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**Cultivation of phototrophic purple bacteria from fermented wine
lees: effect of light regime and hydraulic retention time**

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ABSTRACT

Purple phototrophic bacteria (PPB) received tremendous interests as a promising platform for the simultaneous treatment of organic-abundant wastewater and valorization of value-added bioproducts. This study investigated the combined roles of hydraulic retention time (HRT) and illumination regime on PPB productivity, substrate utilization, ammonium removal, polyhydroxyalkanoate (PHA) and hydroxyvalerate (HV) accumulation, and pigment production using fermented wine lees. Reactor performance was evaluated at HRTs of 2, 4, and 6 days under 12-h intermittent and 24-h continuous illumination to elucidate the relationships between pilot conditions and PPB metabolic responses. The results demonstrated that an intermediate HRT of 4 days provided the most favorable overall conditions for PPB cultivation, particularly under the 12-h illumination mode. PPB productivity reached a maximum level, accompanied by remarkable reductions in soluble COD, ammonium, volatile fatty acids (VFAs), and other organic acids. The improved performance at intermediate HRT was attributed to a favorable balance between biomass retention, substrate availability, and phototrophic activity, whereas short HRT was associated with biomass and substrate washout, and prolonged HRT with substrate depletion and reduced metabolic activity. Illumination regime substantially modified these responses, with continuous illumination generally resulting in less favorable substrate assimilation at intermediate and extended HRTs, potentially because of increased cellular maintenance requirements and altered carbon consumption. PHA and HV accumulation well demonstrated the metabolic flexibility of

PPB under different operating conditions. Short HRT favored intracellular polymer storage, whereas increasing HRT progressively reduced PHA and, more markedly, HV accumulation. Under continuous illumination, HV accumulation increased at the intermediate HRT but declined substantially at 6 days, suggesting that effective polymer production needs a suitable balance between phototrophic activity and readily assimilable carbon supply. Pigment production showed a distinct dependence on biomass retention and HRT. Bacteriochlorophyll formation was highest at intermediate HRT under both illumination regimes, whereas carotenoid accumulation was comparatively stable under 12-h illumination and increased with HRT under continuous illumination, which was attributed to the physiological adaptation to prolonged illumination and substrate limitation. Correlation analysis supported these observations, revealing strong associations between total pigments and their major fractions, as well as between VFA and total organic acids, while PHA showed generally weak relationships with biomass productivity, pigments, and substrate-related parameters. These findings indicate that polymer accumulation is not necessarily proportional to biomass growth or treatment efficiency and is possibly governed by distinct metabolic and environmental controls. This study revealed that HRT and illumination should be optimized together rather than considered as independent operating parameters. An HRT of 4 days under 12-h illumination provided the most balanced condition for PPB growth, organic and nutrient removal, and photopigment production, whereas shorter HRTs were more favorable when maximizing biopolymer accumulation was the principal goal. The study highlights the potential of fermented wine lees as a carbon-rich cultivation substrate for PPB and demonstrates that controlling hydraulic and light conditions can improve the balance between wastewater treatment and simultaneous recovery of biomass and value-added bioproducts.

Keywords: Purple phototrophic bacteria; Hydraulic retention time; Illumination regime; PHA/HV accumulation; Fermented wine lees.

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1. Introduction

1.1. Food Waste as a Global Environmental Challenge

Global estimates show a huge amount of global waste generation, comprising one-third of global food production (1). According to the United Nations' Food and Agriculture Organization (FAO), 1.3 billion tons of produced food are wasted in the supply chain, which also means the loss of water, resources, and energy (2). It is expected that by 2050, a great demand for food production will exist to supply a raised global population of nearly 9.8 billion.

Besides the higher environmental resources required to supply food demand, the use of different chemicals such as pesticides, additives, and fertilizers in cultivation, plastics and paper materials in packaging, and fuel and petrochemicals for transportation and distribution contributes to a significant environmental footprint (3).

1.2. Environmental Sustainability and Resource Management

In recent years, the concept of environmental sustainability and resource management has emerged as a basic principle to overcome the growing concern that human activities impose on our environment and finite resources (4). Population growth, development, industrial expansion, and shifting consumption patterns have increased the demand for raw materials, energy, water, and land while simultaneously increasing the production of solid and liquid wastes (5). As a result, sustainable development strategies are inevitable to switch from traditional approaches that focus mainly on waste disposal toward those minimized resource consumption, reduce waste production, and keep precious materials in productive and lasting systems. In this context, sustainability can

be translated environmentally not only by minimizing environmental impacts but also through the effective production, use, recovery, and regeneration of resources within the entire life cycle.

A key element of this transient stage from traditional toward modern disposal practices is adopting new strategies in waste management. This approach allows for preventing the loss of useful and potentially valuable resources and simultaneously providing secondary environmental benefits. Landfilling, for instance, pollutes and demands considerable land resources and also could lead to releasing greenhouse gases, leachate, noxious odor, and material (6). Even worse, the uncontrolled land and water disposal of assimilable wastes can contribute to loss of oxygen and loss of organic carbon and nutrients that could be valorized in a productive system. These challenges have motivated governments, industries, and researchers to explore sustainability-aligned strategies such as waste prevention, reuse, recycling, material recovery, and energy recovery (7).

The concept of sustainability also provides a transition platform to switch from the traditional linear economy toward the circular economy paradigm (8). Under this comprehensive waste management approach, materials are maintained in a reuse/recycling state, which leads to minimizing environmental footprint.

1.3. From Linear Economy to Circular Economy

The conventional linear economic model is commonly known as a “take–make–use–dispose” order that is illustrated in Figure 1. Under this traditional approach, natural resources are exploited, used for production and manufacturing, consumed by end users, and finally discarded as unwanted waste. Despite the supportive nature of the linear economy concept for economic and industrial growth, it relies extensively on constant extraction of pristine resources. However, increasing

resource shortages, environmental exploitation, climate change, and increasing demands and waste generation highlight the practical limitations of this strategy (9).

The circular economy (CE), on the other hand, as illustrated in Figure 2, aims to decouple economic growth from the constant consumption of limited environmental resources by maximizing the value and functionality of materials and components. Instead of considering waste as the last stage of a manufacturing chain, the CE model aims to prevent waste generation and convey waste materials to productive directions, such as reuse, recycling, recovery, and remanufacturing. In this context, the circular approach could be defined as a systems-oriented model that links resource conservation, waste minimization and prevention, technological development, and environmental sustainability (10).



Figure 1. The conventional linear economic model

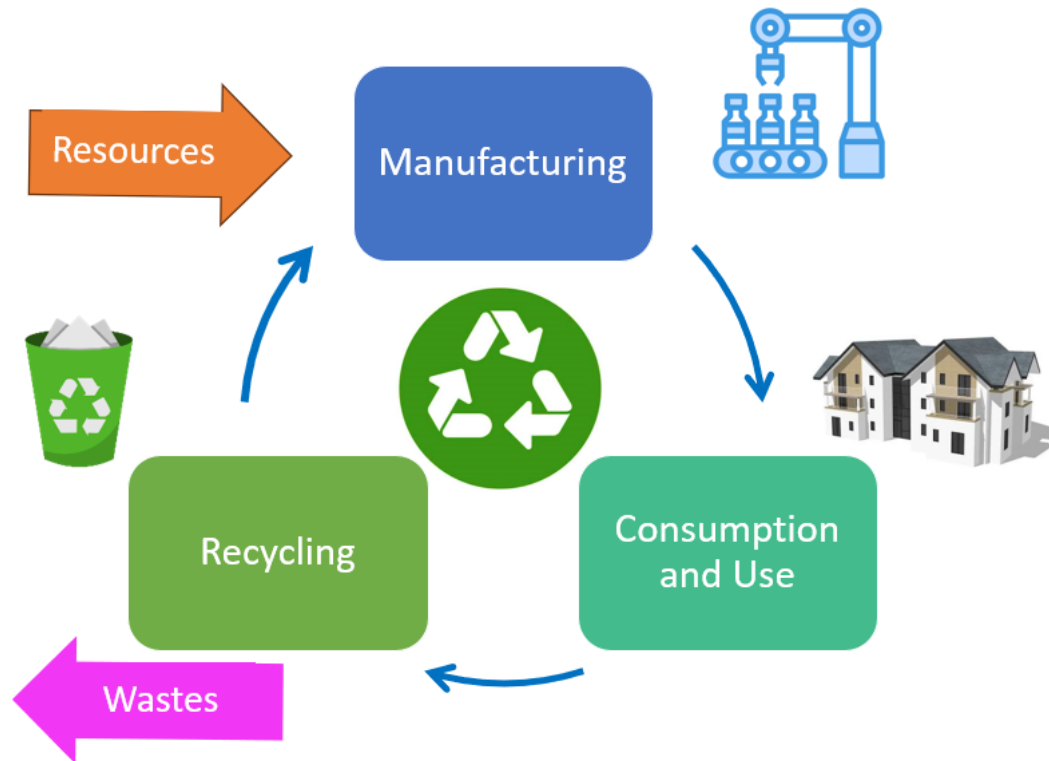


Figure 2. The modern circular economy model

Regarding waste management to align with circularity, strategies such as waste prevention and reducing redundant consumption, increasing product quality and their lifetimes, reusing products, servicing and repairing products, recycling, recovering materials, nutrients and energy, and upcycling waste products into added-value products are within this concept. Within this context, avoid waste generation is considered the favored hierarchy which then followed by practices such as reuse and recovery (11).

Advances in wastewater management have substantially improved the protection of aquatic environments; however, future treatment systems must move beyond conventional pollutant removal toward integrated approaches that minimize energy demand, recover valuable resources, and effectively address emerging contaminants (12). Due to the abundant levels of organics, substrates, nutrients, water, and other precious substances, the concept of circularity is particularly

relevant to wastewater and solid waste management. These approaches treat materials not only as wastes requiring disposal, but transform them into a new resource, which gives advantages for the economy and the environment by reducing virgin resource consumption (13).

1.4. Valorization and Added-Value Recovery

Valorization is a special strategy within the circular-economy model that represents the conversion of waste streams into valuable products, substances, chemicals, fuels, etc., with financial and environmental benefits. In this context, waste disposal is coupled with identifying opportunities through which wastes can become raw materials for new processes. Making or keeping value from resources that would otherwise be lost is the major goal of valorization practices (14).

A close expression, added value, is closely connected with valorization. Under this strategy, the low-economic value materials undergo suitable physical, chemical, biological, or thermochemical modifications to convert into products with significant higher functionality or market potential. The conversion of organic wastes in water and wastewaters into biogas, fertilizers, chemicals, or other valuable products falls within this concept. Similarly, using Agri wastes and industrial wastes as precursors to produce porous adsorbents and catalysts. From an environmental sustainability viewpoint, the most favorable valorization techniques are those contribute to the simultaneous reduction of wastes and replacing pristine raw materials (15).

1.5. Food Waste as a Resource for Circular Economy

As food production in an increasing demand worldwide, and the food production requires tremendous number of resources such as land, water, and fertilizer, it gave a unique opportunity in the context of circularity. Food waste practices and management strategies have become a

growing demand from environmental and socioeconomic viewpoints. Discarding food could be translated in the real world to the wasting of environmental resources consumed for cultivating, processing, conveyance, storage, and distribution. In addition to this, challenges related to waste processing and disposal, such as generating greenhouse gases, water pollution, and health-related issues, should be added. Considering these issues, minimizing food waste production and resource recovery are among the key strategies in sustainable waste-management systems (16, 17).

Although in the past food waste has almost been discarded through landfilling or disposal-oriented approaches, with a growing understanding of the environmental and economic outcomes, regulatory authorities and researchers are motivated to improve a management hierarchy based on prevention, reduction, reuse, recycling, recovery, and disposal. This waste management hierarchy approach seeks to keep the value embedded within food waste instead of being permanently lost.

As food waste consists of a heterogeneous and resource-rich composition, it is particularly appealing for the circular-economy concept. Based on its composition, it can have high levels of minerals, carbohydrates, lipids, fibers, proteins, micronutrients, and water. As a result, it could provide opportunities for recovery of water and precious materials via multiple pathways. The organic and biodegradable fractions could convert to valuable products such as biogas, biofuel, organic acids, microbial biomass, and fertilizers. Consequently, food waste should not only be considered as an unwanted endpoint in the food supply chain but a secondary feedstock for producing energy and new compounds (18).

