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Evaluating bioproducts production in a purple phototrophic biofilm photobioreactor: Fuel-synthesis wastewater vs. simple substrates

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ABSTRACT

This study evaluated the influence of different carbon sources on purple non-sulphur bacteria (PNSB) biofilm formation, and the production of polyhydroxybutyrate (PHB) and other value-added bio-products. Higher PNSB growth and biofilm formation were observed in fuel-synthesis wastewater (FSW), followed by acetate. Both FSW and acetate cultures contained a large proportion of Rhizobiales, while sugar substrates led to yeast enrichment. Protein contents reached 40–41 % for acetate, which is suitable for single cell protein (SCP) use. The maximum PHB content from the suspended and biofilm growth was obtained from glucose (18.1 ± 0.10 %, 12.9 ± 1.11 %), followed by FSW (14.9 ± 0.25 %, 11.2 ± 0.50 %). FSW showed the highest overall productivity for both protein and PHB. Pigment concentrations were low for all substrates. The study underscores substrate influence on microbial community and bioproducts potential. It highlights FSW as a promising substrate for PNSB, suggesting its treatment integration for resource recovery.

1. Introduction

The global demand for sustainable materials has driven significant research and innovation in recovery of biomolecules and other resources from wastewater. This includes biopolymers such as poly-lactic acid, cellulose, and polyhydroxyalkanoates (PHAs) that can be used as bio-based and biodegradable plastics (Kovalcik et al., 2019), high-value biomolecules such as photopigments, and whole-cell applications such as single cell protein (SCP) and biofertilizers. Some of these recovery routes are briefly described.

Polyhydroxyalkanoates (PHAs) are biologically produced biocompatible polyesters with adequate strength, ultraviolet light stability, and excellent gas and water barrier properties, making them popular as biodegradable plastic alternatives (Westlie et al., 2022). Polyhydroxybutyrates (PHBs), a type of PHA, have applications in various fields, including medical packaging, nanotechnology, and agriculture (Sirohi et al., 2020). However, their high production costs have hindered commercialization, making them more expensive than petroleum-based polymers (Manikandan et al., 2021).

SCP is a protein-rich source that can replace conventional animal and human proteins and includes minerals, fats, carbohydrates, and vitamins. Its high areal productivity, low water usage, and reduced greenhouse gas emissions make it a promising solution for food security through use as aquaculture or livestock feed (Shaikh et al., 2023a).

Bacteriochlorophylls (BChls) and carotenoids (Crts) are pigments that convert light energy and serve as natural colorants and health enhancers in food, cosmetics, and pharmaceuticals. Their production from industrial wastewater demonstrates their potential for enhancing wastewater reuse.

Within the circular economy approach to wastewater treatment, a key goal is to maximize recovery through increasing conversion yields. In this respect, purple non-sulphur bacteria (PNSB) have received increasing attention due to their high substrate to biomass yields, which are typically close to 1 gCOD/gCOD (COD, chemical oxygen demand) – courtesy of their photoheterotrophic metabolism. This rare metabolic pathway and photosynthesis in the near infrared range also makes them easy to enrich in mixed cultures (Capson-Tojo et al., 2020). They are capable of producing various value-added bioproducts while treating

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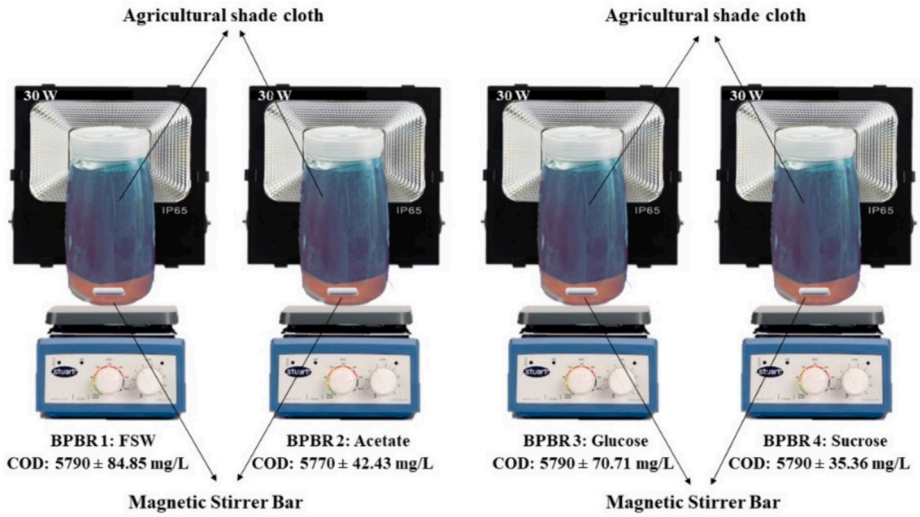

Fig. 1. Biofilm photobioreactor and experimental setup used in this study.

the wastewater, including those described previously (George et al., 2020).

PNSB can utilize various organic carbon sources for PHB production, including biodiesel waste, cheese whey, and various wastewaters (Sali and Mackey, 2021). However, most substrates require pre-treatment to break down complex molecules, adjust pH or remove inhibitory compounds for optimal PNSB growth and biomolecule production.

One industrial wastewater that could lend itself to PNSB-driven treatment and resource recovery is fuel synthesis wastewater (FSW), a by-product of synthetic fuel production. Synthetic fuels are of growing interest as a clean-burning traditional fuel that can be converted from biomass (Ruth and Stephanopoulos, 2023). Feedstocks are typically converted to syngas as an intermediary, which is then synthesized into hydrocarbons using the Fisher-Tropsch process. This process results in roughly one water molecule for every carbon chain elongation. The FSW produced is typically treated using aerobic oxidation, resulting in high energy consumption and significant CO_2 emissions (Surkatti et al., 2020). However, FSW is rich in volatile fatty acids (VFAs) and alcohols, transparent with minimal colloidal particles, and does not require pre-treatment, making it suitable for phototrophic cultures (Wada et al., 2022).

To assess its relative suitability with PNSB biotechnology, the study compares FSW to acetate, the most preferred VFA substrate by PNSB for PHB production (Wu et al., 2012), and two sugars - sucrose and glucose. The two sugars were selected as they both are commonly used carbon sources and have been well studied for PHB production (Jiang et al., 2016; Nascimento et al., 2016).

