Jurnal Biolokus: Jurnal Penelitian Pendidikan Biologi dan Biologi
Vol. 9, Issue 1, June 2026
Pages : 89 – 100

p-ISSN: 2621-3702
e-ISSN: 2621-7538

Implementation of *Chlorella vulgaris* co-culture in open pond systems for sustainable green industry development

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ABSTRACT

Microalgae are promising sources of high-value biomass and renewable energy. *Chlorella vulgaris* is widely recognized for its ability to grow under various trophic conditions. Co-cultivation of microalgae with bacteria has the potential to enhance biomass production, although its effectiveness depends on the cultivation system. This study compared the growth performance and volatile metabolite profiles of *C. vulgaris* co-cultured with an Indole-3-Acetic Acid (IAA)- and vitamin B12-producing bacterial consortium in Open Pond (OP) and Photobioreactor (PBR) systems. From June to August 2025, *C. vulgaris* was co-cultivated with the bacterial consortium in the OP and PBR systems for 14 days. Growth was monitored by measuring cell density at 675 nm with a UV-Vis spectrophotometer, while the volatile metabolite profiles of the biomass harvested at the early stationary phase were analyzed using Gas Chromatography-Mass Spectrometry (GC–MS). The PBR system achieved a significantly higher peak cell density ($9,6 \times 10^7$ cells/mL) than the OP system ($4,0 \times 10^7$ cells/mL). GC-MS identified 136 metabolites, with the PBR system exhibiting a higher abundance of polyunsaturated fatty acids (PUFAs). Conversely, the OP system exhibited higher levels of chavicol (1.02%) and the quorum-sensing molecule farnesol (0.53%), indicating adaptive responses to environmental stress. These findings indicate that functional bacteria benefit both cultivation systems, although the OP system requires further optimization for sustainable industrial applications.

Keywords: *Chlorella vulgaris*, co-culture, IAA-producing bacteria, vitamin B12-producing bacteria, volatile metabolites

INTRODUCTION

Microalgae have broad industrial applications, including food, feed, pharmaceuticals, bioremediation, cosmetics, and renewable energy, due to their diverse biochemical composition (Novoveská et al., 2023). One of the preeminent microalgae species for industrial-scale development is *Chlorella vulgaris*, a unicellular green microalga renowned for its high photosynthetic efficiency and wide adaptability to autotrophic, mixotrophic, and heterotrophic conditions (Ahmad et al., 2013; Ru et al., 2020). These advantages make large-scale cultivation essential.

Open ponds (OP) are widely used in large-scale production due to their low cost and simple operation compared to photobioreactors (PBR) (Morales et al., 2019; Rasheed et al., 2023; Tan et al., 2020). OP are also more energy efficient, consuming up to 90% less energy than closed PBR (Costa et al., 2018). This energy efficiency increases the economic viability of industrial operations. However, to offset the environmental vulnerabilities of open systems, co-culture techniques are essential to mitigate operational constraints.

The application of co-culture techniques to microalgae can significantly increase biomass yield and production of high-value compounds through the metabolic activities of the microbial strains involved, especially bacteria (Berthold et al., 2019; Rosero-Chasoy et al., 2022). In co-culture systems, microalgae release dissolved organic matter (C, N, and P) that stimulates

bacterial growth. Conversely, bacterial remineralization of these compounds into inorganic forms stimulates microalgal productivity (Rasheed et al., 2023).

Several bacterial strains known for their ability to influence microalgae growth include Indole-3-Acetic Acid (IAA)-producing bacteria and Vitamin B12-producing bacteria. Bacterial metabolites, particularly IAA, have been shown to increase lipid productivity in microalgae (Magdouli et al., 2016). Furthermore, microalgal growth is significantly affected by Vitamin B12 synthesized by bacteria. Within microalgal metabolism, Vitamin B12 functions as a vital coenzyme involved in methionine biosynthesis (Prabaningtyas et al., 2021).

C. vulgaris offers sustainable industrial potential through the production of high-value biomass. Previous studies have demonstrated that microalgal–bacterial co-cultures can enhance microalgal growth and metabolite production (Borges Lopes et al., 2025; Gopalakrishnan et al., 2024). However, most studies have focused on biomass productivity under controlled cultivation conditions, whereas comparative studies of bacterial co-cultures in Open Pond (OP) and Photobioreactor (PBR) systems are still limited. Furthermore, little information is available on how cultivation system configuration influences the volatile metabolite profiles of *C. vulgaris* during bacterial co-cultivation. Consequently, this study aims to compare the growth performance and volatile metabolite profiles of *C. vulgaris* co-cultured with an Indole-3-Acetic Acid (IAA)- and vitamin B12-producing bacterial consortium in OP and PBR systems.