As a consequence, energy and material recovery from inevitable food waste streams can address waste accumulation, resource scarcity, greenhouse-gas emissions, and the demand for alternative sources of nutrients, materials, and energy.

1.6. Food-Waste Management within the Circular-Economy Framework

The translation of food-waste management from the circular economy model needs a shift from simple waste treatment toward resource recovery and value creation. Keeping this strategy in mind, suitable handling and treatment practices will depend on the composition, amount, and characteristics of waste streams, and accessible technologies, environmental burdens, and economic viability. The production of valuable substances, chemicals, fuel, energy, and functional materials could be adopted within this context.

Interestingly, the circular-economy concept is recognized by the United Nations Sustainable Development Goals (SDGs) under the 2030 Agenda, which fundamentally address responsible demand and production, climate action, sustainable societies, clean water, and minimum food waste production (19). As a result, enhancing food-waste management approaches is part of a broader evolution toward resource conservation and environmentally sustainable demand/consumption systems.

1.7. Wine-Industry Wastes as Valuable Resources

The economic and environmental challenges related to food waste management have highlighted it as a critical global subject of the 21st century. Under these circumstances, the governmental regulatory authorities shifted their waste management strategies from landfilling to green approaches such as waste reduction, recycling, and reusing them, and recovering energy and materials (16). Nowadays, by understanding the finite nature of environmental resources, the concept of the circular economy has been highlighted more than ever. In the context of food waste

management, the circular economy manifests in the recovery of precious materials and energy generation in the form of electricity and fuel (20).

Of fruits, grapes are the most frequent species that are widely distributed on Earth. A majority of the cultivated grapes (~80%) are consumed for wine production, and about 13% and 7% of these fruits are consumed fresh and used in the fruit juices industry (21).

Wine production is a highly attractive industry as biorefinery and circular economy concepts have been highlighted in recent years. As part of the agro-industrial waste stream, processing one ton of grapes for winemaking typically generates about 0.06 t of wine lees and around 1.65 m³ of wastewater. These estimates highlight both the environmental footprint of the sector and the opportunities for improving sustainability within this key industry (22).

Particularly, Italy ranks among the world's major wine-producing nations, and its vast winemaking sector generates substantial by-products and effluent streams(23). Together with France and Spain, these countries account for nearly half of global wine production, highlighting Italy's strategic weight in the biorefinery context (24).

Upon treatment, the large volume of wastewater produced during wine processing could potentially be a source of water for industrial and irrigation purposes. Besides, the organic constituents in these wastewaters are a unique biodegradable source of chemicals for energy production, nutrient recovery, and extracting bioactive chemicals (25).

Wine lees, which mainly comprise a wide spectrum of microbial and grape biomass, refer to the sediments remaining after fermentation and ripening of the grapes. From the quantitative viewpoint, these sediments rank the second most frequent by-product of the wine industry, just after grape pomace (26). These massive residues are a precious source of carbon and nutrients for

biofuel production, fish and animal feed, and cell cultivation. These sustainability-oriented approaches are advantageous from both an environmental decontamination perspective and a green-production standpoint.

1.8. Purple Phototrophic Bacteria (PPB)

These microorganisms comprise a diverse group from a physiological and ecological standpoint, and are capable of converting light into chemical energy through anoxygenic photosynthesis. This sort of photosynthesis that is unlike oxygenic phototrophs in cyanobacteria and microalgae, does not use water as an electron donor and therefore O_2 is not released within the photosynthesis. Instead, different microorganisms, based on their metabolic pathways and environmental conditions, are very flexible and able to utilize a variety of organic and inorganic substances as electron donors (27). These compounds could widely consist of organic acids, sulfide, thiosulfate, alcohols, hydrogen, and ferrous iron (28). Having such a flexible metabolic pattern, PPB are able to develop in a wide variety of illuminated environments that are different in organic loadings, redox conditions, oxygen levels, and type and abundance of electron donors (29). PPB contain photosynthetic pigments, particularly bacteriochlorophylls and carotenoids, that give them characteristic pink, purple, red, or brown coloration. Besides this distinctive coloration, their photosynthetic pathway also differs fundamentally from that of oxygenic phototrophs.

PPBs are particularly adapted to the utilization of relatively long-wavelength light in the far-red and near-infrared (NIR) regions. Depending on the species, the major light-harvesting pigments in PPB could be bacteriochlorophyll a and b and carotenoids that enable them this light harvesting properties. This ability gives PPB the ability to exploit portions of the light spectrum that are less effectively used by algae and other phototrophic microorganisms. Similar to other photosynthetic

species, the abundance of light with desired wavelength plays a key physicochemical role in regulating their growth rates and metabolic activity, pigment formation, and biomass production (30). Considering this photoreactivity behavior, previous studies have demonstrated the role of light treatment in maximizing the abundance of BBP species in microbial consortia. (31)

Interestingly, PPB cells contain Type II photosynthetic reaction centers, where light irradiation starts electron transport, which leads to the generation of a proton motive force for ATP. This cyclic photosynthetic electron transport does not comprise the oxidation of water. Consequently, photosynthetic pathways can continue under anoxic or limited oxygen environments without oxygen production. PPB obtain the necessary cellular biosynthesis energy by oxidation of desired organic or inorganic electron donors. These special properties distinguish PPB from oxygenic phototrophs and allow them to survive and remain active under harsh ecological environments where light is available, but oxygen is absent (32, 33).

One unique metabolic property of PPB is that they are highly adaptable as a function of environmental conditions. Some PPB species exhibit photoheterotrophic, photoautotrophic, chemotrophic, or even mixed metabolic strategies. Under light irradiation and oxygen-deficient conditions, many PPB species grow photoheterotrophically. This strategy allows them to use organic substrates for carbon and electron sources while efficiently using energy from light. Contrary to photoheterotrophic growth, in photoautotrophic growth, reduced inorganic compounds are used as electron donors. Some PPB species can also switch their metabolism to use chemotrophic pathways in the absence of light. Such metabolic flexibility enables PPB to adapt well to fluctuations in irradiation, oxygen level, substrate accessibility, and redox conditions (34).

From a physiological viewpoint, PPB are normally classified into two major groups of purple sulfur bacteria (PSB) and purple nonsulfur bacteria (PNSB). During phototrophic activity, PSB are adapted to environments with reduced sulfur constituents and are especially competent at the utilization of sulfide and other reduced forms of sulfur compounds as electron donors. The elemental sulfur synthesized during sulfide oxidation could be deposited intracellularly or even outside the cells. The distribution of different species is strongly related to the presence of sulfide-rich environments, sufficient illumination, and oxygen-free environments.

As evident from the name, PNSB generally shows higher metabolic adaptability and is especially effective in environments with organic compounds and low levels of sulfide. *Rhodobacter*, *Rhodopseudomonas*, *Rhodospirillum*, *Rhodovulum*, and *Rhodocyclus* are among the representative genera in this class. Despite these bacteria predominantly relying on reduced sulfur substrates, many PNSB can assimilate a wide range of organic compounds, i.e., volatile fatty acids (VFA), alcohols, etc. The effective performance of simultaneous utilization of light and organic carbon has nominated them as interesting species for treating organic-rich wastewater (34).

The spread of PPB in a particular medium is mainly controlled by different environmental parameters such as light irradiation and intensity, oxygen level, redox potential, pH, water temperature, ionic strength, nutrient accessibility, and the presence and type of electron donors. PSB are usually spread near the transition zone of illuminated and anoxic regions where reduced sulfur compounds are commonly abundant. In real world scenarios, such conditions are commonly found in stratified lakes, bottom sediments, microbial mats, sulfur-rich springs, saline environments, and similar aquatic environments with obvious redox gradients. PNSB, on the other hand, have a relatively wider distribution and being found in freshwater and marine systems,

wetlands, bottom sediments, ponds, organic-rich environments such as wastewaters (30, 35). Their occurrence is particularly favored by illuminated, oxygen-restricted environments, and the abundance of readily biodegradable organic compounds.

The composition and abundance of the PPB community is strongly affected by the interaction between the environmental parameters. For instance, strong sulfide accessibility can support sulfur-oxidizing species, whereas organic-carbon-rich and sulfide-deficient environments may be favored by PNSB. In a similar pattern, light intensity and spectral wavelength impact the depth, and the presence or absence of oxygen determine phototrophic or alternative respiratory metabolisms PPB can establish. As a result, PPB communities are predominantly concentrated at interfaces where light, electron donors, and appropriate redox environments occur concurrently (36).

The supreme metabolic functionalities of PPB have received attentions in environmental remediation, mostly wastewater treatment and resource recovery (37). Most PNSB can decompose organic substrates, and nutrients (nitrogen and phosphorus) into biomass under suitable irradiation and oxygen-deficient conditions (38). This strategy provides an interesting alternative to conventional aerobic treatment, where a significant portion of organic carbon is calcined to end products such as carbon dioxide. Interestingly, in PPB-based treatment systems, organic substances can simply be incorporated into microbial biomass. This technique is exceptionally advantageous as it enables simultaneous pollutant removal and biomass production, which contains valuable compounds such as microbial protein, pigments, lipids, and polyhydroxyalkanoates (39, 40).

Beyond organic wastewater treatment, PPBs are also interesting due to their interactions with potentially toxic components and pathogenic species. Studies revealed that some PPBs can withstand, uptake, accumulate, or transform inorganic and organic pollutants. The excellent metabolic products and cellular components are suggested to have contribution in pollutant immobilization or transformation. Moreover, some PPBs are known to produce biologically active chemicals and antibacterial/antifungal agents that expand their applications beyond wastewater treatment. These features nominate PPB as a promising biological agent not only for wastewater treatment and pollutant removal but also as a fundamental resource recovery approach and the production of value-added products (34, 41).

1.9. Wine-lees residues as cultivating medium for PPB

Cultivating phototrophic purple bacteria (PPB) on mixed feedstocks such as wine-lees residues offers an efficient way to turn these waste streams into value. Anoxygenic PPB stand out for their remarkable metabolic adaptability, using bacteriochlorophyll pigments to harvest near-UV and near-infrared light (42). PPBs are extensively studied for nutrient recovery, bioplastic production, biohydrogen generation, and biomass and pigment production. These microorganisms also demonstrated strong performance in treating wastewater, as they can assimilate COD and ammonia, inorganic electron donors such as iron(II), H_2 , and H_2S , accumulate phosphorus, and remove nitrate through anoxic denitrification (43).

The cultivation of PPB, however, in research and particularly real-scale treatment units is in an early stage (44). Hence, despite the successful application of PPB for treating wastewater in lab studies, there is very limited data for translating these studies into the real world.

Up to day, different studies have been conducted to figure out the role of environmental factors, operational variables, light illumination source, and substrate on the yield of PPB and treated streams. Hülsen et al used PPB grown as outdoor biofilms to convert piggery (PWW) and chicken-processing wastewaters (CWW) into single-cell protein while simultaneously treating nutrients. Using 60–100 L flat-plate reactors, the system reached up to 71% COD removal in PWW and 78% in CWW, with significant N and P reductions and biomass productivities of 14 g TS/m².g. The authors concluded that the purification and biomass production performance closely tracked the availability of volatile fatty acids (45).

2. Materials and methods:

2.1. Experimental Setup

Herein, the valorization of fermented wine lees as an abundant carbon-based food waste was studied. Fermented wine lees is a carbon-rich substrate that could be readily used by heterotrophic phototrophic purple bacteria (PPB). Two parallel laboratory-scale photobioreactors were operated to assess the effect of illumination regime and hydraulic retention time (HRT) on the cultivation yield of PPB. Both reactors were identical in geometry, working volume, and operational configuration to ensure that illumination was the only determining parameter in bacterial growth.

Although sunlight is an abundant and cost-free energy source, its inherent day–night, seasonal, and weather-related fluctuations can result in variable irradiance, whereas artificial lighting provides greater control over illumination at the expense of additional energy requirements (46). Building on this consideration, the present study evaluated PPB cultivation under two controlled illumination regimes, 12-h intermittent and 24-h continuous lighting, to elucidate how illumination duration affects wastewater treatment performance and PPB biomass and bioproduct production. The light source consisted of LED panels positioned around the reactors to ensure uniform irradiation. Light intensity was kept constant across both systems to isolate the effect of photoperiod from light strength.