A major bottleneck for most resource recovery applications is the separation and harvesting of biomass and their biomolecules. For instance, the processing of PHB can account for up to 30 % of PHB production costs due to the difficulty in recovering PHB from non-PHB cell mass (Surendran et al., 2020). This includes biomass separation, pre-treatment, PHA recovery, separation, and purification (Rivero et al., 2019). Common methods like centrifugation, filtration, and sedimentation are energy-intensive or slow with high land requirements (Yu et al., 2020). Biofilm-based systems can reduce these costs by requiring little to no dewatering before downstream processing due to their high biomass concentration (Li et al., 2017). In addition, biofilm reactors provide protection to inhibitory compounds, which is particularly valuable in settings like industrial plants, where fluctuating flows and loads can prevail (Saini et al., 2023). Biofilms also offer the benefit of stability, avoiding biomass washout under such fluctuating conditions. PNSB based resource recovery using biofilms is promising, with Hülsen et al. demonstrating improved SCP quality using a biofilm-based PBR treating pre-settled red meat processing effluent (Hülsen et al., 2020a). However, generally the literature lacks studies focusing on bioresource recovery using biofilm-based PNSB systems.

Therefore, the current study has three aims: (a) assess how different carbon sources can influence PNSB biofilm formation and suspended growth in a biofilm photobioreactor (BPBR), (b) compare the suitability of FSW to other pure carbon sources on production of PHB and (c) evaluate other value added bio-products (Crts, BChls and SCP) from both the suspended and biofilm growth that could be co-produced or used as an alternative product based on fluctuating market demand. To achieve these study objectives, four BPBRs with different carbon sources, including FSW, sodium acetate, D-glucose, and sucrose were used.

2. Materials and methods

2.1. PNSB growth media

A mixed culture enriched with PNSB was used as inoculum which was previously grown on FSW. This culture was then grown on the four different carbon substrates: FSW, sodium acetate, D-glucose, and sucrose. All carbon sources were made to a strength of 5790 mg/L of COD, to match that of the FSW soluble COD (5790 ± 84.5 mg/L) received from a gas-to-liquids plant utilizing the Fischer Tropsch process in Qatar. The other characteristics of FSW, as previously reported in our earlier study (Shaikh et al., 2023a), are presented in Table A1 in the supplementary data of this manuscript. In addition to the carbon source, the growth media contained various nutritional supplements such as KH_2PO_4 (3.03 g/L), $NaHCO_3$ (4.29 g/L), ATCC trace minerals (10 mL/L), and ATCC vitamins supplement (10 mL/L). In this investigation, no nitrogen source was added to the growth media of all carbon sources because our earlier studies demonstrated the advantages of nitrogen-deficiency in promoting PNSB biofilm formation (Shaikh et al., 2023b). The experiment duration was 15 days.

2.2. Biofilm photobioreactors and their operational conditions

Four laboratory-scale BPBRs were operated in batch mode in this study. Each BPBR comprised a glass vessel having a wide mouth with a 2 L volume. Agricultural shade cloth was used as a biofilm support based on previous screening and performance testing (Shaikh et al., 2023b). The shade cloth used was 30.5 cm × 15.2 cm with mesh opening size of approximately 600–800 μm and mesh diameter of approximate 220–240 μm (Figure A1, Supplementary Information). Each BPBR was kept at room temperature, subjected to 300 rpm stirring, and exposed to

an average irradiance of 100 W/m^2 measured at the surface of the bottles. The pH of all BPBRs was adjusted to 7.5 before starting the experiment. The BPBRs were each illuminated by a single 30 W white floodlight placed at a distance of 15 cm away from each BPBR. All BPBRs were nitrogen flushed for 90 s to assure anaerobic conditions were achieved. Fig. 1 illustrates the BPBR and experimental setup used in this study.

2.3. Analytical methods

During the experiment, the absorbance at 420 nm was measured to assess PNSB growth in the suspended biomass using a UV-3600 plus spectrophotometer (Shimadzu, Japan). The wavelength of 420 nm was chosen given that adsorption/scattering is more responsive at this wavelength in PNSB than in relation to other bacteria (Myers et al., 2013). At the end of the experiment, both the suspended and biofilm fractions of the sludge culture were analysed gravimetrically for total suspended solids (TSS) and volatile suspended solids (VSS) according to established protocols (APHA, 2012). For attached biomass quantification, a volume of distilled water sufficient to wash off all the biofilm was used and recorded for each specific reactor. To ensure representative sampling for TSS and VSS measurements, the collected biofilm was homogenized thoroughly in the wash water, ensuring that the biofilm fragments were evenly distributed before sampling. The volume of water used to wash off the biofilm was then multiplied by the measured values of TSS and VSS to obtain the final mass values.

Biofilm thickness was evaluated qualitatively through visual inspection. Photographs of the biofilm on the shade cloth were taken at the end of the experiment, and the relative thickness was assessed based on the visual appearance of the biofilm layers. To facilitate a comprehensive comparison between suspended and biofilm growth, all TSS and VSS values are reported in terms of mass (mg) instead of concentration (mg/L). The pH was measured using a pH meter (Orion Star, Thermo Scientific, USA). Samples of effluent wastewater were centrifuged at 23,400*g* for 10 min in a centrifuge (Sorvall LYNX 6000, Thermo Scientific, USA) to extract the supernatant for COD determination. The supernatant was then filtered using 0.2 µm polyethersulfone syringe filters (Nalgene, Thermo Fisher, USA). The USEPA reactor digestion method (method 8000) and high range COD vials from Hach (USA) were used to measure the COD (mg/L) (HACH, 2021). At the end of the experiment, the extracellular polymeric substance proteins and polysaccharides (EPS-PN and EPS-PS) (mg/g), hydrophobicity (%), Crts (µg/g), BChls (µg/g), SCP (%), and PHB (%) of the suspended and biofilm growth were extracted and analysed. A formaldehyde and heating extraction protocol was used for the extraction of EPS (Liu and Fang, 2002). Samples were treated with formaldehyde, heated for 10 min at 80 °C, centrifuged, and filtered to obtain the EPS extract (Shaikh et al., 2023a). This extract was then analysed for EPS-PS using the phenol-sulphuric colorimetric method using glucose as standard (Dubois et al., 1956), and EPS-PN using the Lowry protein assay method using bovine serum albumin as standard (Lowry et al., 1994).

The Crts were measured using acetone as the solvent, following the method described by Chumpol et al. (2018). A 10 mL sample was centrifuged, the supernatant removed, and acetone added. The mixture was sonicated to break the cells, then centrifuged again. The absorbance of the supernatant at 480 nm was used to quantify the total carotenoids using the formula described in a previous study (Wang et al., 2017).

