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DOI
10.1016/j.biortech.2024.131467

Publication date
2024

Document Version
Final published version

Published in
Bioresource Technology

Citation (APA)
Doki, M. M., Mehta, A. K., Chakraborty, D., Ghangrekar, M. M., Dubey, B. K., Alloul, A., Moradvandi, A., Vlaeminck, S. E., & Lindeboom, R. E. F. (2024). Recovery of purple non-sulfur bacteria-mediated single-cell protein from domestic wastewater in two-stage treatment using high rate digester and raceway pond. *Bioresource Technology*, *413*, Article 131467. https://doi.org/10.1016/j.biortech.2024.131467

Bioresource Technology 413 (2024) 131467

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Recovery of purple non-sulfur bacteria-mediated single-cell protein from domestic wastewater in two-stage treatment using high rate digester and raceway pond

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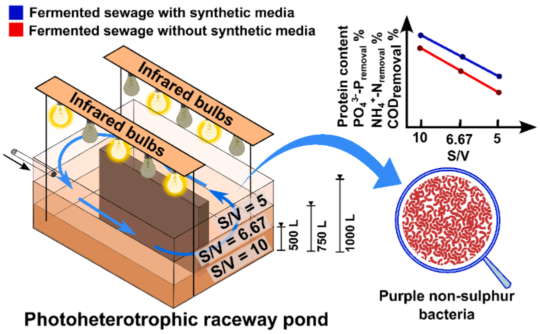
HIGHLIGHTS

- Purple non-sulfur bacteria (PNSB) enriched from anaerobic sludge under infrared light.
- Photoheterotrophic growth and recovery of PNSB was performed using fermented sewage.
- Impact of area/volume ratio on treated wastewater quality and biomass yield evaluated.
- The biomass of 0.9 g VSS/L with protein contents of 43.9 ± 0.2 % *w/w* was obtained.
- Raceway pond having PNSB removed COD (89.0 %), NH_4^+-N (92.5 %), and PO_4^{3-}-P (80.7 %).

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Aquaculture
Photobioreactor
Purple phototrophic bacteria
Resource recovery
Wastewater treatment

ABSTRACT

Wastewater resources can be used to produce microbial protein for animal feed or organic fertiliser, conserving food chain resources. This investigation has employed the fermented sewage to photoheterotrophically grown purple non-sulfur bacteria (PNSB) in a 2.5 m^3 pilot-scale raceway-pond with infrared light to produce proteinaceous biomass. Fermented sewage with synthetic media consisting of sodium acetate and propionic acids at a surface-to-volume (S/V) ratio of 10 m^2/m^3 removed 89%, 93%, and 81% of chemical oxygen demand, ammonium nitrogen, and orthophosphate, respectively; whereas respective removal in fermented sewage alone without synthetic media was 73%, 73%, and 72% during batch operation of 120 h. The biomass yield of 0.88–0.95 g $COD_{biomass}$ /g $COD_{removed}$ with protein content of 40.3 ± 0.3%–43.9 ± 0.2% *w/w* was obtained for

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https://doi.org/10.1016/j.biortech.2024.131467
Received 25 June 2024; Received in revised form 31 August 2024; Accepted 7 September 2024

fermented sewage with synthetic media. The results revealed enhanced possibility of scaling-up the raceway reactor to recover resources from municipal wastewater and enable simultaneous high-rate PNSB single-cell protein production.

1. Introduction

Nitrogen and proteins are essential for the nutrition and health of plants, animals, and humans, yet the environmental impact of proteins in the current food system is unacceptably high, necessitating a protein shift or transition (Verstraete et al., 2016; Spanoghe et al., 2021). A novel class of protein is based on microbes, and a single-cell protein (SCP) is a type of microbial protein that equals the protein-rich whole-cell biomass, generally from single-cell organisms, such as chemotrophic and phototrophic bacteria, eukaryotic microalgae, and yeasts (Verstraete et al., 2016). Single-cell protein can be produced in an axenic condition using pure cultures on a well-defined feedstock, yet production based on open microbiomes or symbiotic cultures on side streams, byproducts, or waste streams for resource recovery has a lower environmental footprint and it is more cost-effective (Alloul et al., 2021).

One interesting group of bacteria for SCP applications is the purple non-sulfur bacteria (PNSB), which, together with the purple sulfur bacteria (PSB), belong to the group of the purple bacteria also termed as purple phototrophic bacteria (PPB), which are an anoxygenic, facultative anaerobes that exhibit phototrophic capabilities (Capson-Tojo et al., 2020; Alloul et al., 2021). Purple non-sulfur bacteria display versatile metabolic characteristics across various culture conditions, including photoheterotrophy, photoautotrophy, chemoheterotrophy, chemoautotrophy and/or fermentative options (Syed et al., 2022). Therefore, they can be cultivated in the presence or absence of light under diverse conditions in terms of carbon sources, electron donors, and acceptors (Alloul et al., 2021). Purple non-sulfur bacteria have high biomass yields ranging from 0.9 to 1.1 g $COD_{biomass}$ /g $COD_{removed}$ (COD − chemical oxygen demand) and growth rates between 0.6 and 3.7 day^{-1} (Alloul et al., 2021). Interestingly, for resource recovery, when PNSB grow in their preferred photoheterotrophic mode, a virtually perfect substrate-C to biomass-C conversion yield is achievable (Cao et al., 2020; Cerruti et al., 2020). Additionally, for open-community non-axenic production, the utilisation of infrared light allows the selective enrichment of PNSB (Cerruti et al., 2020; Segura et al., 2022).

These PNSB have been widely explored for resource recovery and removal of organic matter, nitrogen, and phosphorous from wastewater and other waste streams. Wastewater often contains a wide range of carbon-based substances, including polysaccharides, lipids, proteins, deoxyribonucleic acid, and polyphenolic structures (Dignac et al., 2000). Examples of direct cultivation of PNSB in wastewater are domestic wastewater (Hülsen et al., 2016), acidic wastewater obtained from the food industry (Liu et al., 2016), and breweries (Lu et al., 2019). Yet, volatile fatty acids (VFAs) are an excellent carbon sources for PNSB to grow on, amongst others. Anaerobic fermentation leads to the production of VFAs, such as acetate, butyrate, isobutyrate, propionate, valerate, and isovalerate (Zhu et al., 2022).

These PNSB perform anoxygenic photosynthesis and energy is derived from light, thus the organics serve as carbon source, and the reduction degree of the compound (e.g., VFA) will determine the need for an additional electron source or sink (Wu et al., 2021; Alloul et al., 2021). For instance, a certain amount of inorganic carbon may be required, or H_2 may be produced to reach redox homeostasis while growing on the more reduced longer-chain VFAs. As no COD is respired, in contrast to activated sludge, a lower COD/N ratio can be beneficial for N removal through assimilation. Note that these bacteria can also biologically fix nitrogen (Cerruti et al., 2020). Additionally, these bacteria can store phosphorus as polyphosphate (Wu et al., 2021; Cao et al., 2020), yielding a higher P removal through assimilation than in conventional activated sludge.