2.2. Inoculum and Substrate

The inoculum matrix was obtained from the sediments of Sile river that is located in Treviso, northeast Italy. A batch reactor with a volume of 4 L was used to cultivate BPR. The cultivation begins with centrifugation of the sediments and keeping the supernatant under IR irradiation for

10 days. The temperature of the cultivation medium was kept constant at 25°C, and the nutrient demand was supplied. The PPB growth was monitored continually during the cultivation period using spectra scanning for bacteriochlorophylls (BChl) and carotenoids (Crt_s). Figure 3 illustrates the schematic of the study, from BPR cultivation to WL valorization in an anaerobic digester.

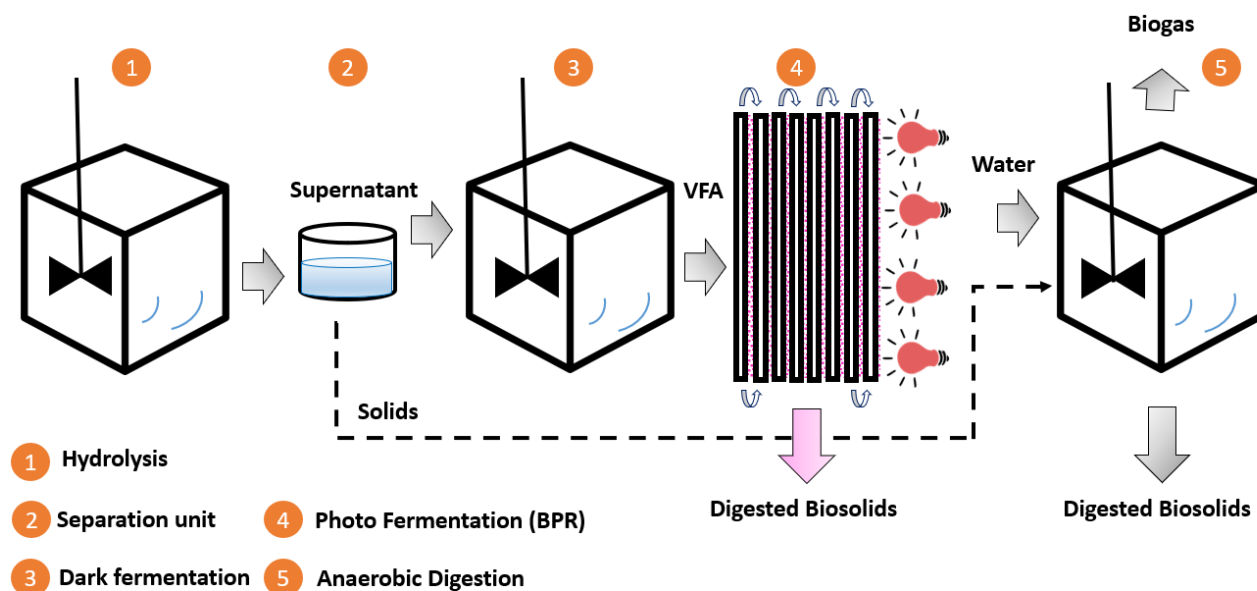


Figure 3. The schematic of the study, from BPR cultivation to WL valorization in an anaerobic digester.

2.3. PPB cultivation

Fermented wine lees were used as the primary substrate, providing a mixture of soluble organic compounds, volatile fatty acids, and nitrogen sources suitable for PPB growth. A local company provided the raw WL, which was kept refrigerated at 4°C until use. Before being processed for fermentation, the WL was hydrolyzed under specific conditions (70°C for 12 h) to solubilize the organic matter, followed by centrifugation to remove the solids. As volatile fatty acids (VFAs), which served as precursors for PPB, was then subjected to an acidogenic dark fermentation to convert the soluble organic matter into VFAs. It is noteworthy that ethanol comprises more than 30% of the soluble COD in the matrix. A 5-L mesophilic (37°C) semi-continuous stirred tank

reactor (CSTR) with an HRT= 4 days was used for the fermentation. The pH was continually monitored and maintained between 6.0 and 6.5 by automatic addition of 3 M NaOH using a peristaltic pump controlled by dedicated software (Idea Bioprocess Technology Srl). The organic loading rate (OLR) ranged from 8.0 to 10.5 g VS/ L. d, depending on the volatile solids (VS) content of the WL substrate. Each day, the fermented effluent rich with VFA was centrifuged (Heraeus Megafuge 40, Thermo Fisher Scientific, Waltham, MA, USA), and the supernatant was stored at 4°C until use. The physicochemical characteristics of the WL before and after fermentation are summarized in Table 1.

Table 1. Physicochemical characteristics of raw wine lees (WL) and acidogenic fermented product.

Characteristic	Unit	Raw WL	Fermented WL
Ammonia	g N-NH ₄ ⁺ /L	0.21 ± 0.06	0.44 ± 0.02
COD_{SOL}	g/L	58.7 ± 0.9	61 ± 1
COD_{VFA}	g/L	8.3 ± 0.2	36.7 ± 0.5
TS	g/L	2.1 ± 0.2	-
Phosphate	g P-PO ₄ ³⁻ /L	0.35 ± 0.02	0.39 ± 0.01
Propionic acid	g COD/L	-	3.2 ± 0.7
Butyric acid	g COD/L	-	10.7 ± 0.3
Ethanol	g COD/L	19.6 ± 0.4	2.2 ± 0.1
pH	-	6.4 ± 0.3	6.0 ± 0.2
Acetic acid	g COD/L	8.3 ± 0.2	23.6 ± 0.4
VS	g/L	1.6 ± 0.3	-

In order to minimize differences associated with microbial adaptation and ensure identical inoculum characteristics, we set up three pilot-scale photobioreactors which operated simultaneously in parallel. Each reactor consisted of a cylindrical plexiglass vessel (1.1 m height and 250 mm internal diameter) equipped with a recirculation pump operating in continuously (24 h/d) to provide complete mixing. Feeding and effluent withdrawal were performed manually once

per day, resulting in a 24 h operating cycle. Reactor performance was monitored using online pH and oxidation–reduction potential (ORP) probes.

Besides, to minimize the buildup of residual organic matter and suppress the proliferation of competing bacterial and algal populations, all cultivating reactors were operated under a low organic loading rate (OLR) of 1.0 g COD/L. d. An irradiance of 140 W/m² using Valoya G2 LED lamps emitting far-red and infrared light was applied to the reactors, which were maintained inside a thermostatically controlled dark chamber at 25°C. Three operating conditions were investigated. PBR-1 and PBR-2 were operated at a HRT of 2.5 and 4 days under continuous illumination (24 h/d). PBR-3 was also operated at an HRT of 4 days but under an intermittent light/darkness regime of 12 h each. Later, the feedstock was introduced at the beginning of each light phase.

2.4. Operational Conditions

Each HRT condition was maintained long enough to reach pseudo-steady-state before sampling. Temperature, pH, and mixing conditions were kept constant across all experiments to avoid confounding effects. To control biomass retention and stable environmental conditions, reactors were fed in semi-continuous mode, with daily withdrawal and replacement of culture volume according to the desired HRT.

2.5. Sampling and Analytical Methods

Samples were taken from the reactors based on the predetermined plan for analyzing the study variables as a function of HRT and illumination condition. Polyhydroxyalkanoate (PHA) content and hydroxyvalerate fraction (HV%), PPB biomass productivity (g VSS/L.d), Pigment concentrations (including bacteriochlorophylls and carotenoids), and removal efficiencies (for

soluble COD, ammonium (NH_4^+), and organic acids), where the main parameters were measured in pilot studies.

Analyses were performed according to standard methods for wastewater characterization and microbial biomass quantification. The analyses performed for the initial characterization of the matrices used as substrates were conducted on samples previously oven dried at 100 °C for 48 h. Once the sample dried, it was finely pulverized using a mortar to obtain a homogeneous material suitable for analysis. PHA and HV fractions were determined using solvent extraction and gas chromatography, and pigments were quantified spectrophotometrically. The experimental data were analyzed to determine the mean values to assess the study parameters. Descriptive statistics were computed for each condition, followed by comparative analysis between illumination regimes and HRT levels.

2.5.1. pH

The pH of the fermented product was measured using a calibrated pH meter equipped with a magnetic stirring system to ensure homogeneous mixing during measurement. The instrument allowed both continuous monitoring and recording of stable pH values. Prior to each measurement, the pH meter (Figure 4 (a)) was calibrated using three control buffer solutions with nominal pH values of 4.00, 7.00, and 10.00 to ensure measurement accuracy.

2.5.2. Chemical oxygen demand (COD)

Within the different HRT, COD was periodically determined to evaluate the conversion of organic matter and subsequently determine the VFA production yield. Soluble COD (COD_{sol}) was quantified using a colorimetric technique with an Analytik Jena Spekol 1500

spectrophotometer (Figure 4 (b)). Prior to analysis, the fermented liquid was passed through a 0.2 µm membrane filter, thereby allowing the determination of the soluble COD fraction.

For the colorimetric analysis, an appropriate aliquot of the prepared sample was mixed with a predetermined volume of potassium dichromate ($K_2Cr_2O_7$) oxidizing solution and an acidic catalytic solution containing sulfuric acid (H_2SO_4), silver sulfate (Ag_2SO_4), and mercuric sulfate ($HgSO_4$). Silver sulfate served as a catalyst, while mercuric sulfate was added to minimize interference caused by chloride ions. The prepared samples were then subjected to acid digestion in a thermoblock at 150 °C for 2 h. Following digestion and cooling, the absorbance of the samples was determined spectrophotometrically at 600 nm. The absorbance values then were used to determine the CODsol concentration, after accounting for the dilution applied during sample preparation. The final CODsol concentration was calculated according to the following equation:

$$sCOD = sCOD_{mis} \cdot F.D$$

where (CODsol) represents the mean COD concentration obtained from the spectrophotometric measurement, while (F.D.) denotes the dilution factor applied during sample preparation. The resulting CODsol values were reported as mg O₂/L.