For BChls, samples were centrifuged, the supernatant was discarded, and the pellets were rinsed with distilled water. After repeating this process two more times, BChls were extracted in an acetone/methanol (7:2 *v*/v) in the dark at room temperature for 1 h. The amount of BChls was calculated using the formula described in a previous study (Wang et al., 2017).

Cellular protein was extracted using a modified alkaline extraction method from Perović et al. (2020). A 15 mg lyophilized sample was agitated with NaOH, sonicated, and digested. After cooling and centrifugation, the supernatant's pH was adjusted first to 3 with HCl, then to 12 with NaOH, and centrifuged again. The protein content was determined using a Lowry protein assay method using bovine serum albumin as standard (Lowry et al., 1994).

PHB was extracted using the sodium hypochlorite dispersion method, followed by washes with distilled water, acetone, and methanol. A 3 mL suspended culture was centrifuged, and the cell pellets were treated with sodium hypochlorite, vortexed, and incubated at 40 °C for 2 h to digest cell components except PHB. After centrifugation, the crude PHB was washed and dissolved in hot chloroform. The PHB-chloroform mixture was heated to evaporate the chloroform, followed by conversion of PHB to crotonic acid with sulphuric acid. The absorbance was measured at 235 nm using sulphuric acid as a blank. Each analysis was conducted in two analytical repetitions.

2.4. DNA extraction, 16S rRNA gene amplicon sequencing and data processing

To understand the microbial community composition in biofilm photobioreactors, samples of suspended and biofilm growth were harvested at the end of the experiment for processing. The DNeasy PowerBiofilm DNA isolation kit (MoBio Laboratories Inc., USA) was used according to the manufacturer's instructions to extract DNA from the PNSB biomass. Understanding the microbial community composition is crucial for optimizing bioprocesses such as COD removal and PHB production in biofilm photobioreactors. Similar methodologies have been effectively used in a range of other studies to analyse microbial dynamics in various environmental and engineered systems (Nocker et al., 2007; Quast et al., 2009; Shade et al., 2013). Amplification, quantitation, and sequencing of hypervariable regions of the 16S rRNA gene were performed using ReadyMade™ 16S rRNA primers as described in Shomar et al. (2020) using the IonS5 platform for high-throughput sequencing. The use of the IonS5 platform for sequencing allows for high-throughput and accurate sequencing of the 16S rRNA gene, providing comprehensive insights into the microbial community composition (Bolyen et al., 2019). The forward and reverse primer sequences were: 16S rRNA For (AGA GTT TGA TCC TGG CTC AG) and 16S rRNA Rev. (ACG GCT ACC TTG TTA CGA CTT) (Integrated DNA Technologies, Coralville, Iowa).

The single-end sequence data obtained from the IonS5 platform was imported into the QIIME 2 (version 2021.4) pipeline (Bolyen et al., 2019). The analysis is as described in George et al. (2020) and reads were analysed using the SILVA database. By utilizing the SILVA database for taxonomic classification, the study ensures reliable identification and characterization of the microbial taxa present in the samples (Kim et al., 2019).

2.5. Statistical analysis

Analysis of variance (ANOVA) was employed to determine if there were statistically significant differences among the means of different treatment groups. The null hypothesis for the ANOVA test posits that there are no differences among group means, and the *p*-value indicates the probability of observing the data assuming the null hypothesis is true (McDonald, 2014). A p-value <0.05 was considered statistically significant, leading to the rejection of the null hypothesis. Following a significant ANOVA result, all pairwise comparisons were made using the Bonferroni correction method, which is a conservative approach to control for Type I error. The Bonferroni post-hoc test adjusts the significance level by the number of comparisons, maintaining the overall significance level at 5 % (Armstrong, 2014). Significant differences identified between individual sample groups by the Bonferroni post-hoc test ($p < 0.05$) are reported. The statistical analysis was conducted using the Statistix 10 software package.