Within the framework of manned life support, PPB such as *Rhodospirillum Rubrum* have been extensively investigated for treatment of domestic wastewater even being part of a recent International Space Station experiment (Ilgrande et al., 2019). However, the proposed axenic PNSB production in closed photobioreactors is expected to face similar scalability and cost-effectiveness considerations as microalgae. Aligned with the work by Ceron-Chafla and Lindeboom (2023), frugal engineering of the CI and CII MELiSSA solution resulted in the conceptualisation of an open raceway-pond reactor as a potential cost-effective and contextualised alternative within the European Union and Department of Science and Technology (India) Saraswati 2.0 programme. Literature reports both batch, continuous lab-scale and demo-scale PPB research on domestic wastewater with the largest PPB enclosed raceway photobioreactor in the world, which was constructed as part of the H2020-BBI-JU project DEEP PURPLE (Sepúlveda-Muñoz et al., 2023).

The present study hypothesised that PNSB can assimilate nutrients utilising fermented domestic sewage under certain operational parameters such as surface area-to-volume (S/V) ratio and COD:N:P ratio. Therefore, this investigation has been focused on investigating the feasibility of a two-stage system contextualised for real Indian conditions, a 100 m^3/day anaerobic fermenter, producing anaerobically fermented sewage, complemented by a pilot-scale raceway pond for the growth and recovery of PNSB. The impact of changing working (culture) volume of raceway pond from 500 L to 1000 L to test the effect of S/V ratio and COD: N:P ratio on the composition of the SCP, growth rate and pollutant removal in two ways: with and without synthetic carbon source media (sodium acetate and propionate to simulate co-digestion of fermented fruit and vegetable waste). The analysis of extracellular parameters, including COD, NH_4^+-N, PO_4^{3-}-P, and VFAs, was performed along with the growth of biomass over time of operation. Subsequently, purple non-sulfur bacterial biomass was collected, and the macromolecular compositions, namely protein, lipid, and carbohydrate, were extracted and analysed. Thus, the hypothesis of present research was this two-stage process can hold a lot of potential to complement existing anaerobic digesters with PNSB raceway ponds and unlock a wide array of PNSB-based resource recovery strategies in India and beyond.

2. Materials and methods

2.1. Enrichment of purple non-sulfur bacteria (mixed consortium)

Purple non-sulfur bacteria (mixed consortium) used in this study was isolated from anaerobic sludge obtained from a 1.35 million litres per day (MLD) sewage treatment plant situated at IIT Kharagpur. Purple non-sulfur bacteria were enriched by mixing 1 mL of the anaerobic sludge (with a volatile suspended solids (VSS) concentration of 0.1 g VSS/L) and 79 mL of the modified medium in a 100 mL serum bottle by incubating in the presence of incandescent infrared light. The synthetic media composition (Cerruti et al., 2020) was modified, which includes CH_3COONa (1 g/L), CH_3CH_2COOH (0.5 mL/L), KH_2PO_4 (0.014 g/L), K_2HPO_4 (0.021 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L), NH_4Cl (0.229 g/L), $CaCl_2 \cdot 2H_2O$ (0.05 g/L), NaCl (0.2 g/L), and 1 mL/L of trace metal solution, which includes $FeCl_3 \cdot 6H_2O$ (2.0 g/L), EDTA-2Na·$2H_2O$ (1.1 g/L), $ZnCl_2$ (0.1 g/L), H_3BO_3 (0.1 g/L), $MnSO_4 \cdot H_2O$ (64 mg/L), $CoCl_2 \cdot 6H_2O$ (100 mg/L), $CuSO_4 \cdot 5H_2O$ (16 mg/L), $Na_2MoO_4 \cdot 2H_2O$ (24 mg/L), $NaSeO_3$ (5 mg/L), and $NiCl_2 \cdot 6H_2O$ (10 mg/L).

The serum bottle (with a working volume of 80 mL) containing culture medium was sparged with nitrogen gas (5–7 min) before tightening the cap and incubated at room temperature in a specially constructed wooden chamber provided with fans on both sides to maintain proper airflow inside the chamber with incandescent infrared light (250 W, E27 230–250 V, IR CL, Philips). The samples from the serum bottle were collected during enrichment for 24 days to analyse the biomass growth (at 660 nm). This was followed by scanning the samples at 300 to 900 nm to identify the carotenoid and bacteriochlorophyll peaks for the preliminary confirmation of PNSB (Hülsen et al., 2014; Alloul et al., 2019). For further confirmation, the developed mixed consortium after two subcultures was sent for metagenomics sequencing with 16S rRNA (v3-v4). The remaining enriched culture was transferred to a reagent bottle (1 L) containing the same media (without autoclaving), whose 40 % volume was replaced weekly with fresh media and treated as parent culture for further experiments.

2.2. *Raceway reactor setup and growth conditions*

The parent culture (5% *v/v* as described in the previous Section 2.1) was transferred to a 5 L conical flask, which was further used to inoculate two 50 L containers having the same media. In this way, the cells were acclimatised, and enough inoculum was developed to perform the experiments in a raceway reactor with total capacity of 2.5 m^3. The raceway reactor was of rectangular geometry with a length of 5 m, a breadth of 1 m, and a height of 0.5 m. The reactor was enveloped in a layer of black polythene, and infrared bulbs (10 no.) were suspended from the ceiling in a grid-like arrangement of rows (5) and columns (2) (Fig. 1). The distance between adjacent bulbs within each row was precisely 1 m, whereas the distance between bulbs in adjacent columns was around 0.5 m. Moreover, the employed ten infrared bulbs arranged in two groups, illuminated in a zigzag pattern controlled by two timers. These timers were programmed to alternate every 30 min (on/off), ensuring continuous illumination over a 12 h period. This configuration provided uniform infrared radiation across the entire surface of the culture broth, with an overall intensity of 250 W/m^2.

In this investigation, fermented sewage was used as a medium for the growth of PNSB. The sewage was fermented in an anaerobic fermenter with the operational conditions as follows: organic loading rate (OLR) of 2.7 kg $COD/m^3.d$; hydraulic retention time (HRT) of 4 h; pH of 5.7 ± 0.3; and temperature of 30 ± 5 °C. The suspended solids from the fermenter effluent were separated using the lamella settler with hydraulic loading rate and HRT as 0.94 $m^3/m^2.h$ and 1.0 h, respectively. The pH of lamella settler effluent was adjusted to 7.0 using Na_2CO_3 in a neutralisation tank (Fig. 1). The required quantity of neutralised fermented effluent was admitted in the raceway reactor for the growth of PNSB and performance was monitored under two scenarios. In the first scenario, neutralised effluent of anaerobic fermenter with additional synthetic media (with composition as shown in Section 2.1) was used; whereas, in the second scenario, only fermented sewage was used as a medium for the growth of PNSB. These two scenarios will be further referred to as fermented sewage with synthetic media and fermented sewage without synthetic media, respectively. The initial pH was adjusted to 7.0 ± 0.3 (in both cases) before adding the inoculum.

In both scenarios, a 10% *v/v* inoculum concentration was employed. This resulted in an initial biomass concentration ranging approximately from 0.02 to 0.05 g of VSS per litre inside the raceway reactor, across all working volumes of 500 L, 750 L, and 1000 L. These volumes corresponded to specific S/V ratios of 10, 6.67, and 5 m^2/m^3, respectively. The reactor was operated for five days when using fermented sewage with synthetic media and for three days when using fermented sewage without synthetic media, maintaining a temperature of 25 ± 5 °C. A photoperiod of 12 h of light and 12 h of dark was provided. The culture broth was stirred every 2 h using paddle mixer, from which 100 mL of homogeneous bacterial cell suspension was taken every 6 h for analysis. Every experiment was run in batch mode for 120 h and repeated twice to ensure reproducibility and data accuracy.