METHOD

Research tools and materials

The equipment used included an analytical balance, an autoclave, a laminar air flow (LAF) cabinet, an incubator, a photobioreactor, a cultivation pond, an aerator, a lighting system with digital timers, and standard microbiological laboratory glassware.

The biological materials consisted of *Chlorella vulgaris* obtained from the Microalgae Culture Laboratory, Greenhouse, Universitas Negeri Malang, an IAA-producing bacterium (*Delftia* sp.), and a vitamin B12-producing bacterium (*Lysinibacillus fusiformis*). The culture media used were Gusrina medium, and Nutrient Broth (NB). Consumables included distilled water, 70% ethanol, and 0,5 McFarland standard solution (equivalent to 1,5 x 10^8 CFU/mL).

Research procedures

1. Media preparation

Microalgae were cultivated in Gusrina medium, which was prepared by dissolving 22,5 g urea, 15 g Triple Super Phosphate (TSP), and 0,5 g $FeCl_3$ in 500 mL of distilled water. The solution was sterilized before aseptic addition of Vitamin B12. For bacterial cultivation, Nutrient Broth (NB) was prepared at a concentration of 8 g/L in distilled water to reach a total volume of 10 L, providing essential carbon and nitrogen sources through beef extract and peptone, respectively. Both media were prepared following standard sterilization protocols.

2. Inoculum and stock preparation

The *C. vulgaris* stock was prepared by inoculating 150 mL of culture into 600 mL of sterile distilled water supplemented with 0,75 mL of Gusrina medium. For bacterial inoculum, two loops of each strain were inoculated into 10 mL of Nutrient Broth (NB) and incubated for 24 hours. Bacterial density was then standardized to 0,5 McFarland (equivalent to 1,5 x 10^8 CFU/mL) to ensure consistent initial concentrations.

3. Co-culture implementation in OP and PBR systems

Co-culture treatments were performed in the OP and PBR systems. Each system was inoculated with a mixture of *C. vulgaris*, Gusrina medium, sterile distilled water, and the bacterial consortium at a volume ratio of 75:0.75:600:75 (mL), respectively. Cultivation was carried out for approximately 14 days until reaching the early stationary phase. In the PBR system,

controlled conditions consisted of an 18:6 h light/dark photoperiod, 4,600 lux light intensity, a constant temperature of 30°C (Lirofiatillah et al., 2020), and 12 h of daily aeration. Conversely, the OP system was maintained in a greenhouse under ambient conditions, with 12 h of daily aeration. The cultivation pond was covered with a transparent plastic roof that allowed natural sunlight while preventing direct rainfall. Small ventilation openings minimized exposure to insects and other animals.

4. Microalgal growth monitoring

Microalgae growth was monitored daily throughout the 14-day cultivation period (Saputri et al., 2025). For each treatment, 1.5 mL samples were collected in duplicate and transferred into microtubes to ensure analytical accuracy. Cell density was determined spectrophotometrically by measuring the optical density at a wavelength of 675 nm, which was selected based on a preliminary spectral scan of the culture samples between 650–700 nm, with reference to previously reported optimal wavelengths (Almomani & Örmeci, 2018; Jui et al., 2024).

5. Biomass harvesting and GC-MS analysis

Biomass of *C. vulgaris* from both PBR and OP systems was harvested by filtering the mixed culture media through fine filter paper. In the OP system, representative 1 L samples were collected from three distinct points to ensure sampling consistency. The obtained microalgal cells were transferred to Petri dishes and dried using a dehydrator until a constant powder form was obtained. The total dry weight (g) was then recorded. Finally, the chemical composition of the powdered biomass was analyzed using Gas Chromatography-Mass Spectrometry (GC-MS) (Moran et al., 2022). Volatile metabolites were identified by comparing mass spectra with the GC–MS system mass spectral library. Only compounds with a similarity index (SI) ≥ 92% were identified. Relative abundance was expressed as composition (%) generated by the GC–MS software.