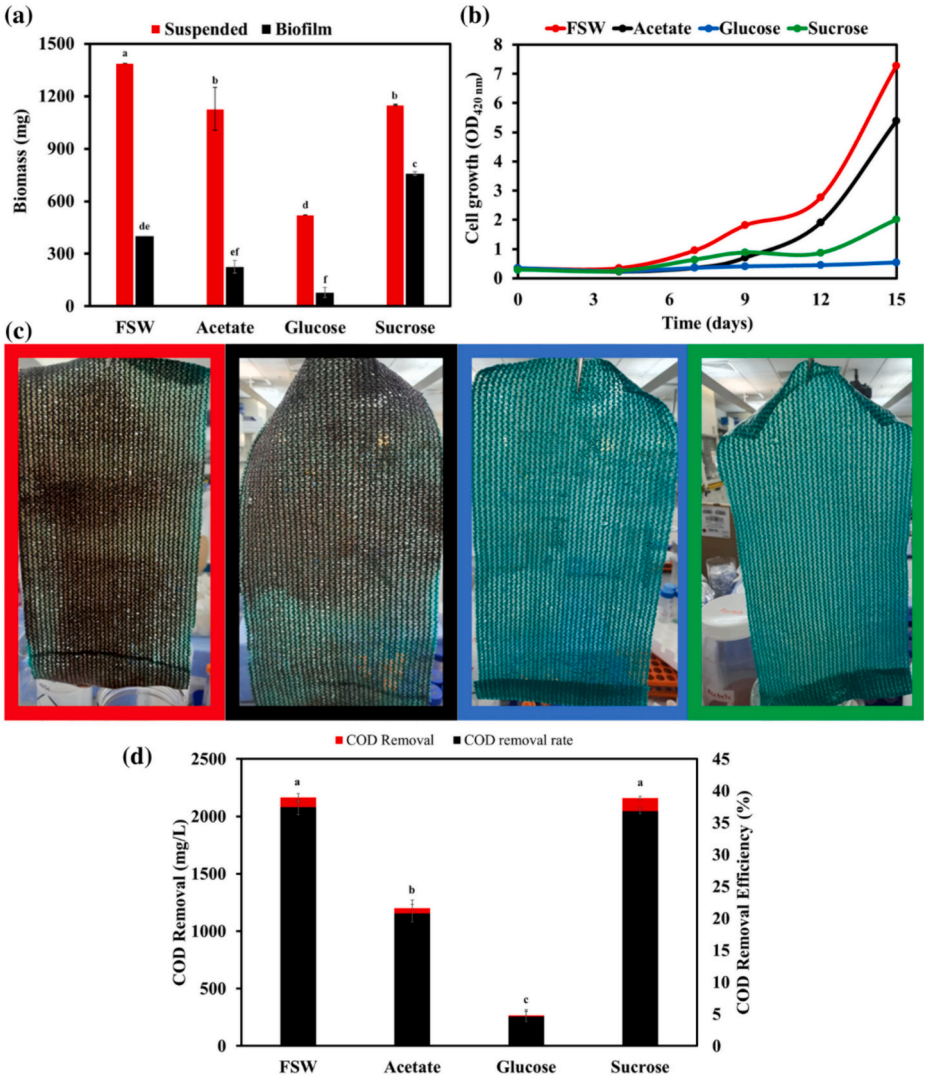

Fig. 2. (a) Final biomass production by VSS determination (b) Suspended cell growth with time by absorbance (c) Biofilm formation in FSW (red border), acetate (black border), glucose (blue border), and sucrose (green border) (d) COD removal and COD removal efficiency. Different alphabetical letters represent significant differences from a Bonferroni post-hoc test conducted following a significant ANOVA test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results and discussion

Four biofilm photobioreactors with different carbon sources (FSW, acetate, glucose, and sucrose) were tested in the batch experiments. This study has been conducted to assess PNSB biofilm formation, PHB production, and other value-added bio-products on different carbon sources, as well as confirming the suitability of this process for FSW treatment.

3.1. Biomass development

The VSS measurement (biomass) results showed that the highest biomass production from suspended growth of 1388.8 ± 0 mg was observed in the FSW condition, followed by sucrose and acetate (almost equal). The glucose condition had the lowest VSS at 520.8 ± 0 mg. No significant difference was observed between sucrose and acetate ($p = 1.0$) while other pairwise comparisons were significant ($p < 0.05$). For biofilm, the highest biomass production was observed in the sucrose substrate at 758.3 ± 11.7 mg condition, followed by FSW, acetate, and glucose. No significant difference ($p = 1.0$) existed between FSW and acetate, and acetate and glucose (Fig. 2a). These results show that, glucose is the least favourable carbon substrate for PNSB growth in both suspension and biofilm. A significant difference was observed ($p < 0.001$) between suspended and biofilm growth of the same carbon substrate, where higher biomass occurred in suspension.

The highest suspended cell growth, measured by absorbance (OD_{420nm}), was observed in FSW (7.28 ± 0.0) followed by acetate (5.40 ± 0.0), sucrose (2.02 ± 0.0), and glucose (0.54 ± 0.0) (Fig. 2b). Values of absorbance were not consistent with VSS and most likely relate to the bias of absorbance to purple phototrophic bacteria (Myers et al., 2013). This is consistent with a purple culture in FSW and acetate conditions and a white yeast in the sucrose and glucose reactors. The culture showed a noticeable lag phase, which is consistent with previous reports of nitrogen-deficient cultures which were used to promote biofilm formation (Shaikh et al., 2023a).

Visual observations of the shade cloth indicated that the most PNSB biofilm (purple in colour) was grown in FSW, followed by acetate. However, when grown in glucose or sucrose, there was white biofilm formation, which was minimal in the glucose condition but consistent and providing full coverage across the sucrose shade cloth. This is indirectly observable in Fig. 2c by the reduced transparency of the sucrose shade cloth compared to other conditions. The white biofilm is

