treatment system, fungal

cocultivation can modify the spatial organization of microbial biomass through hyphal attachment and aggregate formation. The filamentous network observed by SEM in the present study provided a structural framework for the association of algal cells with fungal biomass, potentially increasing the contact between microorganisms and dissolved constituents. Such structural integration is important because the efficiency of microbial consortia depends not only on the metabolic activities of individual organisms but also on their physical organization and extracellular interactions. Previous investigations have demonstrated that fungal-assisted microalgal cultivation can enhance nutrient removal, biomass accumulation, and aggregate formation, with extracellular polymers contributing to the stability of microbial associations (Wu et al. 2023; Mi et al. 2024; Guan et al. 2025). The use of *A. oryzae* in microalgal harvesting has also demonstrated its capacity to establish recoverable fungal–algal structures during wastewater treatment (Khalatbari et al. 2025). In the present system, the simultaneous improvement in pollutant removal and formation of interconnected aggregates suggests that fungal cocultivation established a favorable biological and structural basis for subsequent IAA regulation.

The substantially higher OTC removal in the inoculated treatments than in the abiotic controls further indicates the importance of biomass-associated processes. After 14 days, abiotic OTC loss remained below 12%, whereas the fungal–algal coculture supplemented with 10^{-6} M IAA achieved 92.5% removal. OTC elimination in phototrophic microbial systems can involve adsorption onto cellular and extracellular materials, biological transformation, and abiotic reactions, with their relative contributions varying according to the cultivation conditions (Wu et al. 2022). The presence of fungal hyphae and extracellular polymers may increase the available interfaces for OTC partitioning, while metabolically active microbial biomass provides additional opportunities for contaminant transformation. The contribution of extracellular macromolecules to tetracycline removal and biomass aggregation has been demonstrated in microalgae–fungi consortia, emphasizing the functional importance of the extracellular environment during antibiotic treatment (Wang et al. 2025). Moreover, changes in microbial metabolism and extracellular characteristics have been associated with improved antibiotic removal in other algal-based treatment systems (Ren et al. 2025; Li et al. 2025). These processes offer plausible explanations for the higher aqueous-phase OTC removal observed in the present coculture. Importantly, the concurrent improvements in COD and nutrient removal suggest that fungal cocultivation supported multiple treatment functions rather than enhancing OTC removal alone. The development of an active fungal–algal consortium therefore provided a basis for integrating antibiotic removal with organic matter conversion and nutrient utilization.

IAA supplementation further modified the performance of the consortium, with the most favorable response occurring at 10^{-6} M. The treatment response was concentration-dependent rather than increasing continuously with IAA dosage, indicating that the effectiveness of

phytohormone supplementation depends on the balance between regulatory stimulation and the physiological requirements of the microbial consortium. This behavior is consistent with the concentration-specific effects of phytohormones observed in microalgae-based wastewater treatment systems (Xu et al. 2024; Guo et al. 2024; Chai et al. 2024; Zhao et al. 2026). At the optimal concentration, IAA supplementation increased both biomass accumulation and chlorophyll a content, suggesting that the improvement in treatment performance was accompanied by a favorable growth and photosynthetic response. IAA-associated changes in carbon assimilation and metabolic allocation have been identified in microalgal cultivation, providing a potential physiological basis for increased biomass production under suitable supplementation conditions (Yang et al. 2024). Enhanced growth and pollutant removal following IAA addition have also been observed in *Chlorella*-based wastewater treatment systems, including cultures exposed to antibiotics (Pekkoh et al. 2024; Fayaz et al. 2027). In the present study, the increase in total biomass dry weight was accompanied by higher chlorophyll a accumulation, indicating that the improved performance was not reflected solely in the greater amount of fungal–algal biomass. The simultaneous increase in chlorophyll content and wastewater treatment efficiency is consistent with a favorable response of the phototrophic component within the coculture. These findings suggest that appropriate IAA supplementation can improve consortium performance by influencing both biomass development and physiological activity.