2.3. *Analysis of cell growth and macromolecular compositions*

To monitor the growth of cells, 100 mL of culture broth was collected (every 6 h), and the absorbance was measured at 660 nm in a spectrophotometer (Agilent, Cary 60 UV–VIS, USA). The samples were centrifuged (10,000 rpm; 20 min), washed with phosphate-buffered saline water to remove any residual substrate, dried in an oven, and then a calibration curve (biomass concentration vs. absorbance) was plotted as given by Eq. (1) (Cerruti et al., 2020). The bacterial productivity and specific growth rate (μ) were estimated using Eq. (2) and Eq. (3), respectively.

$$Biomassconcentration(gVSS/L) = 0.4415 \times OD_{660} \tag{1}$$

$$Productivity(gVSS/L.day) = \frac{X_2 - X_1}{t_2 - t_1} \tag{2}$$

$$\mu\left(h^{-1}\right) = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \tag{3}$$

where, X_2 and X_1 are the biomass concentrations at any time t_2 (in h) and t_1 (in h), respectively.

The biomass yield was estimated using the procedure described earlier in the literature (Alloul et al., 2019). The macromolecular compositions, including lipids, carbohydrates, and proteins, were estimated using previously described methods (Mehta and Chakraborty, 2021).

Fig. 1. Set-up of raceway-pond coupled with high-rate anaerobic digester: (a) 1-Feed tank; 2-High-rate anaerobic fermenter; 3-Lamella settler; 4-Neutralisation tank; 5-Raceway pond; (b) 6- Incandescent infrared bulb; 7-Growth of PNSB in raceway pond.

Briefly, for the lipid content of the bacteria, a sample consisting of 50 mg of dried bacterial biomass was mixed with a 2 mL solution composed of n-hexane and isopropanol in a ratio of 3:2 (*v/v*). The extraction process was performed using a rotary shaker at 140 rpm for 7 h. The biomass residue was again mixed with a 2 mL solution of n-Hexane:isopropanol (2:3, *v/v*). The resulting mixture was subjected to extraction and subsequently centrifuged. The supernatant obtained from the centrifugation process was collected after being centrifuged for 5 min at 10,000 rpm at 25 °C. The supernatants from both extraction operations were collected and mixed with an equal volume of water:5N NaCl (9:1) solution. The resulting mixture was then subjected to vortex for 10 min. A vacuum concentrator (Model: CVE-3100, EYELA, Japan) was employed to facilitate the separation and dehydration of the upper phase, which included the lipid dissolved in hexane. Subsequently, the total lipid content was quantified using gravimetric methods.

The Phenol Sulphuric Acid method (Dubois et al., 1951) was used to quantify the carbohydrates. Specifically, 15 mg of dried bacterial biomass was added to 5 mL of distilled water. Subsequently, 2–3 drops of concentrated H_2SO_4 (98%) were added. The solution was heated at 100 °C in a water bath for 30 min. After the mixture was cooled, a gradual addition of 5 mL of concentrated H_2SO_4 was allowed, followed by adding 1 mL of a 5% phenol solution. Subsequently, the mixture remained undisturbed at ambient temperature for 30 min before the commencement of absorbance measurement. The calibration curve was prepared using glucose.

The modified Lowry's method was employed to quantify the total proteins. Specifically, 5 mg of dried bacterial biomass was added in 0.5 mL of 1 N NaOH and subjected to a temperature of 100 °C for 10 min. Following the cooling process, the combination was subsequently combined with a 2.5 mL aliquot of solution C, which consisted of 50 mL of solution A and 2 mL of solution B. The resulting mixture was then vortexed and left undisturbed for 10 min. Subsequently, a volume of 0.5 mL of Folin's reagent with a concentration of 1 N was introduced. Before the absorbance measurement, the solution was subjected to vortex and incubated for 15 min. Solution A was prepared using 5% *w/v* Na_2CO_3, whereas solution B was prepared using 0.5% *w/v* $CuSO_4{\cdot}5H_2O$ in a 1% Na-K tartrate solution. The protein concentration (mg/mL) and its content (*% w/w*) was estimated using Eq. (4) and Eq. (5), respectively.

$$Protein concentration(mg/mL) = 0.666 \times OD_{750} \tag{4}$$

$$Protein content(\%w/w) = \frac{Protein concentration(mg/mL)}{Sample concentration(mg/mL)} \times 100 \tag{5}$$

2.4. Analysis of volatile fatty acids

The VFAs were measured using High-performance Liquid Chromatography (HPLC) (Shimadzu, UFLC, Japan). For this, the samples (10 mL) were centrifuged (10000 rpm, 10 min), and the supernatant was filtered through a 0.22 μm filter. The HPLC analysis was performed using a 300 × 7 mm Agilent Hi-Plex H column with a column temperature of 60 °C. The samples were injected with a volume of 10 μL at a flow rate of 0.7 mL/min. The mobile phase solution used 0.005 mM H_2SO_4 in milli-Q water with isocratic flow.

2.5. Characterisation of wastewater

The preliminary characterisation of sewage was conducted prior to its introduction into the anaerobic fermenter. The samples were analysed for COD, NH_4^+-N, PO_4^{3-}-P, VSS, and VFAs using the Standard Methods (APHA, 2005). Ion chromatography (Metrohm Eco IC 930, Switzerland) was used to measure nitrate (NO_3^-) concentrations. The influent sewage characteristics were as follows: total chemical oxygen demand (COD_T) of 492 ± 70 mg/L; soluble chemical oxygen demand (COD_S) of 368 ± 41 mg/L; pH of 7.14 ± 0.01; alkalinity of 251 ± 32 mg/L of $CaCO_3$; VFAs as 27 ± 11 mg/L; NH_4^+-N as 31 ± 5 mg/L; nitrate-nitrogen (NO_3^--N) as 0.20 ± 0.01 mg/L; PO_4^{3-}-P as 5.0 ± 0.8 mg/L; total suspended solids (TSS) of 122 ± 16 mg/L; and VSS of 88 ± 9 mg/L.

2.6. Statistical analysis

Two-way ANOVA was performed by considering three levels in S/V ratio and two levels in substrate as two factors. Total three different Two-way ANOVA tests were performed for the cellular compositions of protein, lipid, and carbohydrate contents. Further, if the significant differences were found, then Tukey's test was used to assess the multiple comparisons among the factors. The statistical analysis was performed using Microsoft Excel 2019.