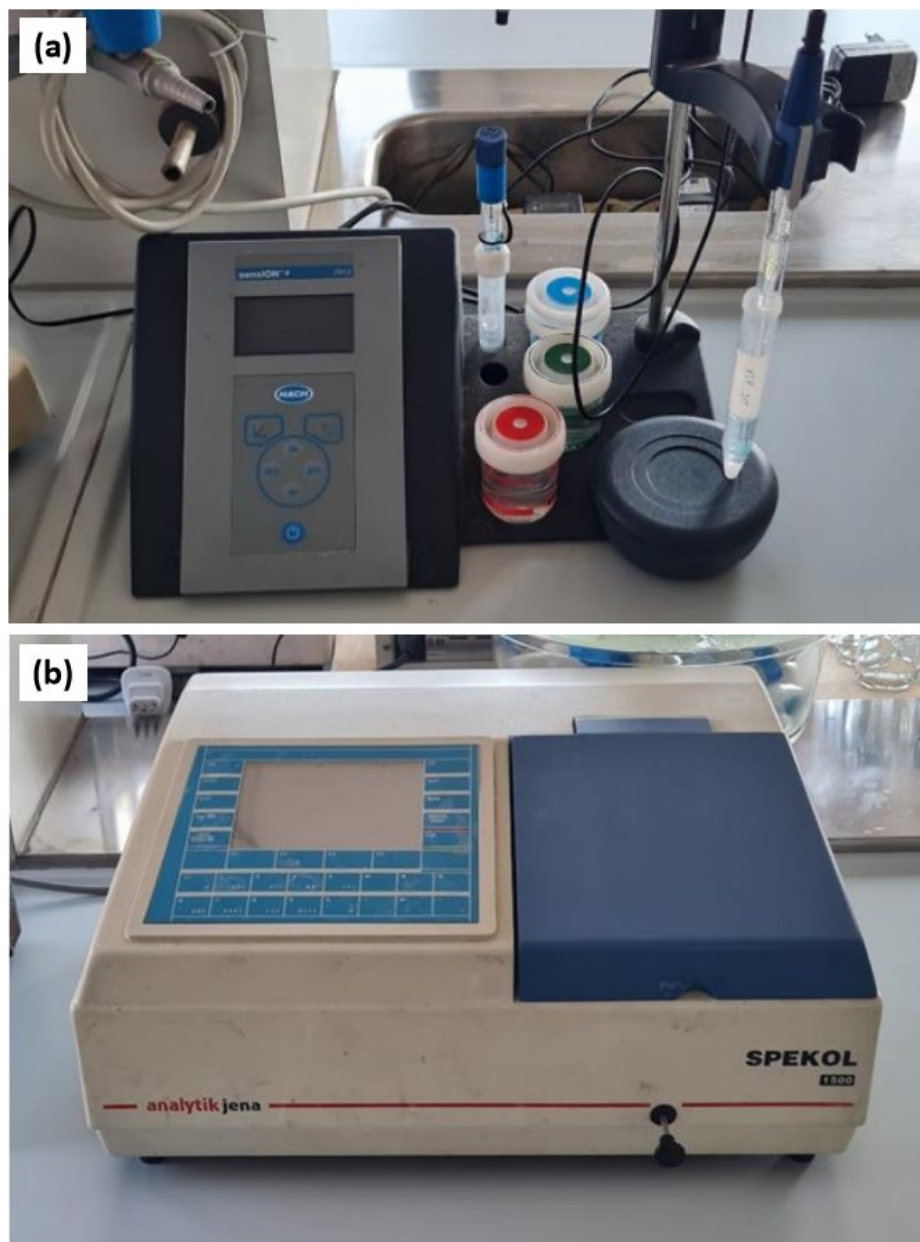


Figure 4. (a) pH meter and (b) spectrophotometer used in laboratory for colorimetric analyzes

2.5.3. Solids

The solids content of the substrate was characterized by determining two important indices i.e. the total solids (TS) and total volatile solids (TVS) according to a standard gravimetric procedure commonly reported in the literature. For the determination of TS, an empty crucible was first weighed to obtain its tare mass in gram (P_0). A known amount of the substrate was

then added to the crucible, and the combined mass of the crucible and wet sample was recorded as (P_1). The sample was subsequently dried in an oven at 100 °C for 48 h to remove the entire moisture. Then, the crucible containing the dried sample was weighed again, giving the dry mass (P_2 - g).

The TS concentration, expressed as g TS per Kg of sample, was calculated using the following equation:

$$TS = \frac{P_2 - P_0}{P_1 - P_0} \times 1000$$

The determination of TVS was subsequently performed using the same dried sample obtained from the TS analysis. The crucible containing the dried residue was placed in a muffle furnace (Figure 5 (a)) and subjected to combustion at 550 °C overnight. This thermal treatment removed the entire organic fraction, leaving only the inorganic ash. After cooling, the crucible containing the remaining ash was weighed, and its mass was recorded as (P_3) (g). The TVS concentration was then obtained from the difference between the dried solids and the residual ash:

$$TVS = \frac{P_2 - P_3}{P_1 - P_0} \times 1000$$

2.5.4. COD of biosolids

COD of solids was determined by measuring the amount of organic matter oxidizable by potassium dichromate under strongly acidic conditions as described in earlier part. For the digestion step, an appropriate aliquot of the pulverized and dried sample was introduced into Teflon® vessels together with the required reagents. The vessels were then placed in a mineralizer (Figure 5 (b)), where the samples experienced a two-step thermal digestion

process. First step was carried out at 175 °C, followed by a second step at 50 °C, with a total digestion time of 1 h. During this treatment, the organic carbon present in the sample was chemically oxidized by the dichromate ions, resulting in the reduction of a portion of the dichromate.

After completing digestion, the remaining excess dichromate was quantified by titration with ferrous ammonium sulfate (FAS), using ferroin as the indicator. A blank determination was accomplished under the same conditions to assess the contribution of the reagents. The COD concentration then calculated from the difference between the FAS volumes required for the blank and sample titrations:

$$COD = \frac{(B) - (C) \times 8 \times \frac{V \times N}{T}}{P}$$

In this equation, B is the volume of FAS liquid consumed during the blank titration (mL), C stand for the volume of FAS liquid spent during the sample titration (mL), N is the normality of the dichromate solution, V is the volume of K₂Cr₂O₇ solution used for digestion (mL), T is the volume of FAS solution corresponding to the titration of the dichromate solution (mL), 8 is the equivalent weight of oxygen (g O₂/ eq), and P is the mass of the dried sample used for the analysis (g). The COD values obtained from this procedure were expressed as mg O₂/ g TS.

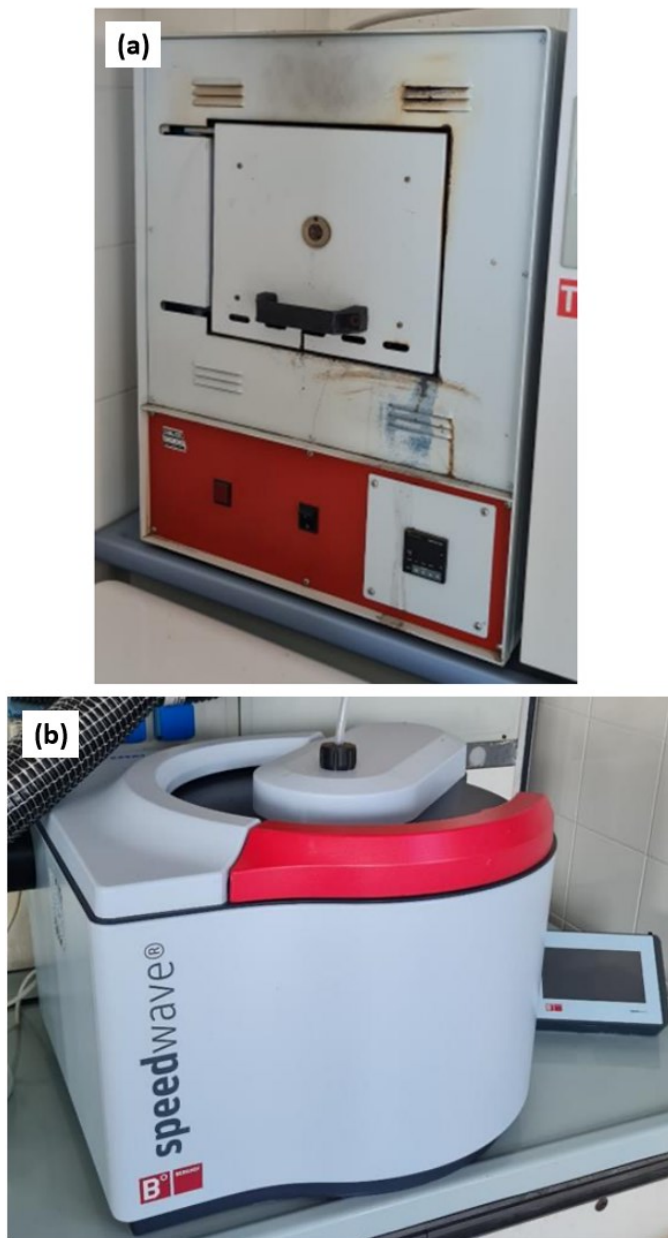


Figure 5. The (a) muffle used for TS-VS measurements, and (b) the mineralizer used for analysis on solid samples

2.5.5. Ammonia Nitrogen

Ammonia nitrogen (N-NH_4^+) was determined using a colorimetric method based on the Nessler reaction. In this procedure, ammonia reacts with an alkaline potassium tetraiodide

solution to produce a yellow-orange-colored complex. The intensity of the resulting coloration is proportional to the ammonia concentration in the analyzed sample.

For wastewater samples, potential matrix interferences were minimized by separating the ammonia from the sample matrix through steam distillation prior to the addition of the reagent. The distillation step was performed using a SCIENTIFIC VELP UDK 129 distillation unit (Figure 6 (a)). The absorbance of both the sample and reagent blank finally was determined at a wavelength of 410 nm.

The ammonia-nitrogen concentration ($\text{mg N-NH}_4^+/\text{L}$) was obtained from the calibration curve established using standard solutions of known ammonium concentrations, considering the dilution factor applied for sample preparation:

$$\text{N-NH}_4^+ = m \cdot \text{ABS} \cdot \text{F.D}$$

Where ABS is the absorbance measured for the sample using the spectrophotometer, F.D. is the overall dilution factor, calculated as the product of any successive dilution steps, and m is the slope of the calibration curve.

2.5.6. Bacteriochlorophyll and Carotenoid

BChls content was determined using organic solvent extraction and spectrophotometric analysis. The PPB biomass was washed twice with distilled water before extraction with a mixture of acetone and methanol (3:1, v/v), followed by centrifugation (4600 rpm for 10 min) to remove cellular debris. The supernatant was then analyzed spectrophotometrically at 770 nm. To prevent photo-oxidation, all extractions and measurements were performed in a dark environment. A similar procedure was followed for Crts quantification, except for the wavelength used, which was set at 480 nm.

2.5.7. Volatile Fatty Acids

VFA concentrations in the samples were determined using a gas chromatography (Agilent 6890N Series GC) equipped with a DB-FFAP capillary column (15 m × 0.530 mm, 1.00 µm film thickness) and a flame ionization detector (FID) (Figure 6 (b)). Quantification was based on a three-point calibration using a standard matrix of the target VFAs, with 2-ethylbutyric acid serving as the internal standard.

The analysis was performed using the “VFA_METODO_FFAP” method, with an oven temperature program ranging from 80 to 200 °C and a final post-run at 220 °C. Before injection, samples were filtered through a 0.2 µm membrane to protect the capillary column from any suspended particle. The analyzed VFAs included acetic (C2), propionic (C3), isobutyric (iC4), butyric (C4), isopentanoic (iC5), pentanoic (C5), isocaproic (iC6), caproic (C6), and heptanoic (C7) acids.

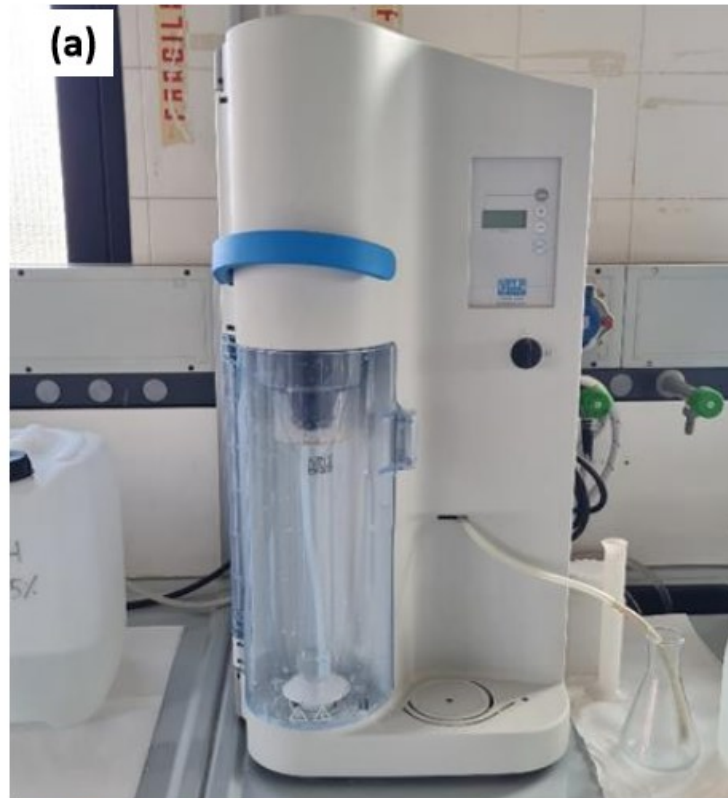


Figure 6. The (a) steam distiller used in laboratory for ammonia and TKN analysis, and (b) Gas Chromatograph used for VFA analyzes

3. Results and Discussion

This chapter presents a comprehensive evaluation of the PPB system operated under different hydraulic retention times (HRT = 2, 4, and 6 days) and illumination regimes (12-hour and 24-hour light). The results are organized to cover major findings in separate sections i.e., biomass productivity, PHA/HV accumulation, pigment formation, and removal efficiencies. Together, these analyses provide an integrated understanding of how operational conditions govern PPB physiology, photophysiology, and wastewater treatment performance.