The antioxidant responses provide further insight into the physiological regulation associated with IAA supplementation. SOD and CAT activities increased in the IAA-treated consortium and reached their highest values during the intermediate cultivation stage, whereas MDA accumulation was lower than in the IAA-free coculture. These changes are particularly relevant under antibiotic exposure because the balance between reactive oxygen species generation and antioxidant protection influences cellular integrity and metabolic activity. SOD catalyzes the dismutation of superoxide radicals, while CAT participates in hydrogen peroxide decomposition. Increased activities of these enzymes can enhance cellular antioxidant capacity, whereas reduced MDA accumulation indicates a lower extent of lipid peroxidation. Accordingly, the combination of higher SOD and CAT activities with lower MDA content suggests that IAA supplementation was associated with a more favorable oxidative balance rather than simply stimulating an isolated antioxidant response. Similar patterns have been observed in phytohormone-regulated microalgal cultures under antibiotic exposure, where improvements in antioxidant defenses were accompanied by enhanced growth and pollutant removal (Song et al. 2026; Fayaz et al. 2027). In the present consortium, the antioxidant response was accompanied by greater chlorophyll accumulation and biomass development, indicating that physiological regulation and growth performance changed in parallel. This relationship is important because sustained cellular activity provides a biological basis for continued nutrient utilization and contaminant removal during prolonged exposure. Thus, the improved

antioxidant status of the IAA-supplemented consortium represents a potentially important physiological component of its enhanced treatment performance.

Extracellular polymer accumulation constituted another major response to IAA supplementation, connecting microbial physiological activity with the extracellular characteristics of the consortium. The measured EPS content in T4 reached 132.04 mg g^{-1} DW, representing a 36.7% increase relative to the IAA-free coculture. Since EPS contents were normalized to biomass dry weight, this increase indicates greater accumulation of the measured extracellular polymers per unit biomass rather than merely reflecting the higher total biomass concentration. Both PN and PS contents increased, with T4 exhibiting a PN/PS ratio of 0.532, suggesting a relatively higher proportion of polysaccharides in the measured EPS composition. EPS composition is relevant to microbial adhesion, polymer interactions, and aggregate organization because extracellular proteins and polysaccharides contribute differently to these processes. Proteins provide functional groups involved in interfacial interactions, whereas polysaccharide-rich matrices can support the development of hydrated extracellular networks and contribute to aggregate cohesion. The functional importance of EPS abundance and composition has been emphasized in microalgal cultivation and biomass separation, where changes in extracellular polymer characteristics can affect aggregation and interactions with dissolved compounds (Zhou et al. 2024; Chen et al. 2025). EPS also participate in microbial responses to environmental stress by modifying the extracellular environment surrounding the cells (Su et al. 2025). In the present study, the simultaneous increases in PN and PS suggest that IAA supplementation influenced extracellular polymer accumulation, while the observed PN/PS ratios provide additional information on their relative composition. Such changes may favor interactions among algal cells and fungal hyphae while providing additional extracellular interfaces relevant to pollutant removal. The improved treatment and clarification performances of T4 therefore coincided with a distinct modification of its extracellular polymeric characteristics.

The variation in EPS composition was further accompanied by changes in the zeta potential of the extracted EPS solutions. The measured potential shifted from −17.45 mV in the IAA-free coculture to −11.36 mV in T4, indicating a reduction in the magnitude of the negative electrokinetic potential. The electrokinetic behavior of EPS is influenced by the abundance, distribution, and ionization of functional groups associated with extracellular proteins and polysaccharides. Consequently, changes in EPS composition may alter the interactions among extracellular constituents and their affinity for microbial surfaces. Previous studies have linked IAA supplementation to changes in microalgal adhesion through modifications of extracellular proteins and interfacial properties (Xie et al. 2024; Tang et al. 2026). The relationship between EPS composition, charged functional groups, and flocculation behavior has also been demonstrated in microalgal cultivation systems (Dai et al. 2025). Moreover, recent

investigations of fungal-assisted bioflocculation have shown that extracellular polymer composition and glycan architecture can be important determinants of microbial attachment and aggregate formation, rather than total EPS abundance alone (Zhang et al. 2025). These findings provide a useful basis for interpreting the present results, in which the increase in EPS content was accompanied by a shift toward a less negative potential in the extracted EPS solutions. Although the measured values characterize EPS extracts rather than the intact fungal–algal aggregates, their variation indicates that IAA supplementation modified the electrokinetic properties of extracellular constituents. The concurrent changes in EPS abundance, composition, and zeta potential suggest that extracellular regulation involved multiple physicochemical characteristics relevant to microbial aggregation.