3. Results and discussion

3.1. Microbial enrichment composition

Purple non-sulfur bacteria were continuously enriched and acclimatised during 24 days of incubation under incandescent infrared light, and with the progress of time, there has been a change in biomass colour in the serum bottle (See Supplementary Material). The OD_{660} of the cell culture increased slowly from 0.05 to 0.43 during 24 days, reached the stationary phase on the 20th day, and stayed there for the rest of the incubation period (See Supplementary Material). The scanning of the culture collected on day 24 showed light absorption peaks (i.e., bacteriochlorophyll) at 806 and 866 nm (See Supplementary Material), confirming the enrichment of phototrophic bacteria (Hülsen et al., 2014; Alloul et al., 2019). Illumina-based metagenomics (v3 − v4) sequencing, followed by Miseq-300 bp PE generation of 0.1 million reads, examined the top 10 genera in the microbial community, as shown in Fig. 2a. Purple phototrophic bacteria (24.7%), *Sphingobacterium* (17.0%), *Muribaculaceae* (16.6%), *Pseudomonas* (10.8%), *Desulphovibrio* (7.3%), *Butyrivibrio* (5.8%), *Arcticibacterium* (5.7%), *Paenibacillus* (4.1%), *Flavobacterium* (4.1%), and *Bacteroides* (3.8%) were dominated in the consortium. Purple phototrophic bacteria are predominantly composed of genera such as *Bradyrhizobium, Magnetospirillium*, *Rhodobacter*, *Methylobacterium*, etc. The categorisation of these purple phototrophic bacteria at the family, genus, and species levels is illustrated using Krona plot (Fig. 2). Operational taxonomic unit (OTU) based analysis revealed that the consortium is primarily composed of *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria.* Among these, *Alphaproteobacteria* are particularly dominated, encompassing bacterial families such as *Bradyrhizobiaceae*, *Rhodobacteraceae*, *Rhodospirillaceae,* which are mainly associated with purple phototrophic bacteria (Fig. 2 and See Supplementary Material). Notably, *Bradyrhizobiaceae* is accounted nearly 22 % of the bacterial consortium, which is capable of storing pigments such as bacteriochlorophyll a, carotenoids and as well known for nitrogen-fixing bacteria (Delamare-Deboutteville et al., 2019) (See Supplementary Material). Moreover, the PNSB (*Rhodobacteraceae*, *Rhodospirillaceae*), which perform photosynthesis in anaerobic environments, were also observed (Fig. 2b and c).

The *Rhodobiaceae* family of *Alphaproteobacteria* is primarily composed of PNSB, including species such as *Rhodobacter sphaeroides* (Fig. 2b), *Rhodobacteraceae* bacterium SH-1, *Rhodobaca barguzinensis*, *Parvibaculum lavamentivorans*, *Rhodobiaceae* bacterium, and *Rhodobacter* sp. CZR27. In contrast, the *Rhodospirillaceae* family within *Alphaproteobacteria* mainly features PNSB like *Magnetospirillum magneticum* (Fig. 2c), *Magnetospira* sp. QH-2, and *Hypericibacter terrae*. The *Rhodobacteraceae* family is characterised by PNSB such as *Rhodovulum sulfidophilum* and *Rhodobaca barguzinensis.* Unlike Alphaproteobacteria, the Gammaproteobacteria phylum, which includes *Chromatiaceae* (See Supplementary Material) and *Ectothiorhodospiraceae* families (See Supplementary Material), is dominated by PSB. The *Chromatiaceae* family contains purple sulfur bacteria like *Nitrosococcus halophilus* and *Nitrosococcus watsonii*, while *Ectothiorhodospiraceae* family features *Halorhodospira halophile*, *Halorhodospira halochloris*, and various *Ectothiorhodospira* species.

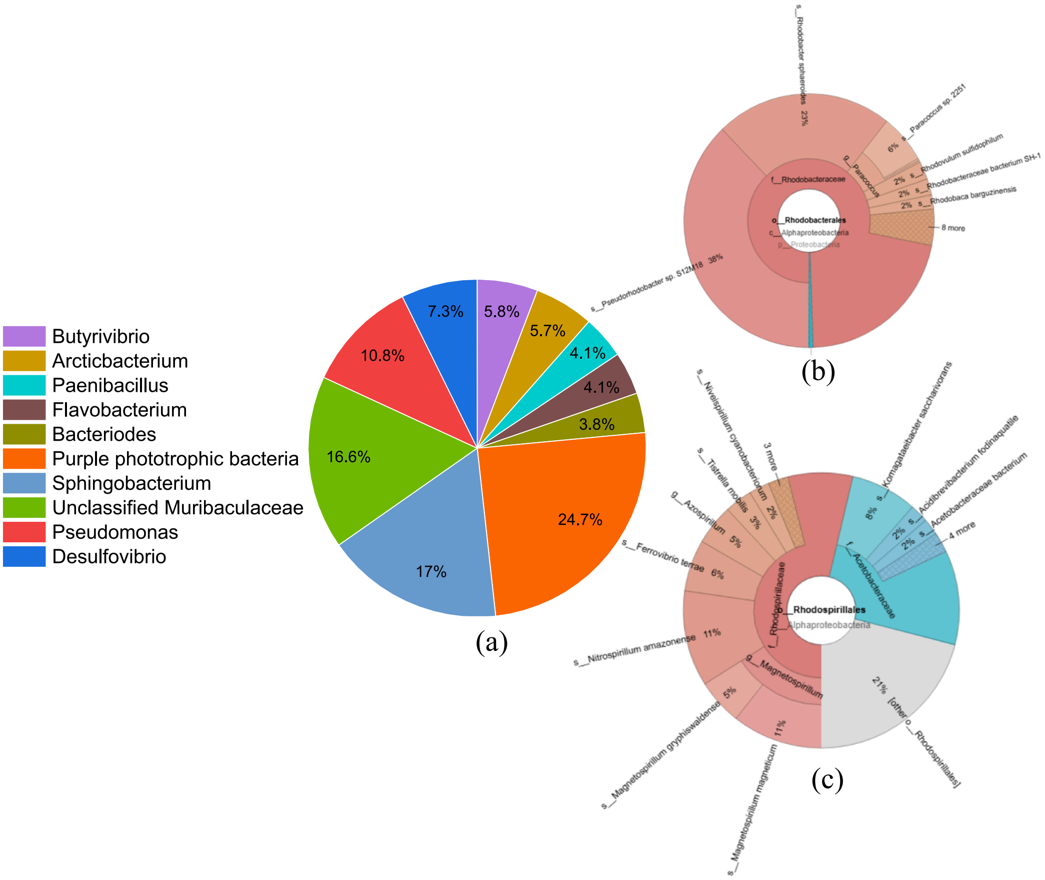

Fig. 2. Microbial population dynamics of the enriched consortium: (a) pie chart illustration of the dominated genera; illustration of (b) *Rhodobacteraceae* and (c) *Rhodospirillaceae* families of PNSB using Krona plots.

Additionally, the inoculum is rich in nitrogen-fixing and plant growth promoting bacteria (e.g., *Methylobacterium* spp., *Nitrospirillum amazonense*, *Azospirillum* spp., *Niveispirillum cyanobacteriorum*). *Desulphovibrio*, *Flavobacterium*, and *Butyrivibrio* were also prevalent in the consortium, responsible for hydrogen sulphide generation, hydrocarbon degradation, and butyric acid production. The presence of nitrifying bacteria in symbiotic relationships with PNSB may enhance the protein content in biomass, making it suitable for use as SCP and biofertiliser. The development of this consortium was most likely due to the stimulation of a simultaneous light, and the dominance of *Alphaproteobacteria*. Purple bacteria consortia have numerous benefits over individual species, including the capacity to convert solar energy into chemical energy, the ability to grow on VFAs, high tolerance to varying growth conditions, nitrogen fixation, and the ability to break complex polymers (Laurinavichene et al., 2014; Lawrence et al., 2023). Previously, for the PNSB consortium higher protein content in their cell wall was reported than the heterotrophic consortium (Laurinavichene et al., 2014).