The results obtained in our study provide a coherent representation of the role of HRT and illumination regime on the metabolic behavior, photophysiology, and treatment performance of PPB. A summary of the results obtained in this study are presented in Table 2 and detailed in the discussion sections.

Table 2. The detailed descriptive statistics of the results for PPB treatment system

Parameter	HRT (h)	Irradiation time (h)	Mean	SD	SEM	Parameter	HRT (h)	Irradiation time (h)	Mean	SD	SEM
PHA %, g/gVSS	2	12	0.11	3.94%	0.70%	VFA mg COD/L	2	12	0	0	0
	2	24	0.10	4.65%	0.88%		2	24	0	0	0
	4	12	0.04	1.96%	0.34%		4	12	83	14	14
	4	24	0.06	1.95%	0.33%		4	24	92	16	16
	6	12	0.03	1.93%	0.32%		6	12	0	0	0
	6	12	0.03	1.85%	0.28%		6	24	0	0	0
HV %, gHV/gPHA	2	12	0.26	10.57%	2.00%	OAS mgCOD/L	2	12	193.09	180	12
	2	24	0.15	12.98%	2.55%		2	24	208.25	185	35
	4	12	0.14	12.74%	2.19%		4	12	118.57	295	51
	4	24	0.22	11.76%	1.99%		4	24	240.52	313	54
	6	12	0.00	0.00%	0.00%		6	12	0.00	0	0
	6	24	0.00	0.00%	0.00%		6	24	0.00	0	0
PPB %, σ/σ_{VS}	2	12	0.20	4.58%	0.81%	OAS (gCO D/L	2	12	0	0	0
	2	24	0.24	4.89%	0.92%		2	24	0	0	0
	4	12	0.28	4.33%	0.74%		4	12	0	0	0

	4	24	0.31	4.66%	0.79%		4	24	0	0	
	6	12	0.13	2.95%	0.48%		6	12	0	0	
	6	12	0.16	2.56%	0.39%		6	24	0	0	
COD mg/L	2	12	310.67	184	51	BCL %, g/g	2	12	0.54	0.09%	0.02%
	2	24	345.12	169	49		2	24	0.85	0.08%	0.01%
	4	12	134.46	772	193		4	12	0.00	0.08%	0.01%
	4	24	1006.07	706	171		4	24	2.80	0.07%	0.00%
	6	12	1122.50	1376	615		6	12	0.00	0.09%	0.00%
	6	24		0	0		6	24	2.01	0.09%	0.00%
NH ₄ mg-N/L	2	12	22.22	6	1	CRT %, g/g	2	12	0.55	62.56%	11.06%
	2	24	17.61	6	1		2	24	1.07	0.09%	0.00%
	4	12	9.86	80	15		4	12	0.00	0.08%	0.00%
	4	24	43.73	77	14		4	24	4.04	0.08%	0.00%
	6	12	12.93	20	3		6	12	0.00	0.09%	0.00%
	6	24	14.75	18	3		6	24	3.12	0.09%	0.00%
VFA (mg COD/L Acetic)	2	12	193.09	180	32	Total pigment %, g/g	2	12	0.04	5.55%	0.98%
	2	24	208.25	185	35		2	24	0.00	0.14%	0.00%
	4	12	82.55	224	38		4	12	0.01	0.12%	0.00%
	4	24	193.66	243	42		4	24	0.01	0.11%	0.00%
	6	12	0.00	0	0		6	12	0.01	0.15%	0.00%
	6	24	0.00	0	0		6	24	0.01	0.14%	0.00%

3.1. PPB Productivity

The average PPB productivity in Figure 4 shows a clear dependence on both the HRT and the illumination time (Table 3). Interestingly, the continuous illumination mode of operation consistently resulted in higher productivity for PPB compared to the 12-hour cycle at all HRTs. This observation indicates that uninterrupted light exposure improves the phototrophic metabolism, allowing PPB to maintain higher growth rates and substrate conversion efficiency. In other words, a synergistic effect was observed at the optimal retention time, where substrate availability is sufficient and constant light is also available in the reactor. This also shows that the highest productivity of 0.31 g/L.d was achieved at HRT = 4 d, suggesting that this operational condition provides the most favorable balance between biomass population, environmental conditions, and metabolic activity. In contrast, the lower productivity observed at HRT of 2 days

is likely due to insufficient retention time for biomass acclimation and growth, and biomass washout. A declining productivity at HRT of 6 days may be associated with substrate depletion in the system or partial biomass washout. Our findings demonstrated that optimizing HRT is essential for maximizing PPB productivity in systems treating fermented wine lees, even under continuous illumination.

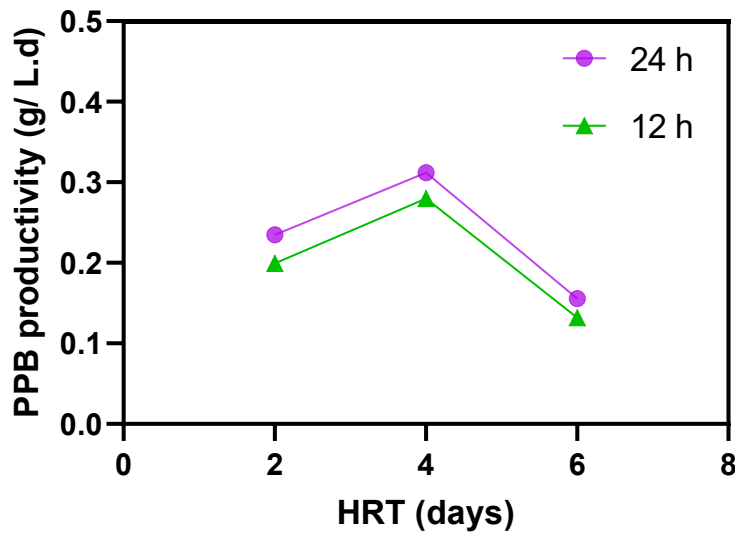


Figure 7. The average PPB productivity under 12 h and 24 h illumination time at different HRTs.

Table 3. Descriptive statistics of PPB production under different hydraulic retention times and illumination regimes

<i>HRT (Days)</i>	<i>Illumination</i>			
	<i>mode</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
2	12	3.99	0.1995	0.001918
4	12	5.5275	0.276375	0.001467
6	12	2.623333	0.131167	0.001033
2	24	4.7	0.235	0.003129
4	24	6.1275	0.306375	0.00246
6	24	3.05	0.1525	0.000511

3.2. PHA and HV Accumulation

Figure 8 shows the accumulation of polyhydroxyalkanoates (PHA) and the incorporation of Hydroxyvalerate (HV) into the polymer structure. The highest PHA content was observed at HRT = 2 days ($0.108 \pm \text{SD}$), particularly under the 12-hour light regime (Table 4). This is followed by a substantial decrease at HRT = 4 days ($0.045 \pm \text{SD}$) and minimal values at HRT = 6 days ($0.029 \pm \text{SD}$). The highest PHA content under 12-hour light irradiation suggests that intermittent illumination may create short dark periods that favor polymer storage by reducing phototrophic energy demand and shifting the culture toward maintenance metabolism. Continuous illumination slightly reduced PHA accumulation, likely due to increased metabolic activity and consumption of stored polymer for maintenance.

As illustrated in Figure 8, HV production is highly sensitive to HRT, even more than PHA. HV exhibited a similar pattern at HRT = 2 days, with the highest HV fraction occurring under the 12-hour regime. At HRT = 4 days, both PHA and HV decreased under the 12-hour regime, reflecting a shift from storage metabolism toward stable growth.

HV content was highest at HRT = 2 days ($0.263 \pm \text{SD}$), decreased markedly at HRT = 4 days ($0.142 \pm \text{SD}$), and was completely absent at HRT = 6 days (Table 5). At HRT = 6 days, both PHA and HV reached minimal levels, with HV effectively absent. This indicates that prolonged retention time limits polymer formation and shifts the culture toward maintenance metabolism.

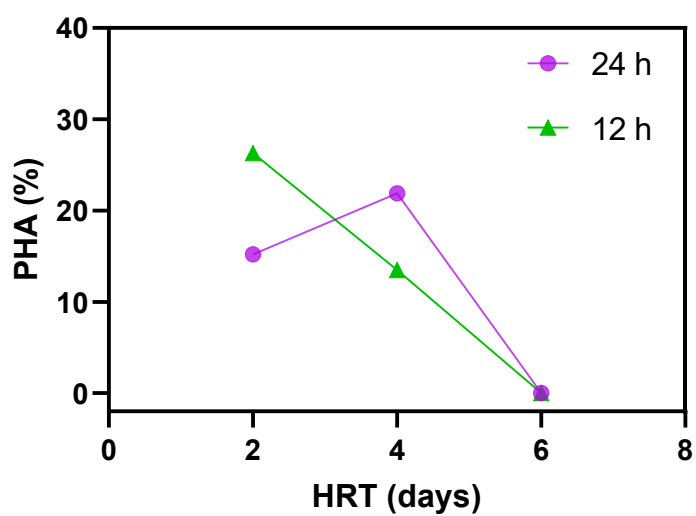
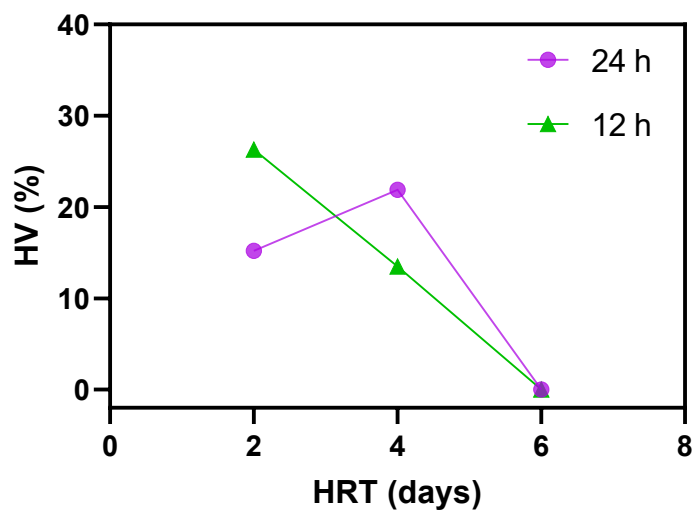


Figure 8. The accumulation of polyhydroxyalkanoates (PHA) and Hydroxyvalerate (HV) content in polymeric structure

Table 4. Descriptive statistics of PHA under different hydraulic retention times and illumination regimes

HRT (Days)	Illumination			
	mode	Sum	Average	Variance
2	12	2.162829	0.108141	0.002052
4	12	0.896088	0.044804	0.000477
6	12	0.573402	0.02867	0.000323
2	24	2.019278	0.100964	0.002816
4	24	1.131466	0.056573	0.000332
6	24	0.593201	0.02966	0.000447

The effect of HRT and illumination regime on PPB yield showed a sharp peak in PPB productivity at HRT = 4 days under the 12-hour irradiation time mode. This finding suggests that a balance between the biomass retention time and substrate availability is crucial for the highest system productivity. Under short HRT, cell washout from the reactor reduced productivity, while reduction in VFAs content and metabolic activity is probably responsible for low PPB under extended HRT. Previous studies demonstrated a trade-off between HRT, biomass production, and pollutant removal in PPB-based systems. In a study by Regueira-Marcos et al., the reported MBC reactor achieved its highest biomass yield at a relatively short HRT of 5 d, whereas extending the HRT to 10 d improved pollutant removal without increasing biomass concentration (47). The study suggested that the 12-hour light regime supports phototrophic growth best under the intermediate HRT and thus optimizing HRT is crucial for maximizing PPB yield in treatment systems. Earlier studies also highlighted the role of appropriate HRT and the washing out of biomass and dilution effect under higher HRT (48). These studies suggested that the washing effect could be controlled via optimizing the design and, if possible, biomass immobilization (49).