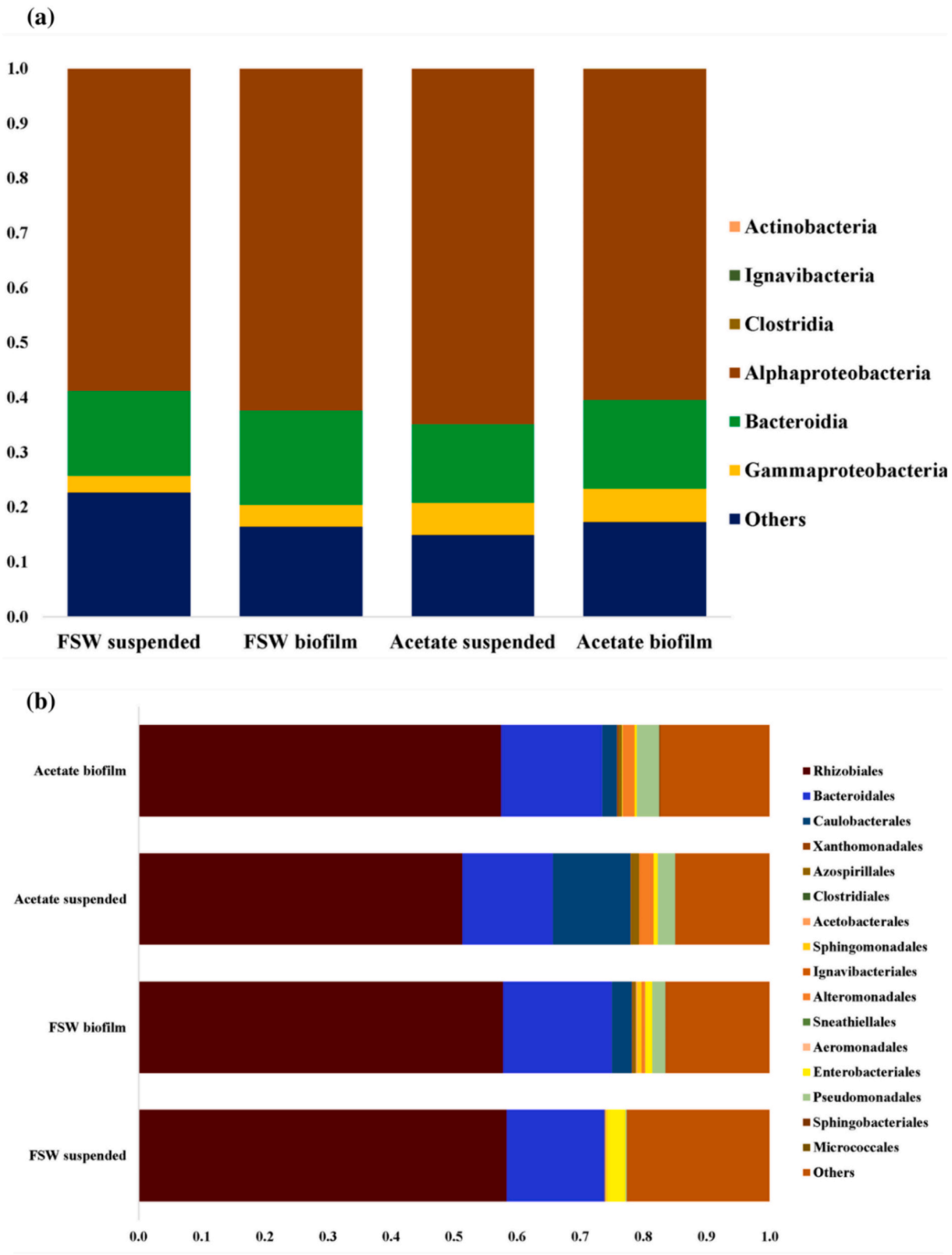

Fig. 3. Relative abundance (% of total OTUs) in terms of (a) phylum and (b) class for the FSW and acetate media, considering both suspended and biofilm growth.

likely due to the presence of yeast, which often forms white or off-white colonies (Wickerham, 1953). Yeast cells were observed as the dominant cell type in the glucose and sucrose conditions under the microscope (Figs. A2 and A3, Supplementary Information), indicating a shift in the microbial community composition.

Only limited studies explore formation of PNSB biofilms. For instance, Hülsen et al. (2020b) reported PNSB dominated biofilm formation on cylindrical photobioreactors tubes and Nhi-Cong et al. (2021) reported single species PNSB biofilm formation on polyurethane form, cinder beads, and coconut fibre. The use of shade cloth provides a low

cost, flexible, and reusable substrate that also provides good penetration of light, making it a promising option for PNSB biofilm formation.

3.2. Organic removal and substrate yield

Higher COD removal of 2165 ± 35.3 mg/L and 2160 ± 14.1 mg/L was observed in FSW and sucrose, respectively. This trend agrees with higher biomass production from these two substrates, although biomass production in FSW was PNSB dominated while in sucrose media was yeast dominated. The COD removal in both cases was similar ($p = 1.0$). After FSW and sucrose, acetate had the next greatest COD removal, followed by glucose. The COD removal by acetate and glucose was statistically different ($p < 0.001$) from each other and from the former two substrates (Fig. 2d).

The highest biomass yield was obtained from glucose with a value of 1.46 ± 0.38 g-VSS/g-COD. This value is well above expected yields for bacterial systems and suggests autotrophic growth may have also been occurring, but also showed high uncertainty due to the variability associated with measuring very low biomass concentrations present in the glucose condition. In contrast, the acetate yield was 0.72 ± 0.04 g-VSS/g-COD, sucrose yield was 0.66 ± 0.01 g-VSS/g-COD, and the FSW yield was 0.55 ± 0.01 g-VSS/g-COD. These values are relatively high by bacterial standards and suggest PNSB dominance in FSW and acetate (Cerruti et al., 2020; Hädicke et al., 2011). The sucrose biomass yield is in line with many studies using yeast (Hagman et al., 2013; Jasman et al., 2015). There was no significant difference ($p > 0.05$) between biomass yields for the different carbon substrates. No inference can be made on the true yields of suspended vs biofilm growth as COD consumption was measured across the reactor as a whole.

3.3. Bacterial community analysis

The bacterial community analysis of the two growth modes in the FSW and acetate conditions is shown in Fig. 3. The glucose and sucrose samples were not analysed because they mainly contained yeast, and we lacked the necessary primers for non-bacterial organisms. The presence of yeast was evident from the microscope images of the biomass (Figs. A2 and A3, Supplementary Information). The reddish-brown colour observed in the FSW and acetate samples, but not in the glucose and sucrose samples, suggests a predominance of PNSB in the former. The most abundant classes found in both suspended and biofilm growth modes for FSW and acetate substrates were Alphaproteobacteria (60–63 %) and Bacteroidia (8–12 %). Bacteroidia are linked to the hydrolysis and fermentation of organic matter. They are commonly identified in wastewater treatment studies, and the majority of Bacteroidia members are anaerobic, with a few genera being aerobic or facultative anaerobic strains (Chen et al., 2022).

At the order level, Rhizobiales had a relative abundance close to 60 % in all samples with the exception of the acetate fed suspended growth which had around 50 %. The presence of the Rhizobiales is consistent with a PNSB dominated culture, since various genera of PNSB including *Rhodobacter* and *Rhodopseudomonas* belong to this order (Fritts et al., 2017). Rhizobiales have ability to degrade a range of recalcitrant and toxic chemicals (Jiang et al., 2019). The relative abundance of Bacteroidales varies from 10 to 15 % in all samples. Bacteroidales are an important group of anaerobic bacteria that play a key role in the decomposition of complex organic compounds to acetate and other volatile fatty acids and cycling of important nutrients in anaerobic microcosms (Lv et al., 2017). These hydrolyzed products are then available for PNSB. The presence of these organisms is surprising given most of the substrates tested are simple substrates, and suggests that they may exist on the soluble microbial products produced by other organisms in the culture, such as from PNSB. Enterobacterales were slightly more dominant in FSW biomass (both suspended and biofilm) as compared to the acetate biomass and are able to ferment a wide range of substrates. This includes the capacity to consume different types of alcohols, such as methanol and ethanol (Gnan and Abodreheba, 1987; Rani et al., 2020), which are prevalent in FSW.