3.2. Growth of purple non-sulfur bacteria using fermented sewage with synthetic media

The cultivation of photoheterotrophic PNSB involves the utilisation of organic carbon in the presence of infrared light. Therefore, the cultivation of PNSB was performed in a raceway reactor with working volumes of 500 L, 750 L, and 1000 L at their respective S/V ratios of 10, 6.67, and 5 m^2/m^3, respectively, to study the transformation of carbon and nutrients available in the fermented sewage with media while keeping all other parameters constant. The initial concentration (i.e., $t = 0$) of COD, NH_4^+-N, and PO_4^{3-}-P was 1555 ± 14 mg/L, 84 ± 0.4 mg/L, 12.2 ± 0.1 mg/L, respectively, in the culture medium. Fig. 3 shows the variations in biomass concentrations, the biomass yield, and the extracellular concentrations of acetate and propionic acid within the raceway reactor. When the cultivation was performed with a working volume of 500 L (10 m^2/m^3), the cells exhibited slower growth for first 24 h, and further progressed exponentially (Fig. 3a) with a specific growth rate of 1.99 day^{-1}. Similar trends were observed with working volumes of 750 L (6.67 m^2/m^3) and 1000 L (5 m^2/m^3) in the raceway reactor, with specific growth rates of 1.78 day^{-1} and 1.49 day^{-1}, respectively (Fig. 3a). Alloul et al. (2021) reported similar growth rate for *R. capsulatus* with a specific growth rate of 1.75 day^{-1}. For working volumes of 500 L, 750 L, and 1000 L, maximum biomass concentrations of 0.90, 0.85, and 0.81 g VSS/L, respectively, was observed (Fig. 3a), with biomass productivity of 0.17, 0.16, and 0.15 g VSS/L.day, and biomass yields of 0.95, 0.91, and 0.88 g of $COD_{biomass}$/g of $COD_{removed}$, respectively (Fig. 3b). The biomass concentrations ranging from 0.94 to 1.41 g VSS/L were achieved by utilising mixed consortia of PNSB (Wu et al., 2021), which corroborated our results.

The biomass concentration showed a slight decline as the culture volume in the raceway reactor increased from 500 L to 750 L to 1000 L. This was due to the significant impact of various parameters such as light intensity, reactor size, and concentrations of carbon and nutrients on the growth of PNSB (Wada et al., 2023). The phenomenon of light attenuation in phototrophs occurs due to the gradual rise in biomass concentration, resulting in diminished efficiency of light energy distribution across the entire culture volume (Wada et al., 2023; Sali and Mackey, 2021).

Similarly, a study conducted by Capson-Tojo et al. (2022), explored light attenuation in enriched PNSB cultures, highlighting the substantial influence of factors like biomass concentration, biomass pigment concentration, and reactor configuration on attenuation. The study further

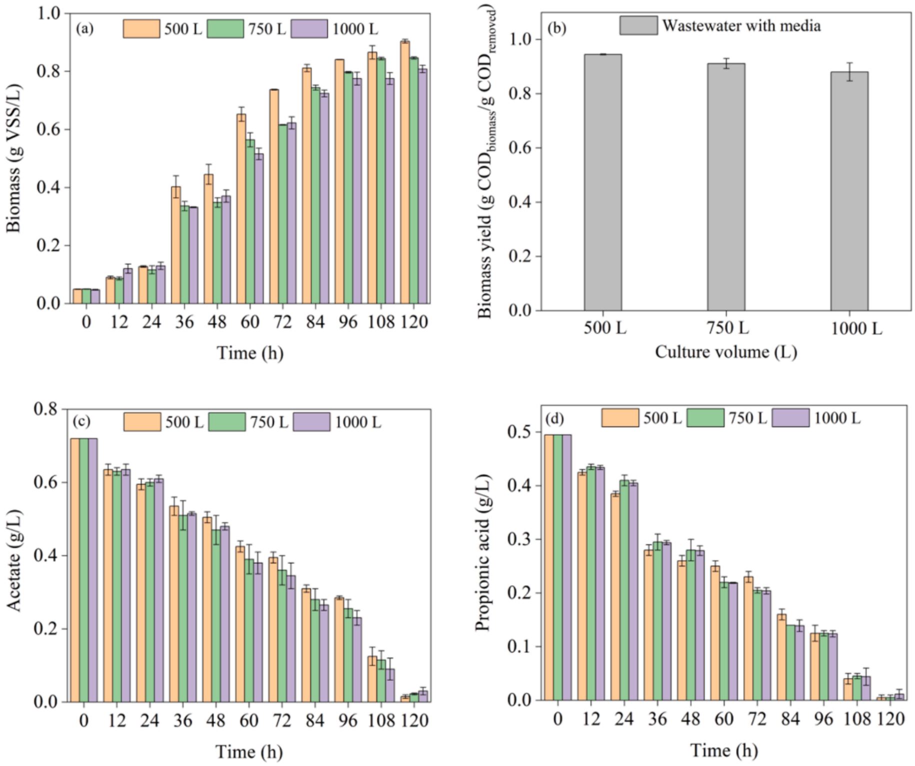

Fig. 3. Growth of PNSB in raceway reactor fed with fermented sewage and synthetic media demonstrating specific growth rates of 1.99 day^{-1}, 1.78 day^{-1}, and 1.49 day^{-1} for volume of 500, 750, and 1000 L, respectively. Temporal variations include: (a) biomass concentration; (b) biomass yield; (c) acetate in culture; (d) propionic acid in culture. All data are presented as Average ± SD=2.

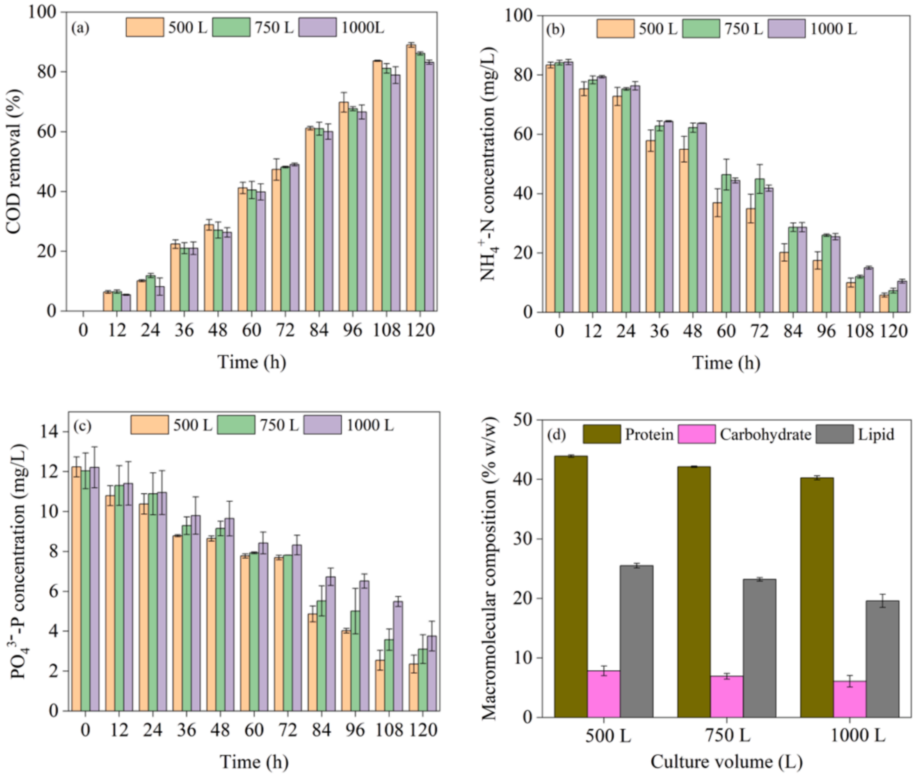

Fig. 4. Growth of PNSB in raceway reactor with fermented sewage with synthetic media: temporal variations include (a) chemical oxygen demand in culture, (b) ammonium nitrogen concentration in culture, (c) orthophosphate concentration in culture; (d) macromolecular composition (*% w/w*) with culture volume. All data are presented as Average ± SD=2.

indicated that effective light penetration in dense cultures is limited to approximately 5 cm. Furthermore, the highest biomass concentration was attained when the S/V ratio reached its maximum value of 10 m^2/m^3, leading to improved light availability (Alloul et al., 2021). In present study, it was observed that the S/V ratios for culture volumes of 500 L, 750 L, and 1000 L corresponds to 10, 6.67, and 5 m^2/m^3, respectively. This increase in volume resulted in a decrease in the intensity of light reaching the cells, consequently leading to decline in the biomass concentrations.