The role of illumination source, light wavelength, pilot configuration, and operation mode also been studied extensively. Wang et al. demonstrated that increasing the light frequency and duty cycle significantly improved both biomass and protein accumulation by purple non-sulfur bacteria (PNSB) grown on sugar-rich wastewater. Metabolomic analysis attributed this improvement to simultaneous electron transport and enhanced carbon and nitrogen metabolism (50). Light intensity is a critical operational parameter for phototrophic systems because there is evidence that both insufficient and excessive illumination can constrain microbial growth. For PPB, reported optimum ranges vary significantly depending on species and pilot conditions. For instance, *Rhodospseudomonas* cultures have shown increasing biomass with increasing light intensity up to

an optimum, followed by a decline at higher intensities due to the photoinhibition effect (51). In this context, some researchers examined the role of flashing lighting on COD conversion and biomass production. Lu et al. demonstrated that flashing light increased biomass by 10.4%-41.5% compared with constant lighting (52). In another study, Dalaei et al. demonstrated that although the PPB can effectively treat municipal wastewater even under relatively low infrared irradiances of 1.4–3.0 W/m², a lower irradiance resulted in reduced growth kinetics compared with higher-light conditions. These findings highlight that PPB performance depends not only on the availability of light but also on how illumination is provided (43). A long-term outdoor photobioreactor study by Hülsen et al. demonstrated that PPB systems can maintain effective treatment efficiency under naturally fluctuating environmental conditions, including temperature and light exposure. This study emphasized the importance of environmental fluctuations in temperature and irradiance when evaluating and optimizing PPB performance in real conditions (53).

The critical functionality of PPB in the accumulation of PHA and HV as a function of HRT under the 12-hour photoperiod reflects the metabolic flexibility of PPB. The significant reduction in PHA accumulation with increasing HRT under continuous illumination highlights the strong influence of substrate availability and metabolic balance in PPB. At short HRT, the presence of higher organic loading and also intermittent illumination both favor carbon storage. As HRT increases to 4 days, substrate availability dropped, and PPB shifted toward steady growth, which led to a decrease in PHA levels to half the level. The negligible PHA accumulation at HRT = 6 days suggests that extended residence could limit the metabolic drivers required for polymer production. It is noticeable that although constant light maintains phototrophic activity, it also increases maintenance energy demand, reducing the carbon flux toward polymer storage at longer retention

times. The significant role of HRT and illumination highlights the importance of optimizing HRT when PPB cultivation is aimed at biopolymer production rather than solely wastewater treatment. Our findings confirm that short HRTs are essential, regardless of whether the light is intermittent or continuous, for maximizing biopolymer production in PPB cultivated on fermented wine lees.

The impactful decline in HV accumulation with increasing HRT under the 12-hour photoperiod, that revealed to be more than that for PHA, highlights the dependence of HV production on both substrate availability and metabolic kinetics. Short HRT provides higher levels of VFAs, which led to rapid substrate conversion and HV formation. However, extended HRT consumes VFAs and causes a net shift of PPB toward maintenance metabolism and suppression of HV production at HRT = 6 days. These findings demonstrate that HV formation is highly sensitive to operational conditions and that short retention times are essential for maximizing HV production.

Studying the HV pattern under continuous illumination (24 hours) showed that the light regime strongly controls PPB metabolism. Constant illumination for an intermediate HRT of 4 days improved HV formation from VFAs conversion to the highest level. However, as the presence of substrate availability diminished and energy demand for cell maintenance increased, HV production decreased significantly at long HRT (6 days). Thus, HV formation seems to require a balance between VFAs turnover and phototrophic activity.

Table 5. Descriptive statistics of HV content under different hydraulic retention times and illumination regimes

<i>HRT (Days)</i>	<i>Illumination</i>		<i>Sum</i>	<i>Average</i>	<i>Variance</i>
	<i>mode</i>				
2	12		4.735229	0.263068	0.006715
4	12		2.844885	0.142244	0.017845
6	12		0	0	0
2	24		2.739821	0.152212	0.012962
4	24		4.239958	0.211998	0.009029
6	24		0	0	0

3.3. COD removal efficiencies

Removal efficiencies for soluble COD were monitored and showed a clear pattern according to operational conditions (Table 6). At HRT = 2 days, COD and OAs removal were relatively high, reflecting rapid substrate turnover. The 12-hour regime provided more stable removal performance, while continuous illumination occasionally enhanced COD removal but introduced variability. Ammonium removal was limited at this retention time due to insufficient residence time for nitrogen assimilation.

The average COD concentration was recorded as 345 mg/L and 1050 mg/L for HRT = 2 and HRT=6 days, respectively. These results showed that nonstop illumination does not develop a clear HRT-dependent trend in COD removal.

Previous reports highlighted the role of setting a suitable COD level for the cultivation matrix, at around 1700-2000 mg/L as is favorable for purple bacteria such as *Rhodovulum sulfidophilum* (54). The presence of nutrients and pH, that is in close relationship with COD, are also critical for a supportive treatment system (55). Other studies also reported that PPB bacteria are efficient in the removal of COD in a mixed wastewater matrix. Yu et al. used a photo-sequencing batch reactor with a COD removal performance of ~84 %, under 2 d HRT and an OLR of 0.9 kg COD/m³/d. They also showed that PPB accumulated promising values of proteins (39.91 %) and pigments (5.34 %) in their granular structures (56).

Table 6. Descriptive statistics of soluble COD removal under different HRT and illumination regimes

HRT (Days)	Illumination		Sum	Average	Variance
	mode				
2	12		2485.35	310.6688	38373.1
4	12		1242.59	138.0656	2683.135
6	12		5612.5	1122.5	1892323
2	24		3106.04	345.1156	32339.21
4	24		9450.32	1050.036	605588.3
6	24		0	-	-

3.4. Ammonium removal

The descriptive statistics, i.e., the average and variance of ammonium concentration, are presented in Table 7. Under different HRT and illumination regimes, N-NH_4^+ concentration decreased from 22.2 mg/L at HRT = 2 days to 9.4 mg/L at HRT = 4 days, followed by a slight increase to 12.9 mg/L at HRT = 6 days. This observation suggested that under the experimental 12 h light condition, intermediate HRT (4 d) is optimal for ammonium removal. N-NH_4^+ concentrations were 17.6 mg/L at HRT = 2 days, increased to 49.2 mg/L at HRT = 4 days, and decreased to 16.6 mg/L at HRT = 6 days. However, the variability within groups prevented statistical significance.

Table 7. Descriptive statistics of ammonium concentration under different HRT and illumination regimes

<i>HRT (Days)</i>	<i>Illumination</i>		<i>Sum</i>	<i>Average</i>	<i>Variance</i>
	<i>mode</i>				
2	12		288.8111	22.21624	17.82406
4	12		150.4482	9.40301	28.52495
6	12		258.3928	12.91964	112.719
2	24		228.9195	17.6092	29.13318
4	24		934.314	49.17442	9475.847
6	24		314.5218	16.55378	686.9023

A significant reduction in N-NH_4^+ was observed at HRT = 4 days under the 12-hour illumination mode, which indicated an optimal link between PPB growth and nitrogen assimilation. A short retention time of 2 days limits ammonium removal due to insufficient treatment time, while an extended HRT of 6 days is associated with an increase in N-NH_4^+ , probably through reduced cell activity or biomass aging.

Under continuous illumination, no significant HRT effect was observed on N-NH_4^+ , which shows that nitrogen assimilation by PPB is strongly influenced by illumination regime. Continuous illumination increases metabolic rate and energy demand. This condition could reduce ammonium

uptake and promote nitrogen accumulation. This observation is consistent with findings of Sali et al., who investigated the effects of light wavelength and nutrient availability on PHA accumulation in PPB. The authors reported an inverse relationship between biomass growth and PHA storage under nutrient-limited conditions. Specifically, reduced biomass formation was accompanied by enhanced PHA accumulation, indicating a shift in cellular metabolism from growth toward intracellular carbon storage when nutrients became limiting (57).

The different observations for the 12-hour illumination period highlighted the key role of optimizing the light regime for effective nitrogen assimilation in PPB systems. Besides, at HRT = 6 days, removal efficiencies declined across all parameters. The difference between illumination regimes became minimal, reflecting reduced growth rates and lower metabolic activity under prolonged retention. Matinfar et al. reported that lower COD/N ratios enhanced PHA-related parameters, including PHB and PHV production, despite reduced NH_4^+ depletion, suggesting a trade-off between nitrogen assimilation and carbon storage (58).

3.5. Organic acids (OAs) (mg COD/L)

The concentration of OAs monitored in 12-hour and continuous modes of illumination and the results are presented in Table 8. As shown for 12 hours illumination system, OAs concentration decreased from 193.1 mg COD/L at HRT = 2 days to 106.7 mg COD/L at HRT = 4 days and reached complete depletion at HRT = 6 days.

Table 8. Descriptive statistics of OAs removal under different HRT and illumination regimes

<i>HRT (Days)</i>	<i>Illumination</i>			
	<i>mode</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>
2	12	3861.85	193.0925	33107.01
4	12	2134.227	106.7114	24299.89
6	12	0	0	0
2	24	4164.9	208.245	36390.98
4	24	4537.327	226.8664	118792.3

6	24	0	0	0
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A strong reduction in OAs concentrations observed at HRT = 4 days under the 12-hour illumination that reflect the best balance between fermentation-derived substrate availability and PPB metabolic activity. This observation is supported by reported PPB characteristics that are commonly known for their effective organics recovery from wastewater with a high biomass yields of around 1 g COD_{biomass}/g COD_{removed} (59). At short HRT, high substrate loading is available in the reactor that limits complete consumption. This phenomenon could attributed to the washing effect of organic matters and microorganisms from the system under high hydraulic loading (60). Study the concentration of OAs as a function of HRT and illumination mode showed that organic acid dynamics are strongly influenced by HRT and that intermediate retention times provide the most favorable conditions for PPB assimilation.

This reactor behavior is consistent with previous modeling results showing that PPB productivity is highly dependent to the availability and composition of fermentation-derived VFAs, with readily assimilable substrates such as acetate supporting higher biomass production (61).

HRT showed a significant impact on VFA consumption in the PPB reactor, and under short HRT, limited consumption of VFA was observed, which increased to complete depletion at high HRT. A strong reduction in acetic acid at HRT = 4 days under the 12-hour illumination period showed that an optimal balance between substrate availability and PPB metabolic activity exists.

The importance of acetate as a metabolically active carbon source is consistent with previous observations in marine purple non-sulfur bacteria, where acetate addition induced metabolic changes associated with TCA-cycle activity and intracellular carbon storage (62).

The decrease in acetic acid reduction is slight for continuous mode of illumination, which can be attributed to the increased maintenance energy demand. At prolonged HRT, acetic acid depletion

shows substrate deficiency and reduced fermentation input. This study revealed that VFA dynamics are strongly influenced by both HRT and light regime, with continuous illumination providing less optimal conditions for acetic acid consumption compared to intermittent illumination method. Besides, the findings demonstrated that intermediate retention times provide the most favorable conditions for PPB assimilation of acetic acid in systems treating fermented wine lees.

As a subclass form of OAs, acetic acid concentration (as mg COD/L) was also monitored under a 12-hour light regime showed a strong and statistically significant effect of HRT. The mean concentration in Table 9 declined from 193.09 mg/L at HRT = 2 days to 71.32 mg/L at HRT = 4 days and was completely depleted at HRT = 6 days.

A similar pattern was observed under continuous 24-h illumination, although the decrease at the intermediate HRT was less pronounced. Acetic acid concentration fell from 208.2 mg/L at HRT = 2 days to 178.0 mg/L at HRT = 4 days, reaching complete depletion at HRT = 6 days.

Table 9. Descriptive statistics of VFA removal under different HRT and illumination scenarios

<i>HRT (Days)</i>	<i>Illumination</i>		<i>Sum</i>	<i>Average</i>	<i>Variance</i>
	<i>mode</i>				
2	12		3861.85	193.0925	33107.01
4	12		1426.367	71.31835	12377.55
6	12		0	0	0
2	24		4164.9	208.245	36390.98
4	24		3382.757	178.0398	70760.78
6	24		0	0	0

3.6. Bacteriochlorophyll (BChl)

BChl is a direct indicator of PPB phototrophic biomass production and pigment synthesis. BChl, an important indicator of PPB phototrophic biomass production and pigment synthesis, reflects the light-harvesting capacity and physiological activity of the biomass. Under the 12-h illumination

regime, BChl concentration increased from 0.00240 mg/L at HRT = 2 days to 0.00318 mg/L at HRT = 4 days before slightly declining to 0.00308 mg/L at HRT = 6 days (Table 10).