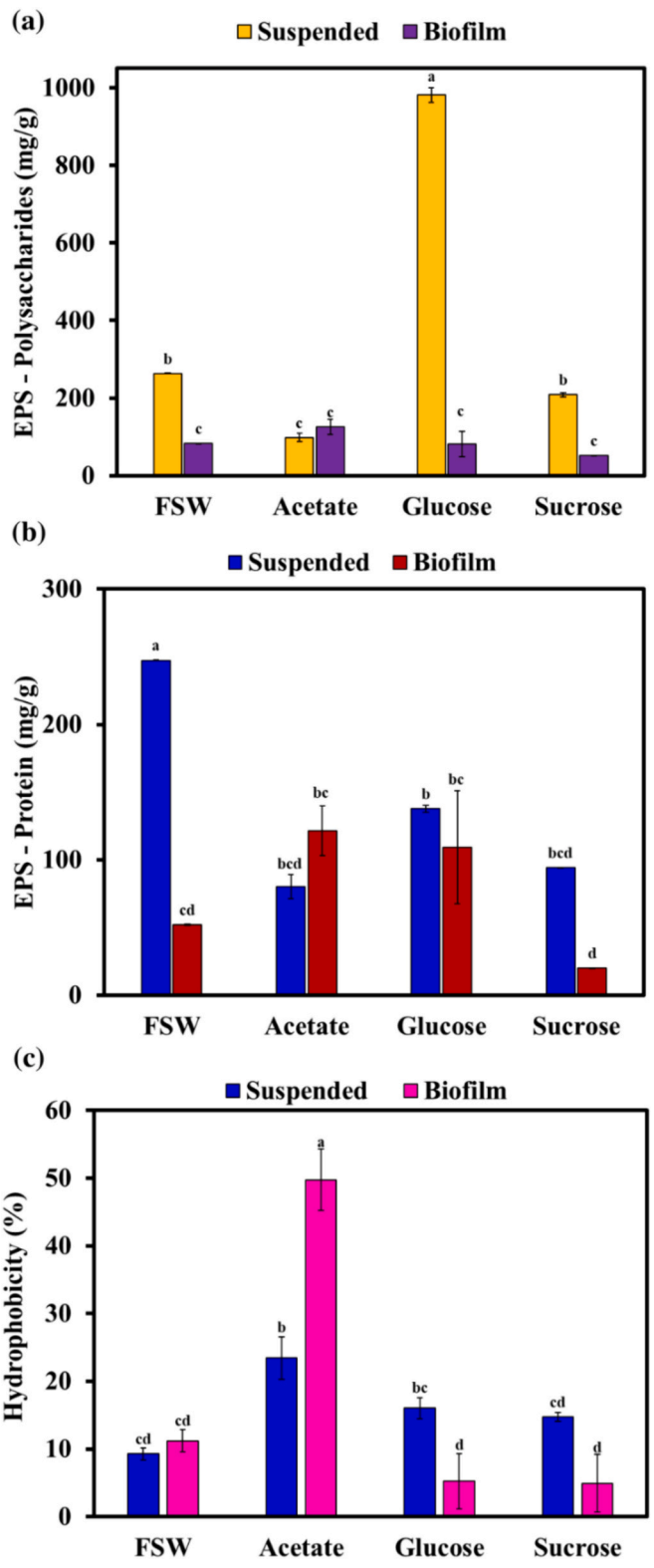

Fig. 4. (a) EPS-PS (b) EPS-PN and (c) hydrophobicity of suspended and biofilm biomass in each BPBRs. Different alphabetical letters represent significant differences from a Bonferroni post-hoc test conducted following a significant ANOVA test.

Certain groups were notably absent in the FSW suspended samples but were present in all other conditions. For example, Caulobacterales

Table 1
The production of Crts and BChls by photosynthetic bacteria cultivated on various carbon substrates. Different alphabetical letters represent significant differences from a Bonferroni post-hoc test conducted following a significant ANOVA test.

Pigment	Carbon source	Pure/mixed culture	Light source	N^+/N^-	Suspended (μg/g)	Biofilm (μg/g)	Reference
Crts	RMPW	Mixed	Infra-red	N^+	4.3–6.1	9.9–12.4	(Hülsen et al., 2020b)
Crts	NS medium	Pure	White LED	N^+	605	–	(Kuo et al., 2012)
Crts	ASW	Pure	–		276–4889	–	(Zhou et al., 2014)
Crts	ABW	Pure	Sunlight	N+	1930–2530	–	(Meng et al., 2018)
Crts	FSW	Mixed	White LED	N^-	0.63 ± 0.03^{a}	3.05 ± 0.02^{bc}	This research
Crts	Acetate	Mixed	White LED	N^-	0.35 ± 0.03^{a}	2.62 ± 0.37^{bc}	This research
Crts	Glucose	Mixed	White LED	N^-	0.14 ± 0.00^{b}	0.92 ± 0.36^{bc}	This research
Crts	Sucrose	Mixed	White LED	N^-	0.03 ± 0.00^{c}	0.07 ± 0.00^{c}	This research
BChls	RMPW	Mixed	Infra-red	N^+	10–13. 2	22.2–28.2	(Hülsen et al., 2020b)
BChls	ASW	Pure	–	N^+	487–3318	–	(Zhou et al., 2014)
BChls	ABW	Pure	Sunlight	N^+	9790–10,750	–	(Meng et al., 2018)
BChls	FSW	Mixed	White LED	N^-	5.84 ± 0.40^{a}	4.41 ± 0.03^{b}	This research
BChls	Acetate	Mixed	White LED	N^-	3.83 ± 0.34^{bc}	2.98 ± 0.45^{c}	This research
BChls	Glucose	Mixed	White LED	N^-	0.50 ± 0.00^{d}	0.11 ± 0.04^{d}	This research
BChls	Sucrose	Mixed	White LED	N^-	0.11 ± 0.00^{d}	0.00 ± 0.00^{d}	This research

RMPW = Red meat processing wastewater, ASW = Artificial sugar wastewater, ABW = Artificial brewery wastewater, FSW = Fuel-synthesis wastewater, N^- = Nitrogen deficient, N^+ = Nitrogen sufficient.

and Xanthomonadales were identified in both biofilm and suspended growth in acetate, as well as in FSW biofilm, but were not detected in FSW suspended samples. Additionally, relatively abundant families such as Sphingobacteriales and Ignavibacteriales were observed across all conditions except for FSW suspended biomass. These differences highlight the distinct microbial community structures and functional potentials between FSW and acetate conditions, and between suspended and biofilm growth modes. The unique composition of microbial communities in FSW suspended samples, characterized by fewer organisms, may indicate an inhibitory element to the FSW, such as the high proportion of short-chain alcohols. This further supports the suitability of PNSB, and their plausible enrichment, in this substrate.