Light-harvesting complexes absorb the photons, ultimately leading to the creation of a transmembrane proton gradient. This gradient is then employed by ATP synthase to facilitate the production of ATP (Monroy and Buitrón, 2020). The energy generated in the form of ATP is utilised by PNSB to assimilate organic carbon, such as acetate and propionic acid (Wada et al., 2023), which serve as electron donors and carbon for biomass synthesis. Consequently, reduced ATP dissipation enhances the incorporation of degradable COD and minimising carbon loss as carbon dioxide. This optimisation promotes the assimilation of macronutrients, particularly nitrogen and phosphorus (Hülsen et al., 2016). The concentrations of acetate (0.72 g/L) and propionic acid (0.495 g/L) decreased rapidly after cultivation initiation, reaching near depletion after 114 h (Fig. 3c, d). Specifically, at the end of the cultivation period (at 120 h), the COD removal was 89%, 86%, and 83% for culture volumes of 500 L, 750 L, and 1000 L, respectively, in the raceway reactor (Fig. 4a).

The transportation of VFAs, specifically acetate and propionic acid, from the culture medium to the bacterial cell membrane is facilitated by diffusion. This transportation mechanism involves using transport proteins, including uniporters and channel proteins (Kemavongse et al., 2007). Once transported, these VFAs are then transformed into acetyl-CoA within the bacteria. This acetyl-CoA is metabolised via two central metabolic pathways: glycolysis and the tricarboxylic acid (TCA) cycle (Petushkova et al., 2019).

The TCA cycle encompasses two distinct metabolic processes, namely catabolism and anabolism. This mechanism enables the production of reducing equivalents necessary for ATP synthesis during respiration by catalysing the complete oxidation of acetyl-CoA to CO_2. Concurrently, it provides metabolites and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) to various biosynthetic pathways (Petushkova et al., 2019). The synthesised biomass functions as an electron acceptor for PNSB, since it has the ability to assimilate NADPH and intermediates of the TCA cycle (Wu et al., 2022). Carbon dioxide produced during acetyl-CoA oxidation in the TCA cycle can be utilised in the pyruvate synthase reaction, pyruvate carboxylation, and autotrophic CO_2 fixation through the Calvin cycle (Petushkova et al., 2019; Hädicke et al., 2011).

Interestingly, the acetate and propionic acid concentrations declined in conjunction with utilising NH_4^+-N and PO_4^{3-}-P (Fig. 4b, c). The percentage of NH_4^+-N removal at S/V ratios of 10, 6.67, and 5 m^2/m^3 was found to be 93%, 91%, and 88%, respectively (Fig. 4b). Similarly, the corresponding percentages of PO_4^{3-}-P removal was determined to be 81%, 74%, and 69%, respectively (Fig. 4c). The consumption of nitrogen and phosphorus primarily occurs during the light period, resulting in a decrease in their concentrations. However, under dark conditions and in the absence of oxygen, these bacteria are capable of growing through fermentation or anaerobic respiration (Hädicke et al., 2011). These bacteria can store phosphorus as polyphosphate, whereas nitrogen is mostly utilised for synthesising cellular components (Hülsen et al., 2016).

The enzyme expressed by the gene ammonium transporter (amt) facilitates the cellular uptake of extracellular ammonium ions. The conversion of 2-oxoglutarate (2-OG), an intermediate of the TCA cycle, with ammonium results in glutamine production (Wu et al., 2022), a precursor for protein synthesis. For phosphorous conversion, it has been observed that PNSB possesses both high-affinity (Pst) and low-affinity (Pit) Pi transporters, which transport phosphate across the cell membrane of PNSB. The polyphosphate kinase and exopolyphosphatase genes were implicated in the biosynthesis and catabolism of polyphosphate, respectively. Additionally, polyphosphate can undergo degradation, producing phosphate that can be utilised for ATP synthesis through the action of V- and F-type ATPases (Wu et al., 2022). Consequently, the carbon and nutrients are transported through the bacterial cell membrane and subsequently assimilated intracellularly to synthesise biomass and metabolites.

3.3. Macromolecular compositions of purple non-sulfur bacteria using fermented sewage with synthetic media

The potential for successful SCP recovery is closely tied to the protein content present in the biomass. Microbial biomass, often considered a viable substitute for protein, generally has protein contents of > 20% of its dry biomass weight (Capson-Tojo et al., 2020). A protein content of 43.9% *w/w* was achieved in the present experiment by employing a culture volume of 500 L (10 m^2/m^3) in a raceway reactor, utilising fermented sewage with synthetic media (Fig. 4d). The protein contents showed a decreasing trend as S/V ratios decreased from 10 to 6.67 to 5 m^2/m^3. Correspondingly, the protein contents decreased from 43.9 ± 0.2% *w/w* to 42.1 ± 0.1% *w/w* to 40.3 ± 0.3 % *w/w* (Fig. 4d). Previous research reported protein contents of 60.1% *w/w* and 42 to 43.6% *w/w*, which are comparable while examining the utilisation of municipal wastewater and modified shrimp feed as feedstock (Saejung and Thammaratana, 2016; Chumpol et al., 2018).

In the present investigation, the protein contents of 40 to 43.9 % *w/w* were obtained, possibly due to the intracellular assimilation of nitrogen and organic carbons. The 2-oxoglutarate (2-OG) generated during the TCA cycle serves as intermediates for nitrogen assimilation in the form of ammonium ions, leading to the production of glutamine and asparagine, which are essential precursors for protein synthesis (Mehta and Chakraborty, 2021). Moreover, the intracellular acetyl-CoA serves as a precursor for synthesis of various metabolites, including carbohydrates, fatty acids, carotenoids, and vitamins (Mehta and Chakraborty, 2021). The carbohydrate contents of 6.1 ± 0.9% *w/w* to 7.8 ± 0.8% *w/w* were obtained in a raceway reactor (Fig. 4d). Carbohydrate metabolism in bacteria is facilitated through three distinct processes: the Embden-Meyerhof-Parnas system, commonly referred as glycolysis, the Entner-Doudoroff pathway, and the pentose phosphate pathway (Tang et al., 2011).