RESULTS AND DISCUSSION

C. vulgaris growth in OP and PBR co-culture

Distinct growth patterns of *C. vulgaris* were observed between the photobioreactor (PBR) and open pond (OP) systems. In the PBR system, *C. vulgaris* exhibited a stable exponential phase, with cell density increasing from 0,7 x 10^7 cells/mL on day 0 to a peak of 9,6 x 10^7 cells/mL by day 14. In contrast, the OP system showed unstable and significantly lower cell densities, which did not exceed 4,0 x 10^7 cells/mL. A further drastic decrease in density was observed in the OP system starting from day 10, with values approaching zero (Figure 1). This phenomenon aligns with the technical challenges reported by Cho et al. (2017), which state that open systems are highly susceptible to fluctuations in abiotic parameters and invasion by wild microbial species. In contrast to previous studies that primarily focused on improving microalgal biomass production, the present study demonstrated that the growth performance of *C. vulgaris* co-cultured with an IAA- and vitamin B12-producing bacterial consortium was highly dependent on cultivation system configuration, with significantly greater performance in PBR than in OP system.

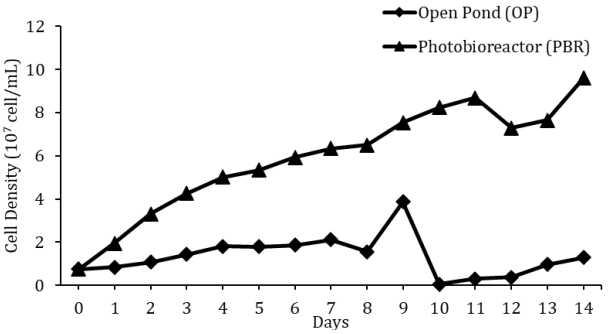

Figure 1. Cell density of *C. vulgaris* in co-culture with IAA-producing and Vitamin B12-producing bacteria

The high cell density observed in the PBR system suggests that the integration of IAA-producing and Vitamin B12-producing bacteria works synergistically in a controlled, closed environment. According to Yao et al. (2019), the presence of functional symbiotic bacteria in

closed systems facilitates efficient metabolite exchange, where bacteria provide essential cofactors that accelerate algal growth kinetics. Specifically, bacterial inoculation stimulates microalgal proliferation by producing growth promoters and vitamins (Dao et al., 2018). In this study, IAA-producing bacteria that produce auxin promoted microalgal growth, while Vitamin B12-producing bacteria supplied essential nutrients (Tong et al., 2023). This interaction forms a complex nutrient cycle, microalgae produce O_2 through photosynthesis which serves as an electron acceptor/carbon-processing aid for bacteria, while bacteria produce CO_2 and growth cofactors that further support the microalgae (Guo & Tong, 2014; Makut et al., 2022).

The low microalgal cell densities observed in OP systems are likely caused by competition with the native or opportunistic microbes introduced through atmospheric exposure, including competing microalgae, zooplankton, fungi, bacteria, and viruses (Ding et al., 2020). These coexisting biological contaminants trigger intense competition for essential nutrients (Lam et al., 2018). Such biotic contamination can significantly reduce biomass yield and quality, thus reducing overall microalgal productivity (Jerney & Spilling, 2018; Shah et al., 2016). In OP systems, nutrient-producing bacteria may be outcompeted by native or invasive microbes. In contrast, the controlled environment of PBR systems promotes the dominance of functional bacteria, enhancing microalgal growth (Rasheed et al., 2023; Skifa et al., 2025).

Higher cell densities in the PBR system were supported by optimal light penetration, stable temperatures, and controlled nutrient distribution. In contrast, unregulated OP systems are more susceptible to weather conditions, resulting in lower CO_2 mass transfer, inefficient mixing, higher evaporation, contamination, and variable light intensity (Faried et al., 2017). Outdoor OP systems expose microalgae to fluctuating irradiance, temperature, and weather fluctuations. Suboptimal daytime and nighttime temperatures reduce productivity by causing photoinhibition and increasing dark respiration (Borowitzka & Vonshak, 2017). Maintaining optimal temperatures is essential for maximizing microalgae productivity. Lower temperatures can reduce carboxylase activity, whereas high temperatures negatively impact enzymatic functions and photosynthetic proteins (Borowitzka & Vonshak, 2017; Skifa et al., 2025). Over the course of the day, these environmental factors can limit photosynthetic efficiency to levels that could potentially inhibit the growth of outdoor microalgae cultures (Skifa et al., 2025).