The consistent maximum at HRT = 4 days under both illumination regimes suggests that intermediate retention provides the most favorable conditions for BChl synthesis and phototrophic biomass development. The subsequent decline at HRT = 6 days, despite prolonged biomass retention, may indicate the onset of substrate limitation or reduced metabolic activity. Overall, the BChl pattern was consistent with the trends observed in PPB productivity, HV formation, and acetic acid consumption, supporting HRT = 4 days as a favorable condition for active phototrophic metabolism.

Table 10. Descriptive statistics of BChl concentration under different HRT and illumination regimes

<i>HRT (Days)</i>	<i>Illumination</i>		<i>Sum</i>	<i>Average</i>	<i>Variance</i>
	<i>mode</i>				
2	12		0.048024	0.002401	9.51E-07
4	12		0.063664	0.003183	5.22E-07
6	12		0.061614	0.003081	1.13E-06
2	24		0.035958	0.001798	5.32E-07
4	24		0.046376	0.002319	4.95E-07
6	24		0.045191	0.00226	2.77E-07

Pigments such as Chls, BChl used by photosynthetic bacteria to receive and convert light into chemical energy (63). The study of BChl formation as a function of HRT showed that under both short and long HRT, pigment formation was diminished. The synthesized pigments mostly washout at low HRT, while substrate depletion reduces metabolic activity and pigment formation under extended HRT. The increase in BChl at HRT = 4 days under the 12-hour photoperiod highlights the optimal balance between biomass retention, substrate availability, and phototrophic activity.

A same trend was observed for BChl production under continuous illumination mode. These findings demonstrate that pigment dynamics are strongly influenced by HRT and that intermediate retention times provide the most favorable conditions for PPB phototrophic performance. Earlier

reports confirmed that the aggregation of pigments by photosynthetic bacteria is highly dependent to the environmental condition (64).

3.7. Carotenoid

The carotenoid (Crt) production concentration was monitored over different hydraulic and illumination scenarios, and the results are presented in Table 11. As seen, Crt increased slightly from 0.00211 mg/L at HRT = 2 days to 0.00257 mg/L at HRT = 4 days and 0.00287 mg/L at HRT = 6 days.

Table 11. Descriptive statistics of Crt production under different HRT and illumination regimes

<i>HRT (Days)</i>	<i>Illumination</i>		<i>Sum</i>	<i>Average</i>	<i>Variance</i>
	<i>mode</i>				
2	12		0.042262	0.002113	2.64E-06
4	12		0.051465	0.002573	4.27E-07
6	12		0.057454	0.002873	7.14E-07
2	24		0.044604	0.00223	9.42E-07
4	24		0.065833	0.003292	7.23E-07
6	24		0.069629	0.003481	6.4E-07

The global demand for naturally derived carotenoids is increasing due to their applications as colorants, antioxidants, animal feed, cosmetics, and nutraceutical products, with growing attention toward non-plant and non-microalgal sources such as PPB (65). In PPB, carotenoids extend beyond their nutritional importance by functioning as integral components of the photosynthetic apparatus, broadening the range of light captured by the cells and complementing bacteriochlorophyll absorption, while also contributing to photoprotection and the stabilization of photosynthetic membranes (66).

Herein, we studied the production of carotenoids under different light and HRT modes. The results showed no significant HRT effect on carotenoid concentration under the 12-hour photoperiod. This observation could be attributed to the stable nature of carotenoid production within the operational

conditions. The slight increase observed at HRT = 6 days may reflect stress-induced pigment accumulation under substrate limitation rather than enhanced phototrophic performance.

In contrary to 12-hours light regime, continuous illumination of the reactor was associated with HRT regime. The steady increase in carotenoid concentration with increasing HRT could be a function of prolonged exposure to stress-induced continuous light and substrate deficiency.

Different strategies were applied to maximize carotenoid production as a valuable byproduct. Recent findings by Grassino et al indicated that light conditions can substantially influence both carotenoid productivity and composition in PPB consortia. They showed that lower light intensity promoted greater carotenoid accumulation, while infrared illumination favored carotenoid production (67).

3.8. Total Pigment

Under 12-h illumination, pigment content (g P/g VS (%)) increased progressively from 0.00451% at HRT = 2 days to 0.00576% at HRT = 4 days and 0.00595% at HRT = 6 days (Table 12). The table also shows a similar trend under continuous 24-h illumination, where total pigment content increased from 0.00403% at HRT = 2 days to 0.00561% at HRT = 4 days and 0.00574% at HRT = 6 days. Pairwise comparisons using Tukey's HSD showed that all HRT levels differed significantly, confirming the progressive increase in pigment accumulation with extended retention time. Although the overall pattern was similar under both photoperiods, the progressive increase in pigments suggests that longer biomass retention favors pigment accumulation, particularly at HRT = 6 days. This response may reflect not only enhanced pigment biosynthesis but also physiological adaptation to prolonged retention and associated substrate limitation, especially at the longest HRT.

Table 12. Descriptive statistics of total pigment concentration under different HRT and illumination regimes

<i>HRT (Days)</i>	<i>Illumination</i>			
	<i>mode</i>	<i>Sum</i>	<i>Average</i>	<i>Variance</i>

2	24	0.080562	0.004028	2.34E-06
4	24	0.112209	0.00561	1.93E-06
6	24	0.114819	0.005741	1.41E-06
2	12	0.090286	0.004514	3.60E-06
4	12	0.115129	0.005756	1.30E-06
6	12	0.119067	0.005953	3.06E-06

Pigment dynamics under both 12-h and continuous illumination well reflected the mutual effects of phototrophic performance, biomass retention, and protective physiological responses in PPB species. A net increase in total pigment content with increasing HRT under both illumination regimes was observed that shows the pigment formation is strongly influenced by biomass retention and substrate availability. At short HRT, biomass washout limits pigment accumulation, whereas longer retention provides more favorable conditions for synthesis of bacteriochlorophylls and carotenoids. The highest pigment levels at HRT = 6 days likely reflect stress-induced carotenoid accumulation under substrate limitation, with prolonged continuous illumination potentially contributing to additional physiological stress. Orlandi et al reported that carotenoids play a key role in shielding cells from photo-oxidative stress that cells experience during the photodynamic reactions (63). Earlier reports documented not only the role of illumination time but also the wavelength and type of illumination. In this context, Saejung et al. reported that white-light illumination (465 nm) enriched a natural microbial consortium to 76% PPB, predominantly *Rhodopseudomonas sp.*, resulting in enhanced bacterial biomass, polyhydroxybutyrate accumulation, and COD removal. In contrast, red light (616 nm) limited PPB proliferation but increased the specific yields of photosynthetic pigments and protein, highlighting a metabolic trade-off between microbial growth and the production of value-added bioproducts (31).

3.9. Correlation Analysis

The correlation analysis revealed strong associations between the pigment fractions, with total pigments closely related to both Crt and BChl, indicating coordinated pigment production within the reactor. In addition, the moderate relationship between PPB productivity and HV suggests a possible association between biomass production and HV-enriched PHA accumulation. However, the generally weak correlations of PHA with pigment parameters and substrate-related variables indicate that polymer accumulation may be regulated by factors other than substrate availability or photopigment production.

Earlier studies also showed that PHA accumulation is strongly dependent on the illumination regime and does not necessarily coincide with expected outcome such as enhanced COD removal (57, 68). The strong correlation between VFA and total organic acids is consistent with the metabolic pathway that explain the dependence of PPB on readily biodegradable OAs. Previous studies have demonstrated that VFAs, particularly when supplied as mixtures, can support biomass production as it serve as effective carbon and electron sources for PNSB (59).

4. Statistical Insight

4.1. PPB, PHA Productivity

The maximum productivity was observed at HRT = 4 days ($0.276 \pm \text{SD}$), followed by lower values at HRT = 2 days ($0.200 \pm \text{SD}$) and HRT = 6 days ($0.131 \pm \text{SD}$). As statistically showed in Table 13, under the 12-hour light cycle, PPB productivity differed significantly across hydraulic retention times ($F = 71.67$, $p < 0.001$).

Besides, with $F = 58.33$, $F_{\text{crit}} = 3.16$, and a p-value of 1.63×10^{-14} , statistical analysis using one-way ANOVA shows a very strong effect of HRT on PPB productivity under continuous illumination (24 h light). The highest productivity was observed at HRT = 4 days ($0.306 \pm \text{SD}$), followed by lower values at HRT = 2 days ($0.235 \pm \text{SD}$) and HRT = 6 days ($0.153 \pm \text{SD}$). These findings indicate that continuous illumination improves PPB growth, particularly at average retention time.

Table 13. One-way ANOVA of the effect of HRT on PPB production under 12-h illumination

Illumination time 12 h						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.21109	2	0.10554	71.6662	2.77E-16	3.15884
Within Groups	0.08394	57	0.00147			
Total	0.29504	59				
Illumination time 24 h						

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.23718	2	0.11859	58.3267	1.63E-14	3.15884
Within Groups	0.11589	57	0.00203			
Total	0.35308	59				

4.2. PHA and HV Accumulation

In order to statistically analyze the role of HRT under 24-hour illumination, a one-way ANOVA was performed. Having $F = 21.63$, $F_{crit} = 3.16$, and $p = 1.02 \times 10^{-7}$ ($p < 0.001$) in Table 14 shows a strong and statistically significant effect of HRT on PHA accumulation. Because $F \gg F_{crit}$ and the p-value is extremely small, the differences among HRT 2, 4, and 6 days are statistically significant. In addition, the values of 0.1, 0.05, and 0.02 for HRT 2 d, HRT 4 d, and HRT 6 d clearly show a decreasing trend in PHA accumulation as HRT increases. These results indicate that continuous illumination does not prevent the decline in PHA synthesis associated with increasing HRT.

Statistical analysis in Table 15 revealed that the 12-hour light regime irradiation led to the accumulation of different levels of HV across hydraulic retention times ($F = 32.99$, $p < 0.001$). These results indicate that HV synthesis is a highly dynamic bioproduct strongly favored at short retention times and becomes fully suppressed as the substrate diminishes at high HRT.

Under 24-hour irradiation, HV accumulation differed significantly across HRT ($F = 25.31$, $p < 0.001$). HV content increased from HRT = 2 days ($0.152 \pm \text{SD}$) to HRT = 4 days ($0.212 \pm \text{SD}$), and then dropped at HRT = 6 days. These results indicate that continuous illumination promotes HV synthesis at intermediate retention times, but do not support HV production at extended HRT.

Table 14. One-way ANOVA of the effect of HRT on PHA content under different HRT and illumination scenarios

Illumination time	12 h					
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.07058	4	0.03529	37.1303	4.74E-11	3.15884
Within Groups	0.05417	8	0.00095			
Total	0.12476	2				
Illumination time	24 h					
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.05186	1	0.02593	21.6331	1.02E-07	3.15884
Within Groups	0.06832	2	0.00119	9		
Total	0.12018	3				

Table 15. One-way ANOVA of the effect of HRT on HV content under different HRT and illumination scenarios

Illumination time	12 h					
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.58631	8	0.29315	32.9897	6.4E-10	3.17879
Within Groups	0.45320	5	0.00888	1		
Total	1.03952	3				
Illumination time	24 h					
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.39670	2	0.19835	25.3054	2.56E-08	3.18261
Within Groups						

Within	0.39191		0.00783
Groups	3	50	8
	0.78861		
Total	5	52	

4.3. COD removal

Present work revealed a strong association between COD removal in PPB systems treating fermented wine lees with both HRT and the illumination regime. Under the 12-hour photoperiod, the pronounced reduction in COD_{SOL} at an HRT of 4 days suggests that this condition provided a favorable balance between substrate availability and PPB metabolic activity. In contrast, under 24-hour illumination, the effect of HRT on COD removal was more pronounced, with elevated COD levels observed at an HRT of 4 days. This difference suggests that the light regime may substantially affect substrate utilization and overall process performance. Continuous illumination may increase cellular energy and maintenance requirements, potentially reducing substrate uptake, which led to the accumulation of soluble organic compounds. The elevated COD under prolonged illumination may also indicate physiological stress or the release of intracellular compounds. Conversely, insufficient HRT may limit the extent of organic substrate degradation, while reduced metabolic activity and biomass decay could further contribute to soluble organic accumulation. The absence of a similar response under the 12-hour photoperiod highlights the importance of optimizing the illumination regime together with HRT to achieve effective COD removal in PPB systems.