In addition to carbohydrates and proteins, a considerable proportion of the dry biomass consists of lipids. As the cultivation progresses towards the stationary phase, the decrease in essential nutrients like nitrogen (N) or phosphorus (P) leads to increased carbon availability (Carlozzi and Touloupakis, 2021). This increase stems from the limitation of carbon utilisation in cell growth and nitrogen metabolism. The hydrolytic release of carbon skeletons from protein and other cellular compounds further boosts the carbon availability, shifting in carbon flux towards the production of storage compounds, such as carbohydrates and lipids, while simultaneously reducing the protein content (Varshney et al., 2018; Carlozzi and Touloupakis, 2021). The lipid contents of 25.5 ± 0.4% *w/w*, 23.2 ± 0.3% *w/w*, and 19.6 ± 1.1% *w/w* were achieved at S/V ratios of 10, 6.67, and 5 m^2/m^3 in the raceway reactor (Fig. 4d). Purple non-sulfur bacteria have been documented to contain nutritionally beneficial lipid components, such as mono- and poly-unsaturated fatty acids and 5-aminolevulinic acid, potentially enhancing their feed value (Wada et al., 2023). These findings align with previous research by Wada et al. (2023), which has reported the recovery of lipid contents ranging from 17.0% *w/w* to 26.5% *w/w* using fuel-synthesis process water as the feedstock in a 65 L photobioreactor employing PNSB. Overall, these results suggest that fermented sewage with synthetic media holds promise as a viable substrate for resource recovery using PNSB.

3.4. Growth and macromolecular compositions of purple non-sulfur bacteria using only fermented sewage without synthetic media

The cultivation of PNSB with fermented sewage without media was performed at S/V ratios of 10, 6.67, and 5 m^2/m^3 with their corresponding working volumes of 500 L, 750 L, and 1000 L in the raceway reactor, keeping all other parameters constant. When the cultivation was performed at an S/V ratio of 10 m^2/m^3 in a raceway reactor, the cells had a logarithmic growth phase without any lag phase (Fig. 5a), with a specific growth rate of 0.68 day^{-1}. During this period, the PNSB utilised the organic carbon and other nutrients present in the fermented sewage. As a result, a maximum biomass concentration of 0.11 g VSS/L was achieved at 36 h (Fig. 5a). However, when the cultivation periods extended beyond 36 h, the depletion of VFA concentration (from 31.7 mg/L at 36 h to 4.5 mg/L at 72 h) within the raceway reactor led to cell death, ultimately reducing the biomass concentration to 0.03 g VSS/L at 72 h (Fig. 5a, b). Similar trends were observed with working volumes of 750 L and 1000 L in the raceway reactor with maximum biomass concentration of 0.10 and 0.09 g VSS/L at 36 h and specific growth rate of 0.65 day^{-1} and 0.64 day^{-1}, respectively (Fig. 5a). In contrast, the cultivation of PNSB using fermented sewage with synthetic media resulted in consistent increase in cell growth with biomass concentrations of 0.90, 0.85, and 0.81 g VSS/L for S/V ratios of 10, 6.67, and 5 m^2/m^3, respectively, at 120 h (Fig. 3a), owing to the presence of significant carbon and nutrients in the extracellular medium.

The PNSB microorganisms can uptake carbon (C), nitrogen (N), and phosphorus (P) from wastewater with a COD:N:P ratio of 100:7:2 (on mass basis) (Cerruti et al., 2020). The COD removal at S/V ratios of 10, 6.67, and 5 m^2/m^3 was 73%, 71%, and 69%, respectively (Fig. 5c), with biomass yields of 0.85, 0.84, 0.82 g of $COD_{biomass}$/g of $COD_{removed}$, respectively (See Supplementary Material). The COD removal at 36 h was 70 %, which was further enhanced to 73% at 72 h for a culture volume of 500 L (Fig. 5c). The gradual increase in COD removal seen after 36 h might have been attributed to the gradual reduction in extracellular concentrations of VFA beyond 36 h (Fig. 5b, c). The measurement of COD serves as a crucial indicator for assessing the effectiveness of wastewater treatment systems, where COD assimilation typically facilitates microbial biomass growth (Rashid et al., 2022). In an earlier study, the COD removal varied from 36 to 89% during the treatment of citrus juice wastewater using PNSB (Rashid et al., 2022).

The extracellular concentrations of nitrogen and phosphorous followed a similar trend as that of COD removal after 36 h (Fig. 5c, d, and Fig. 6a), which might have been attributed to the gradual decrement of the concentration of VFA (Fig. 5b). Nitrogen plays a crucial role in facilitating the growth of microorganisms and the utilisation of carbon (Wu et al., 2021). Purple non-sulfur bacteria can uptake nitrogen through the assimilation of ammonium ions. Ammonium ions are transported from the extracellular media into the bacterial cell membrane and assimilated directly, whereas nitrate is transferred from the extracellular medium into the bacterial cells and then converted into ammonium ions before assimilation (Zhu et al., 2019). Subsequently, the conversion of ammonium ions takes place intracellularly into amino acids. In the present investigation, the 73 % removal of NH_4^+-N was achieved using PNSB from fermented sewage with a culture volume of 500 L (Fig. 5d). As the S/V ratios decreased from 10 to 6.67 to 5 m^2/m^3, the removal of NH_4^+-N decreased from 73% to 65% to 61% (Fig. 5d). Earlier research reported the percentage removal of COD (96%), N (77%), and P (73%), respectively, during the cultivation of PNSB employing 1.5 L sequencing batch photo-bioreactor using synthetic media (Cerruti et al., 2020).

The removal of PO_4^{3-}-P by 72% was observed in a working volume of 500 L (10 m^2/m^3) in the raceway reactor, which decreased to 71% for 750 L (6.67 m^2/m^3) and 68 % for 1000 L (5 m^2/m^3) of culture volumes (Fig. 6a). The formation of precipitates, specifically hydroxyapatite and struvite, has been observed as a result of the presence of Ca^{+2} and Mg^{+2} ions in the extracellular medium with a pH of above 8.0 (Posadas et al., 2017). Based on the measured pH value (≤8.05) (See Supplementary Material) in the culture medium after cultivation, it can be inferred that

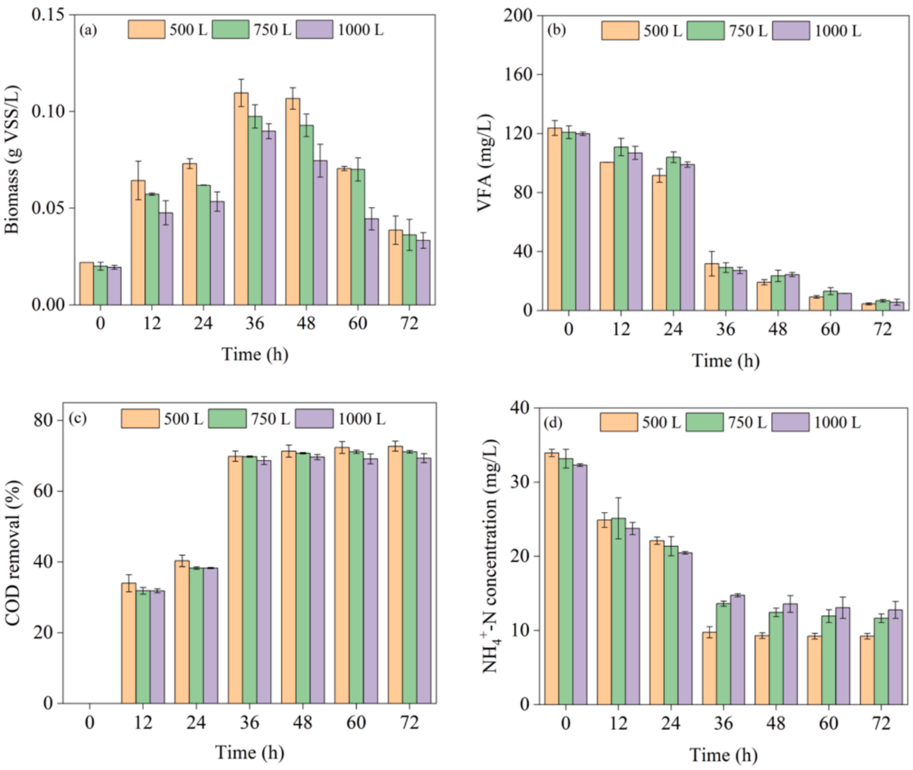

Fig. 5. Growth of PNSB in raceway reactor with fermented sewage without media: temporal variations include (a) biomass concentration, (b) volatile fatty acids in culture, (c) chemical oxygen demand removal, (d) ammonium nitrogen concentration in culture. All data are presented as Average ± SD=2.