Volatile profiles and symbiotic dynamics in OP and PBR systems

GC-MS analysis of *C. vulgaris* biomass revealed a total of 136 identified metabolites. The data indicate that the PBR system provides a more stable environment for *C. vulgaris* growth compared to the OP system. This stability is reflected in the higher abundance of polyunsaturated fatty acid (PUFA) profiles in the PBR-treated biomass, including linoleic acid, α-linolenic acid, γ-linolenic acid, stearidonic acid, eicosadienoic acid, eicosatetraenoic acid, arachidonic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) (Table 1).

Physiologically, enhanced lipid biosynthesis is linked to Vitamin B12 supplied by the symbiotic bacteria, which serves as a cofactor for mitochondrial methylmalonyl-CoA mutase involved in odd-chain fatty acid catabolism (Pettinato et al., 2025). The higher prevalence of odd-chain fatty acids, such as tridecanoic acid (C13) in the PBR system (0,55%) compared to the OP system (0,29%), suggests that the biosynthesis of these fatty acids is optimized under controlled conditions (Table 1). These findings may indicate more efficient odd-chain fatty acid metabolism under controlled cultivation conditions.

In addition to the energetic support provided via the succinyl-CoA pathway, the enhanced PUFA profiles can be attributed to the supplementation of indole-3-acetic acid (IAA) by

the associated bacteria. This auxin hormone can stimulate lipid production and optimize fatty acid composition for high-quality biodiesel production (Lin et al., 2022). These findings are consistent with previous reports indicating that in the genus *Chlorella*, IAA supplementation can increase the percentage of PUFAs, specifically linoleic acid, α-linolenic acid, and γ-linolenic acid (Chen et al., 2020; Salama et al., 2014).

However, the presence of PUFAs in microalgae is highly susceptible to climatic fluctuations (Zakaria et al., 2025), as evidenced by the superior PUFA yields in the controlled PBR system compared to the OP system. The lower PUFA profile in the OP system may result from photooxidation induced by direct sunlight exposure, where intense ultraviolet and visible light radiation generate free radicals and singlet oxygen that degrade unsaturated fatty acids (Tao, 2015). Consequently, this induces severe oxidative damage, significantly compromising the PUFA yields within the OP system.

Table 1. Volatile metabolite profile of microalgae *C. vulgaris*

Compound Category	Compound Name	Composition (%)	
		PBR	OP
Fats (Lipids and Fatty Acids)	propionic acid	1.38	0.73
	butyric acid	0.39	0.20
	valeric acid	0.55	0.29
	hexanoic acid	1.06	0.56
	heptanoic acid	0.71	0.38
	octanoic acid	0.66	0.35
	nonanoic acid	1.41	0.74
	decanoic acid	0.72	0.38
	undecanoic acid	1.03	0.55
	dodecanoic acid	1.08	0.57
	tridecanoic acid	0.55	0.29
	myristic acid	2.55	1.34
	palmitic acid	2.15	1.13
	octadecanoic acid	0.97	0.51
	heptadecanoic acid	1.31	0.69
	nonadecanoic acid	0.50	0.26
	tetracosanoic acid	1.10	0.58
	palmitoleic acid	3.67	1.93
	stearidonic acid	2.59	1.36
	α-linolenic acid	3.45	1.82
	γ-linolenic acid	1.84	0.97
	linoleic acid	2.65	1.39
	oleic acid	5.10	2.68
	eicosapentaenoic acid	1.68	0.88
	arachidonic acid	1.13	0.59
	eicosatetraenoic acid	1.20	0.63
	eicosadienoic acid	0.79	0.42
	gondoic acid	0.84	0.44
	docosahexanoic acid	2.01	1.06
	erucic acid	1.47	0.77
Terpenoid	p-cymene	1.30	0.69
	β-phellandrene	0.75	1.68
	α-terpinolene	0.53	0.28
	α-thujene	0.56	1.26
	α-pinene	0.84	0.44
	Camphene	0.50	1.12
	Limonene	1.65	0.87
	δ-3-carene	0.58	1.30
	α-terpinene	0.68	0.36
	γ-terpinene	0.25	0.13