3.4. Suspended and biofilm biomass characterization

3.4.1. Extracellular polymeric substances

One of the key elements in establishing and maintaining the biofilm structure and properties is the extracellular polymeric matrix. The extracellular polymeric matrix comprises water and EPS, primarily polysaccharides (PS), proteins (PN), and DNA (Martino, 2018). For the biofilm growth, the acetate biomass showed the highest EPS-PS content of 125 ± 19 mg/g, followed by FSW (83 ± 0.1 mg/g), glucose (81 ± 32 mg/g) and sucrose (51 ± 0.9 mg/g). In contrast, for the suspended growth, the highest EPS-PS content was 981 ± 19 mg/g which was observed in the glucose condition. In contrast, the FSW sample had 263 ± 0.8 mg/g, sucrose had 209 ± 5.7 mg/g and acetate had 98 ± 11 mg/g. The EPS-PS of the glucose suspended biomass was different ($p < 0.001$) from all other samples and almost twice the nearest value (Fig. 4a). As previously mentioned, the low biomass concentration in this reactor may have led to an erroneous VSS measurement, leading to both a high yield and EPS quantification. However, it should also be noted that PS measurements are in relation to glucose, and different sugars have very different responses to the reagent. For instance, Dubois et al. (1956) while testing different sugars observed an approximately 5 times variation in absorbance response per mass of sugar, across the different sugars tested.

Only the biofilm of acetate showed higher PS production than the corresponding suspended growth. One reason for higher PS production in biofilm growth could be excess light stress, since the experimental setup had the shade cloth rotating near the edge of the bottle. It has been shown that increasing light intensity can enhance EPS production in photosynthetic microorganisms up to the light saturation point, above which the production declines (Babiak and Krzemińska, 2021). The FSW biofilm, in comparison to acetate, was thicker which could provide some attenuation of light to layers of biomass below the biofilm surface, giving a lower average EPS-PS in the biofilm. Substrate complexity and mass transfer into the biofilm may also play a role. In the case of glucose with very little biofilm growth, the shade cloth provided little shading against the light, and so the suspended growth may have increased its production of EPS-PS due to light stress. The glucose culture showed very limited growth compared to other substrates, and may have been due to energy directed towards this excessive PS production.

The highest EPS-PN production among the biofilms of 122 ± 18 mg/g was observed in the acetate condition, followed by glucose, FSW and sucrose. In contrast, the highest EPS-PN production from the suspended growth was observed in FSW at 247 ± 0.4 mg/g, which was followed by glucose, sucrose, and then lastly acetate at 80.23 ± 8.7 mg/g (Fig. 4b). There was a significant difference ($p < 0.001$) between the suspended and biofilm growth of FSW but no significant difference ($p > 0.05$) between the suspended and biofilm growth of other carbon substrates. This may be attributed to the development of the biofilm in the FSW case, and therefore the more significant differences between fixed and suspended biomass micro-environments.

3.4.2. Hydrophobicity

Hydrophobicity plays a crucial role in microbial attachment and biofilm formation, as it influences the interactions between bacterial cells and surfaces (Tobergte and Curtis, 2013). In our study, we investigated how hydrophobicity varies between cells grown in suspended and biofilm growth modes, providing insights into their surface colonization abilities. The hydrophobicity of biofilm (11.2 ± 1.6 %) and suspended biomass (9.2 ± 1.6, %) in the FSW condition was comparable, while for the acetate biomass the biofilm hydrophobicity was much greater (49.7 ± 4.5 %) than the suspended biomass (23.4 ± 4.5 %). For the two sugar substrates the opposite was found, with suspended biomass showing higher hydrophobicity (Fig. 4c). Although acetate and glucose conditions showed significant difference between their biofilm and suspended growth, both were similar ($p = 0.09$) to the respective biomass in the FSW and sucrose conditions. Acetate values were significantly greater than either sugar substrate. In one study with yeast, different strains of the same genus showed vastly different responses in terms of hydrophobicity to the same substrates (Porfírio et al., 2017), while in this study, the two sugar substrates showed similar hydrophobicity but different biofilm formation ability, possibly influenced by growth on the substrates. The relationship between hydrophobicity and biofilm formation varies with the type of strain and microorganism, being either directly or inversely proportional. Choi et al. (2015) observed that while *Bacillus cereus* exhibited minimal biofilm formation despite its high hydrophobicity, *Escherichia coli* O157:H7 demonstrated significant biofilm development accompanied by low hydrophobicity

Table 2
The production of SCP and PHB content by photosynthetic bacteria cultivated on various carbon substrates. Different alphabetical letters represent significant differences from a Bonferroni post-hoc test conducted following a significant ANOVA test.

SCP/PHB	Carbon source	Pure/mixed culture	Condition	Suspended (%)	Biofilm (%)	Reference
SCP	RMPW	Mixed	N^+	62.1	64.3	(Hülsen et al., 2020b)
SCP	ASW	Pure	N^+	35–40	–	(Cao et al., 2021)
SCP	CM medium	Pure	N^+	62.7	–	(Patthawaro and Saejung, 2019)
SCP	FSW	Mixed	N^-	36.1 ± 0.1^a	37.7 ± 0.0^b	This research
SCP	Acetate	Mixed	N^-	41.7 ± 0.0^c	41.0 ± 0.1^d	This research
SCP	Glucose	Mixed	N^-	30.1 ± 0.3^e	25.5 ± 0.1^f	This research
SCP	Sucrose	Mixed	N^-	8.5 ± 0.1^g	7.8 ± 0.0^h	This research
PHB	Acetate	Pure	S^-/S^+	2.5–20	–	(Touloupakis et al., 2021b)
PHB	Acetate	Pure	N^+	25.1 ± 1.0	–	(Carlozzi and Touloupakis, 2021)
PHB	Lactate	Pure	N^+	27.1 ± 2.6	–	
PHB	Malate	Pure	N^+	8.7 ± 1.5	–	
PHB	Succinate	Pure	N^+	13.7 ± 3.1	–	
PHB						
PHB	FSW	Mixed	N^-	15.0 ± 0.3^a	11.3 ± 0.5^{be}	This research
PHB	Acetate	Mixed	N^-	11.5 ± 0.7^{be}	9.2 ± 0.3^b	This research
PHB	Glucose	Mixed	N^-	18.1 ± 0.1^c	12.9 ± 1.1^{ae}	This research
PHB	Sucrose	Mixed	N^-	4.2 ± 0.9^d	3.4 ± 0.3^{cd}	This research

RMPW = Red meat processing wastewater, CM = Chicken manure, ASW = Artificial sugar wastewater, FSW = Fuel-synthesis wastewater, N^- = Nitrogen deficient, N^+ = Nitrogen sufficient, S^- = Sulphur deficient, S^+ = Sulphur sufficient.

levels. In this study, different carbon sources have been used causing the biomass to behave differently. With FSW, a high level of biofilm formation has been observed with relatively low hydrophobicity, whereas for a similar microbial community in the acetate condition the substrate has led to a higher hydrophobicity and correspondingly, a lower level of biofilm formation.