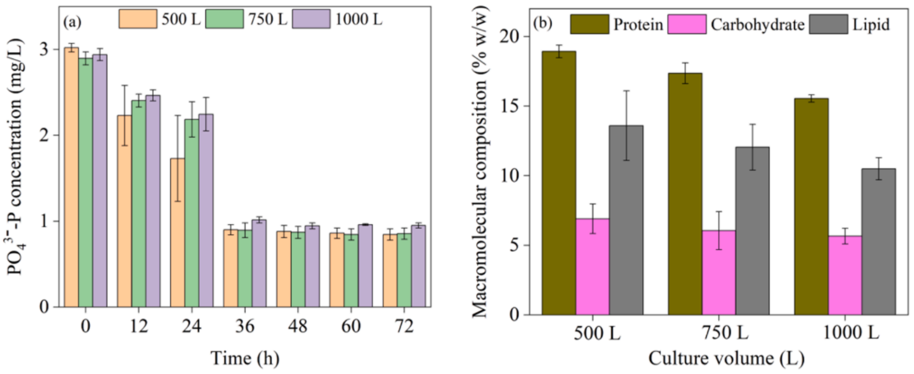

Fig. 6. Growth of PNSB in raceway reactor with fermented sewage without media: temporal variations include (a) orthophosphate concentration in culture; (b) macromolecular composition (% *w/w*) with culture volume. All data are presented as Average ± SD=2.

most of the PO_4^{3-}-P is taken up through metabolic uptake rather than being caused by precipitation. Phosphorus is transported across the bacterial cell membrane and subsequently undergoes photophosphorylation, a process that converts adenosine diphosphate (ADP) to ATP through the synthesis of organic compounds. Further, the synthesised ATP can be utilised for bacterial cell growth and other metabolic pathways (Wu et al., 2021).

The protein, lipid, and carbohydrate contents in dry biomass were found to be 18.9 ± 0.4% *w/w*, 13.6 ± 2.5% *w/w*, and 6.9 ± 1.1% *w/w*, respectively, when cultivating PNSB using fermented sewage without additional media with a working volume of 500 L (10 m^2/m^3) in a raceway reactor (Fig. 6b). Conversely, these percentages were measured at 43.9 ± 0.2%, 25.5 ± 0.4%, and 7.8 ± 0.8% for the fermented sewage with synthetic media (Fig. 4d). Although the carbohydrate contents of the both scenarios (fermented sewage with synthetic media and without media) are similar, the former exhibited a higher protein and lipid content compared to the latter, which may be attributed to the depletion of VFA from culture medium (Wu et al., 2021; Carlozzi and Touloupakis, 2021).

A two-way ANOVA analysis (See Supplementary Material) revealed significantly higher protein and lipid contents in samples with synthetic media compared to those fermented without synthetic media (p-value < 0.01), as well as a significantly higher protein and lipid content at an S/V ratio of 10 m^2/m^3 (p-value < 0.01 and p-value $= 0.04$, respectively). Conversely, no significant difference was detected in carbohydrate content across different S/V ratios (p-value $= 0.33$) or substrate variations (p-value $= 0.37$), indicating that carbohydrate content is not influenced by these factors. Additionally, no significant interaction effects between S/V ratio and substrate content were found in any of the three cellular compositions, suggesting that these factors do not jointly affect cellular concentrations, although the significant individual effects have been noted. Further, utilising fermented sewage without media eliminates the need for expenses related to media. While the recovery of biomass and metabolites from fermented sewage without media is lower, it can be improved by utilising fermented sludge that has high COD and nutrient contents.

3.5. Overview of feasibility and challenges for scaling up an outdoor raceway reactor for purple non-sulfur bacteria cultivation on fermented sewage

The batch study of fermented sewage augmented with synthetic media for additional carbon sources and necessary nutrients has revealed the feasibility of upscaling raceway reactors in real-time field-scale applications. The productivity of PNSB was greatly influenced by various parameters such as S/V ratios, light intensity, COD:N:P ratio, etc. The increase of S/V ratio from 5 to 10 m^2/m^3 is evident in enhancing biomass production, nutrient removal, and protein content. Furthermore, complementing with COD:N:P ratio through the synthetic medium, removal efficiencies could be enhanced from 72 to 89 % in the batch experiments.

The additional carbon source required could be sourced from the onsite sludge produced through sludge fermentation or by adding protein-rich waste such as the organic fraction of municipal solid waste, fruits and vegetable waste, kitchen waste, etc. Further research will be required for operating the raceway reactor in optimising the operational strategies, such as HRT and SRT, for different influent feedstocks and their validation by appropriate mechanistic models with sensitivity analysis for identifying the major influential parameters (Puyol et al., 2017). Additionally, raceway reactors offer promising solutions by minimising capital and operational expenditures and promoting the recovery of resources through natural illumination and adopting effective operational strategies that can reduce skilled labour requirements.

4. Conclusions

The PNSB consortia have proficiently assimilated organic components and nutrients from fermented sewage in the proposed two-stage configuration of an anaerobic fermenter, followed by recovery from an open raceway reactor on real Indian domestic sewage. The biomass yield was 0.82 to 0.85 g $COD_{biomass}$ /g $COD_{removed}$ for fermented sewage and increased to 0.88 to 0.95 g of $COD_{biomass}$/g of $COD_{removed}$ for fermented sewage with additional VFA as carbon source. These results on water quality, biomass yield and composition pave the way for upscaling two-stage anaerobic fermentation and phototrophic resource recovery strategy for outdoor cultivation of PNSB using domestic wastewater.

CRediT authorship contribution statement

Manikanta M. Doki: Writing – original draft, Visualization, Validation, Software, Investigation, Formal analysis, Conceptualization. **Arun Kumar Mehta:** Writing – original draft, Visualization, Validation, Software, Investigation, Formal analysis, Conceptualization. **Debkumar Chakraborty:** Writing – review & editing, Investigation, Formal analysis. **Makarand M. Ghangrekar:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Brajesh K. Dubey:** . **Abbas Alloul:** Conceptualization. **Ali Moradvandi:** Writing – review & editing, Conceptualization. **Siegfried E. Vlaeminck:** . **Ralph E.F. Lindeboom:** .

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgment

The authors duly acknowledge the financial support provided by the Department of Science and Technology, Ministry of Science and Technology, Government of India (File No. DST/IMRCD/India-EU/Water-call2/SARASWATI2.0/2018).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2024.131467.

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