Compound Category	Compound Name	Composition (%)	
		PBR	OP
	Sabinene	0.36	0.19
	1,8-cineole	0.77	
	1-terpinen-5-ol	0.48	0.25
	Carvone	0.82	1.83
	Citral	1.23	2.76
	Phellandral	0.54	1.20
	(Z)-β-ocimenol	0.47	
	Linalool	0.19	0.43
	trans pinocarveol	0.56	1.25
	1-isopropyl-4-methylene 2 cyclohexen 1 ol	0.56	1.24
	cis-2,8-menthadien-1-ol	0.35	0.78
	p-mentha-1-ene-6-one	0.41	0.91
	trans (-)-p-mentha-1(7),5-dien 2-ol	0.43	0.96
	trans-1(7),8-p-menthadien-2-ol	0.64	1.43
	(+)-cis-p-mentha-1(7),8-dien-2 ol	1.07	2.40
	p-mentha-1,8-dien-6-ol	0.65	1.45
	α-terpineol	0.67	0.35
	cis-p-2-menthen-1-ol	1.10	2.46
	Isoborneol	0.47	1.05
	Nerol	1.14	2.55
	Cuparene	0.50	1.12
	Calamine	0.56	1.25
	β-farnesene	0.57	1.27
	β-bourbonene	1.05	2.34
	α-bergamotene	0.54	1.21
	β-sesquiphellandrene	0.50	1.11
	β-bisabolene	0.83	1.85
	β-caryophyllene	0.49	1.10
	β-elemene	0.45	1.01
	Viridiflorene	0.42	0.22
	Aromadendrene	0.77	0.41
	α-selinene	0.50	1.12
	β-selinene	0.47	1.05
	δ-cadinene	0.47	0.25
	Farnesol	0.24	0.53
	juniper camphor	0.92	2.07
	Bisacumol	0.47	1.05
	Curlone	0.32	0.72
	germacrone-13-al	0.73	1.64
	Viridiflorol	0.66	0.35
	Sesquisabinene	0.47	1.05
	α-copaene	0.72	1.61
	α-santalene	0.59	1.32
	Spathulenol	0.47	1.06
	β-eudesmol	0.58	1.29
	Nerolidol	0.40	0.90
	Dehydrocurdione	0.86	1.92
	4-hydroxybisabola-2,10-diene 9-one	0.44	0.99
	Bisacurone	0.32	0.72
	4-methoxy-5-hydroxybisabola 2,10-diene-9-one	0.47	1.05
	Campesterol	0.55	1.23
	β-sitosterol	0.64	1.43
	α-amyrin	0.46	
Phenolic Compounds	phenol, 2-methyl-	0.37	0.19
	phenol, 3-methyl-	0.44	0.23
	phenol, 3,5-dimethyl-	0.85	0.45

Compound Category	Compound Name	Composition (%)	
		PBR	OP
	phenol, 4-ethyl-	1.06	0.28
	2-(hydroxymethyl) Phenol	0.54	0.29
	phenol, 2,3,6-trimethyl-	0.77	0.40
	2-methoxy-4-methylphenol	0.28	0.15
	2-methoxy-6-methylphenol	0.18	
	phenol, 4-ethyl-2-methoxy-	0.15	0.08
	2,6-dimethoxyphenol	0.17	0.09
	ethanone, 1-(2,4,6 trihydroxyphenyl)-	0.19	0.10
	Guaiacol		0.09
	3,4-dimethoxyphenol		0.10
	Chavicol	0.46	1.02
	benzene, 1-ethenyl-4-methyl-	0.41	0.22
	benzene, 2-propenyl-	0.51	0.27
	1,2-benzenediol, 4-methyl-	0.17	0.09
	4-ethyl-1,3-benzenediol	0.13	0.07
	1,2,3-trimethoxybenzene	0.28	0.15
	1,2-benzenediol		0.13
Secondary & Volatile Metabolites	hexanal	0.23	
	heptanal	0.57	
	Nonanal	0.23	
	heptadeca 8,11,14 trienal	0.45	0.24
	1-penten-3-one	0.28	
	6-methyl-5-hepten-2-one	0.28	0.62
	2-nonanone	0.21	
	2-(2-methyl-2-propenyl)-2 cyclohexen-1-one	0.17	0.09
	ethanone, 1-(2,4,5 trimethylphenyl)-	0.23	0.12
	γ-butyrolactone	0.19	
	furan, 2-ethenyl-	0.52	0.08
	ethanone, 1-(2-furanyl)-	0.18	0.10
	benzoic acid methyl ester	0.93	0.49
	3-methyl-2-cyclopenten-1-one	0.30	
	1,2-cyclopentanedione, 3 methyl-	0.33	0.18
	2-cyclopenten-1-one, 2 hydroxy-3-methyl-	0.25	0.13
	2-cyclopenten-1-one, 2,3,4 trimethyl-	0.36	0.19
	1-buten-3-yne, 2-methyl-	0.24	0.13
	6-methyl-3-heptyne	0.31	0.16
	1,5-octadien-3-ol	0.50	0.26
	1-octen-3-ol	0.17	
	2,5-dimethyl-1,4 benzoquinone		0.18