The improvement in static clarification provides a functional perspective on these extracellular changes. The IAA-supplemented consortium achieved clarification efficiencies of 76.4% and 88.2% after 15 and 30 min, exceeding the corresponding values of the IAA-free coculture by 22.2 and 16.6 percentage points, respectively. The difference decreased to 12.4 percentage points after 60 min, indicating that the advantage of IAA supplementation was particularly pronounced during the early settling period. This temporal pattern is important because clarification performance is determined not only by the final degree of suspended biomass removal but also by the rate at which a clarified supernatant can be obtained. Fungal-assisted aggregation can improve gravitational separation by organizing dispersed algal cells into larger microbial structures, while extracellular polymers contribute to the interactions that maintain aggregate integrity. The importance of fungal morphology, extracellular constituents, and interfacial interactions has been demonstrated in studies of fungal-assisted *C. vulgaris* flocculation and *A. oryzae*-based microalgal harvesting (Tölle and Kuenz 2025; Jing et al. 2025). EPS-associated flocculation also depends on polymer composition and cultivation conditions, which influence the development and settling behavior of microbial aggregates (Nishad et al. 2025). In addition, electrostatic interactions and solution chemistry can substantially influence microalgal flocculation performance (Zhang et al. 2024). In the present study, the interconnected fungal structures observed by SEM, increased EPS accumulation, and altered electrokinetic properties of the EPS extracts were accompanied by faster clarification. These findings support an association between IAA-mediated extracellular regulation and the early-stage separation behavior of the consortium. From a process perspective, the pronounced improvement within the first 30 min is particularly valuable because faster clarification may reduce the settling time required to obtain a biomass-depleted supernatant.

The combined improvements in pollutant removal, physiological responses, extracellular characteristics, and biomass clarification highlight the potential of IAA regulation as an integrated strategy for fungal–algal wastewater treatment. The results suggest that the response to IAA supplementation involved several connected processes: enhanced growth and

chlorophyll accumulation were accompanied by favorable antioxidant responses, while increased EPS accumulation and changes in extracellular electrokinetic properties coincided with improved early-stage clarification. The integration of microbial interactions, structural organization, and wastewater purification has become an important direction in microalgae-based treatment technologies (Takahashi et al. 2024; Yang et al. 2025). Recent developments in phytohormone-assisted phototrophic reactors have further demonstrated the potential of coupling antibiotic removal with nutrient recovery under continuous-flow conditions (Yao et al. 2026). Within this broader context, the present study identifies a complementary approach based on IAA-regulated fungal–algal cocultivation, where enhanced wastewater treatment performance was accompanied by rapid gravitational clarification. Its main contribution lies in connecting physiological responses with extracellular polymer characteristics and separation performance within the same treatment system. This coordinated improvement provides a basis for further developing fungal–algal processes in which efficient pollutant removal and convenient biomass separation are considered together.

5. Conclusions

This study demonstrated that appropriate IAA supplementation improved both wastewater treatment performance and biomass clarification in an OTC-exposed *Chlorella vulgaris*–*Aspergillus oryzae* coculture. The response was concentration-dependent, with 10^{-6} M IAA providing the most favorable overall performance. Beyond enhanced pollutant removal, IAA supplementation was associated with greater biomass accumulation, improved antioxidant responses, and increased EPS production. Increased EPS accumulation and a shift in the zeta potential of extracted EPS solutions coincided with faster gravitational clarification, particularly during the initial settling period. These findings suggest that the benefits of IAA regulation extend beyond microbial growth stimulation to encompass physiological and extracellular characteristics relevant to wastewater treatment and biomass separation. Overall, this study provides an integrated perspective on phytohormone-assisted fungal–algal cultivation and supports its potential application in antibiotic-containing wastewater treatment where efficient biomass recovery is also required.

Declarations

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Availability of data and material

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability

Not applicable.

Authors' contributions

Likai Wu: Conceptualization, Methodology, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. Rilin Tan: Investigation, Data curation, Validation, Writing – review & editing. Mingyu Han: Methodology, Investigation, Project administration, Writing – review & editing. Xinran Zhang: Investigation, Data curation, Validation. Xinrui Dong: Investigation, Data curation, Validation. All authors read and approved the final manuscript.

Ethics approval

Not applicable. This study did not involve human participants or animals.

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