The one-way ANOVA was performed on COD removal data, and the results are shown in Table 16. With $F = 3.96$, $F_{crit} = 3.52$, and $p = 0.036$ ($p < 0.05$), and average COD levels of 310.7 mg/L, 138.1 mg/L, and 1122.5 mg/L, the differences among HRT 2, 4, and 6 days are statistically significant for the system operated under the 12-hour light regime. Besides, this pattern shows a

non-linear and operationally important trend for COD reduction in the system. At HRT = 4 days, removal efficiencies reached their highest levels. Continuous illumination resulted in superior COD and OAs removal, indicating that sustained phototrophic activity supports more complete substrate utilization. A sharp peak in COD content was observed at HRT = 6 days (1122.5 mg/L), which suggests that intermediate retention time provides optimal conditions for COD removal by PPB. On the other hand, the table shows $F < F_{crit}$ and $p > 0.05$, confirming that the ANOVA test didn't reveal any statistically significant effect of HRT on soluble COD (SOL) under 24 h light illumination.

Table 16. One-way ANOVA of the effect of HRT on soluble COD removal under different HRT and illumination scenarios

Illumination time	12 h					
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3278083	2	1639041	3.962377	0.036452	3.521893
Within Groups	7859369	19	413651			
Total	11137452	21				

Illumination time	24 h					
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2236105	2	1118052	3.286186	0.065532	3.68232
Within Groups	5103420	15	340228			
Total	7339525	17				

4.4. Ammonium removal

Ammonium removal was studied under the 12-hour light regime, which shows that $N-NH_4^+$ concentrations differed meaningfully ($F = 10.22$, $p < 0.001$) across different HRTs (Table 17)

However, with an average COD level of 17.6 mg/L, 49.17 mg/L, and 16.55 mg/L for HRT of 2 days, 4 days and 6 days, the one-way ANOVA shows no statistically significant effect ($F = 1.62$, $p = 0.209$) of HRT on N-NH_4^+ conversion under continuous illumination.

Table 17. One-way ANOVA of the effect of HRT on ammonium removal under different HRT and illumination regimes

Illumination time	12 h					
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1236.27	5	618.137	10.2155	0.00021	3.19958
Within Groups	2783.42	3	60.5092	9	3	2
Total	4019.69	8				

Illumination time	12 h					
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	12363.1	3	6181.56	1.61892	0.20874	3.19072
Within Groups	183279.	1	3818.31	5	1	7
Total	195642.	2				

4.5. Organic acids (OAs)

Under the 12-h light regime, total OAs varied significantly with HRT ($F = 9.78$, $p < 0.001$) (Table 18). Tukey's HSD confirmed significant differences among all HRT levels, indicating progressively enhanced VFA consumption with increasing retention time and complete utilization at $\text{HRT} = 6$ days.

Once continuous mode of illumination was applied to reactors. OAs also differed significantly among HRTs ($F = 6.13$, $p < 0.01$). Unlike the 12-h regime, OAs increased slightly from 208.2

mg COD/L at HRT = 2 days to 226.9 mg COD/L at HRT = 4 days before declining to zero at HRT = 6 days. Tukey's HSD confirmed significant differences among the HRT levels, with the complete depletion at HRT = 6 days indicating enhanced substrate consumption at prolonged retention. The transient accumulation of OAs at HRT = 4 days under continuous illumination, in contrast to their decline under 12-h illumination, suggests that the illumination regime influenced organic-acid assimilation, with continuous light potentially imposing greater physiological stress and reducing assimilation efficiency at intermediate HRT.

Table 18. One-way ANOVA of the effect of HRT on OAs removal under different HRT and illumination regimes

Illumination time 12 h						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	374224.9	2	187112.4	9.778221	0.000223	3.158843
Within Groups	1090731	57	19135.63			
Total	1464956	59				
Illumination time 24 h						
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	634540.5	2	317270.3	6.133463	0.003868	3.158843
Within Groups	2948482	57	51727.76			
Total	3583023	59				

As shown in Table 19, under this operation mode, HRT had a significant effect on acetic acid concentration ($p = 2.99 \times 10^{-5}$). Tukey's HSD indicated significant differences among all HRT

levels, demonstrating progressively greater acetic acid consumption as retention time increased.

The overall effect of HRT was significant ($F = 7.18$, $p < 0.01$), and Tukey's HSD identified significant differences between each pair of HRT levels. These results indicate that extending HRT to 6 days enabled complete acetic acid utilization under both illumination regimes.

Table 19. One-way ANOVA of the effect of HRT on VFA removal under different HRT and illumination scenarios

Illumination time	12 h					
Source of Variation	SS	Df	MS	F	P-value	F crit
Between Groups	381333.1	2	190666.5	12.57569	2.99E-05	3.158843
Within Groups	864206.5	57	15161.52			
	124554					
Total	0	59				

Illumination time	12 h					
Source of Variation	SS	Df	MS	F	P-value	F crit
Between Groups	504040.6	2	252020.3	7.18181	0.001673	3.161861
Within Groups	196512.3	56	35091.47			
	246916					
Total	3	58				

4.6. Bacteriochlorophyll (BChl)

These variations in BChl were statistically analyzed, and Table 20 showed a significant difference existed among HRTs ($F = 4.16$, $p < 0.05$). Tukey's HSD confirmed significant differences among all HRT levels, with HRT = 4 days showing the highest BChl accumulation.

A similar trend was observed under continuous 24-h illumination ($F = 3.74$, $p < 0.05$). BChl increased from 0.00180 mg/L at HRT = 2 days to 0.00232 mg/L at HRT = 4 days and then decreased slightly to 0.00226 mg/L at HRT = 6 days. Tukey's HSD also indicated significant differences between all HRT levels.

Table 20. One-way ANOVA of the effect of HRT on BChl concentration under different illumination times

Illumination time	12 h					
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	7.22E-06	2	3.61E-06	4.158912	0.020608	3.158843
Within Groups	4.95E-05	57	8.69E-07			
Total	5.67E-05	59				

Illumination time	24 h					
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3.25E-06	2	1.63E-06	3.743666	0.029677	3.158843
Within Groups	2.48E-05	57	4.34E-07			
Total	2.8E-05	59				

4.7. Carotenoid

The one-way ANOVA in Table 21 shows no statistically significant effect of hydraulic retention time on Crt concentration under the 12-hour light regime; the variability within groups is high enough that ANOVA does not reveal a significant difference between the mean values ($F = 2.32$, $p = 0.107$).

Under the 24-hour light regime, however, Crt concentrations were statistically different across hydraulic times ($F = 11.84$, $p < 0.001$). Crt increased from 0.00223 mg/L at HRT = 2 days to

0.00329 mg/L at HRT = 4 days and reached 0.00348 mg/L at HRT = 6 days. These results indicate that continuous illumination promotes carotenoid synthesis, particularly at longer retention times.

Table 21. One-way ANOVA of the effect of HRT on Crt production different HRT and illumination regimes

Illumination time		12 h				
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	5.86E-06	2	2.93E-06	2.324981	0.106991	3.158843
Within Groups	7.18E-05	57	1.26E-06			
Total	7.76E-05	59				

Illumination time		24 h				
Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	1.82E-05	2	9.09E-06	11.83859	5.01E-05	3.158843
Within Groups	4.38E-05	57	7.68E-07			
Total	6.2E-05	59				

4.8. Total Pigments

Table 22 showed that total pigment content was significantly affected by HRT under both illumination regimes ($p < 0.05$). Tukey's HSD confirmed significant differences among all HRT levels, with each increase in retention time resulting in significantly greater pigment accumulation.

Table 22. One-way ANOVA of the effect of HRT on total pigment under different HRT and illumination regimes

Illumination time		12 h				
Source of Variation	SS	df	MS	F	P-value	F crit

	5.86E-		2.93E-	2.32498	0.10699	3.15884
Between Groups	06	2	06	1	1	3
Within Groups	7.18E-	5	1.26E-			
	05	7	06			
Total	7.76E-	5				
	05	9				

Illumination time	24 h					
<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	1.82E-		9.09E-	11.8385	5.01E-	3.15884
	05	2	06	9	05	3
Within Groups	4.38E-	5	7.68E-			
	05	7	07			
Total		5				
	6.2E-05	9				

4.9. Correlation Analysis

Correlation analysis presented in Table 23 revealed coherent relationships among PPB productivity, pigment parameters (BChl, Crt, and total pigments), and polymer accumulation. This study indicated a strong correlation between total pigments and Crt ($r = 0.83$) and BChl ($r = 0.75$), confirming the tight coupling between pigment fractions.

Organic acids (VFA and OAs) also showed an almost perfect correlation ($r = 0.98$), reflecting their shared metabolic origin. Moderate correlations were observed between PPB productivity and HV ($r = 0.48$). In contrast, PHA showed weak correlations with most parameters, suggesting that PHA accumulation was not directly linked to pigment formation or substrate availability.

Table 23. Pearson correlation matrix of polymer accumulation, PPB productivity, soluble COD, organic acids, and pigment parameters

<i>Parameter</i>	<i>PHA %</i>	<i>HV%</i>	<i>PPB productivity</i>	<i>CODS OL</i>	<i>N-NH4+</i>	<i>VFA</i>	<i>OAs (TOT)</i>	<i>BChl</i>	<i>Crt</i>	<i>Total Pigments</i>
PHA %	1.00									
HV %	0.59	1.00								
PPB productivity	0.18	0.49	1.00							
CODSOL	-0.24	-0.02	0.08	1.00						

N-NH ₄ ⁺	0.01	0.04	0.06	0.58	1.00					
VFA	0.35	0.33	0.37	0.31	0.49	1.00				
OAs	0.27	0.29	0.36	0.35	0.53	0.98	1.00			
BChl	0.03	-0.10	-0.11	-0.12	-0.12	-0.16	-0.14	1.00		
Crt	-0.07	-0.21	-0.07	0.07	-0.10	-0.17	-0.13	0.26	1.00	
Total										
Pigments	-0.03	-0.20	-0.11	-0.03	-0.14	-0.21	-0.17	0.76	0.83	1.00

5. Conclusion

The aim of this study was to evaluate the effects of hydraulic retention time (HRT) and illumination regime on PPB growth, substrate removal, pigment synthesis, and biopolymer production during the treatment of fermented wine lees, and to identify operating conditions that optimize both treatment performance and bioproduct recovery. Our findings demonstrated that HRT strongly modulates PPB metabolism, whereas the light regime determines the direction and intensity of the subsequent physiological response. Intermittent 12-h illumination provided a more balanced environment for PPB cultivation, with HRT = 4 days emerging as the metabolic optimum, supporting efficient COD removal, VFA uptake, ammonium assimilation, pigment synthesis, and PHA/HV production. Increasing HRT to 6 days further increased pigment accumulation but coincided with depletion of readily available substrates, which turned the system into substrate limitation mode and less metabolic activity. In contrast, continuous 24-h illumination produced a clear metabolic shift toward a weaker substrate assimilation and PHA/HV formation and a greater accumulation of total pigments, particularly carotenoids. The pronounced pigment accumulation at HRT = 6 days under continuous illumination was mostly associated with a protective response to prolonged illumination and substrate limitation. As our final conclusion, this study revealed that intermittent illumination is more favorable for simultaneously achieving effective fermented wine lees treatment and PPB bioproduct production, while continuous illumination may be better suited when pigment enrichment is the primary objective. Thus, appropriate direction of illumination regime and HRT represents a key operational strategy to direct PPB system toward either treatment efficiency or bioproduct recovery.

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