Note: All reported metabolites showed a similarity index (SI) of ≥ 92% using the Mass Spectral Library available in the GC-MS system.

Nevertheless, GC-MS identification revealed that hexanal, a byproduct of oxidative PUFA degradation, was exclusively detected in the PBR system (Table 1). Hexanal is a major oxidative degradation product of omega-6 fatty acids, particularly linoleic acid (Hashem et al., 2022). The data show that linoleic acid levels in the PBR reached 2,65%, nearly double the concentration found in the OP system (1,39%) (Table 1). Consequently, the higher substrate abundance in PBR likely promoted hexanal formation. Unlike the OP system where hexanal is volatile (Lane et al., 2020), the closed design of PBR enhanced its retention within the biomass matrix. This may explain the absence of hexanal in the OP system despite the presence of precursor substrates.

Beyond primary nutritional aspects, the symbiotic interaction mediated by bacterial Indole-3-Acetic Acid (IAA) influences the

diversity of secondary defense metabolites. IAA stimulation is known to trigger the phenylpropanoid pathway in microalgae (Fan et al., 2022). This activation accounts for the elevated levels of phenylpropanoid-derived compounds, such as chavicol, observed in the OP system (1,02%) (Table 1). Chavicol plays a crucial role in algal defense mechanisms due to its potent antimicrobial and antioxidant properties (Ninkuu et al., 2025). These findings suggest enhanced self-defense responses to environmental stress, including microbial contamination and unpredictable weather in the OP system. In contrast, the lower chavicol content in the PBR system (0,46%) (Table 1) reflects the high environmental stability of the closed cultivation system.

Besides its defensive role, IAA may act as a diffusible signal mediating interspecies communication through a Quorum Sensing (QS)-like mechanism, enabling microalgae to respond to extracellular IAA and competitor density (Chung et al., 2018). Within the phycosphere, IAA functions as a cross-kingdom signaling molecule between microalgae and bacteria, potentially influencing microalgal growth and population dynamics (Cheng et al., 2023). The bacterial signaling was reflected in the microalgal volatile organic compound (VOC) profiles, with the oxygenated sesquiterpene farnesol being more abundant in the OP system (0,53%) than in the PBR system (0,24%). Farnesol is a well-known QS regulatory molecule in fungi, serving multiple functions including cell cycle maintenance, preventing overcrowding to avoid nutrient depletion, and regulating resilience to environmental stress (Padder et al., 2018). These results suggest that the OP system prioritizes survival over growth. The higher abundance of farnesol may reflect a stress-adaptive strategy, contributing to lower cell densities compared to the PBR system.

Collectively, these findings demonstrate that cultivation system configuration influences both biomass productivity and volatile metabolite profiles of *C. vulgaris* during co-cultivation with an IAA- and vitamin B12-producing bacterial consortium. These findings provide new insights into microalgal-bacterial interactions under industrial cultivation systems. They also support controlled PBR cultivation for sustainable production of high-quality biomass.

CONCLUSION

The closed PBR system offered superior stability and efficiency for *C. vulgaris*, producing a peak density of 9,6 x 10^7 cells/mL and an enriched PUFA profile. In contrast, the OP system exhibited lower growth and increased defense metabolites, such as chavicol (1,02%) and farnesol (0,53%), due to environmental stress. Despite these challenges, the OP system remains industrially relevant due to its cost-effectiveness. Future optimization through semi-closed configurations and more competitive bacterial consortia is recommended to reduce contamination and stabilize biomass quality.

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