3.5. Value-added bio-products and PHB production

3.5.1. Carotenoids and bacteriochlorophylls (Crts and BChls)

It has been observed that higher Crts and BChls were obtained from FSW followed by acetate, glucose, and sucrose (Table 1). The Crts content from suspended and biofilm growth of all BPBRs varied in the range 0.032–0.63 μg/g and 0.07–3.05 μg/g, respectively. The Crts content of biofilm growth of the FSW and acetate were different ($p < 0.001$) from the glucose and sucrose suspended growth. However, no significant difference ($p > 0.15$) was observed between the BPBRs for suspended growth.

All BPBRs have a BChls content ranging from 0.0092 to 5.84 μg/g in suspended growth and 0.026–4.41 μg/g in biofilm (Table 1). In the FSW condition the BChls content differed between the suspended biomass and biofilm ($p = 0.012$) but for all other carbon substrates the suspended biomass and biofilm within the same condition were similar ($p > 0.24$). This most likely correlates to the thicker biofilm in the FSW condition and light shading effects. For the glucose and sucrose conditions the BChls content was less than the Crts content. The same BChls levels were an order of magnitude less than those in the acetate and FSW conditions. This can be explained by the yeast dominance in these substrate conditions. BChls are limited to the anoxygenic phototrophic bacteria, while carotenoids are produced by a small number of yeast species and by other bacteria (Gómez et al., 2014).

From these results it is evident that Crts were higher in the biofilm growth whereas BChls were higher in the suspended growth. A major use of Crts by photosynthetic organisms is to dissipate excess photonic energy during photosynthesis. Since the shade cloth and biofilm growing on it were at the edge of the photobioreactor wall, this provided shading to the suspended growth. The cells present in the biofilms were then exposed to higher light intensities, driving biofilm to synthesize greater quantities of Crts (Liu et al., 2022). By the same process, the lower light intensity reaching the centre of the reactor drives a higher BChl production by suspended growth in order to capture the limited light energy passing beyond the shade cloth and biofilm. This observation is partially in line with the observation of the study reported by Hülsen et al. (2020b). Hülsen et al. (2020b) found higher Crts and BChls from the biofilm growth as compared to the suspended growth. The variation in results for BChls is attributed to the thicker biofilm buildup in their study, which hindered light penetration, along with poor mixing.

The Crts/BChls ratio varies depending on the growth conditions. The dominance of yeast results in higher Crts/BChls ratios for both sugar substrates in suspended growth compared to FSW or acetate. It is worth noting that both sugars have lower Crts and BChls concentrations. For the suspended growth the Crts/BChls ratio lies in the range of 0.09 to 0.28 while for the biofilm it varies between 0.69 and 8.32. The high Crts/BChls ratio observed in glucose biofilm is likely due to limited growth, resulting in higher light exposure. In comparison, the ratios are lower for suspended growth, as the shade cloth and biofilm offer some protection. These findings are consistent with the study of Meng et al. (2017) but can only be compared to FSW and acetate-based BPBRs since they used a pure culture of PNSB (*Rhodopseudomonas*). Hülsen et al. (2020b) reported pigments in PNSB-dominated biofilms with Crts/BChls ratios ranging from 0.43 to 2.2. Those values are approximately four times lower than the values reported in this study and may result from the lower light intensity used in that study of 5.8–16 W/m^2, which is around 6 times less than the light intensity of this study.

Overall, the concentration of Crts and BChls from suspended biomass and biofilm of all the BPBRs were found to be lower than the values reported in the literature (Table 1). The primary reasons for this are the use of a mixed culture and the non-optimised culture conditions such as use of a white LED rather than infra-red to aid enrichment of PNSB. However, an important finding is that FSW has the potential to produce higher Crts and BChls than other more expensive pure carbon sources such as acetate. The Crts and BChls production can be maximized through critical optimization to enhance PNSB dominance in the culture.

3.5.2. Single cell protein (SCP)

Among all carbon sources, acetate showed the highest protein content of 41.73 ± 0.0 % from suspended growth and 40.97 ± 0.0 % from biofilm growth. FSW was the next highest with 37.73 ± 0.0 % and 36.1 ± 0.0 % from biofilm and suspended growth, respectively. Significant differences ($p < 0.001$) were observed between protein content of the suspended and biofilm biomass of the same BPBR as well as for the same growth modes between different BPBRs (Table 2). Protein content was higher in the suspended growth of all the BPBRs except the FSW condition, where biofilm had a higher protein content. This could be due to a higher biomass production from the biofilm of FSW as compared to the other BPBRs leading to a more active culture on the shade cloth than the

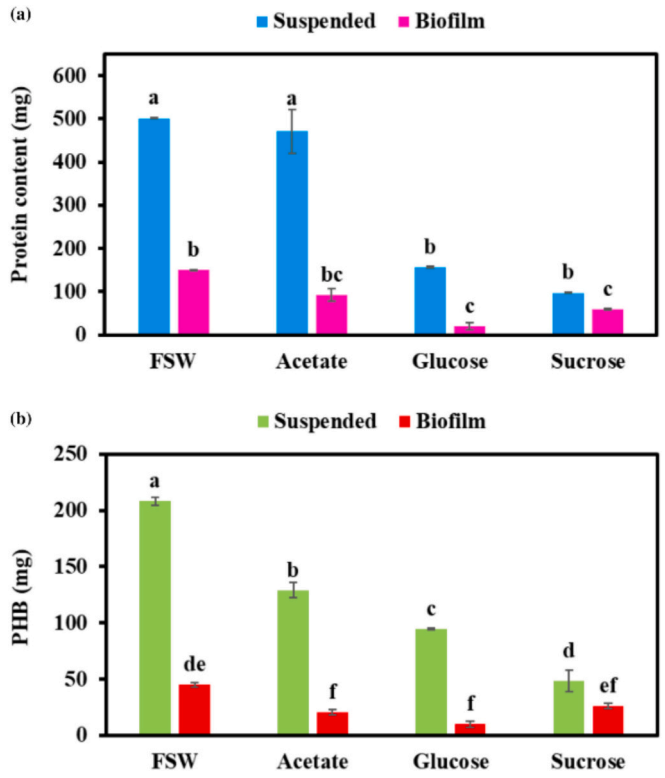

Fig. 5. (a) Protein content and (b) PHB mass of suspended and biofilm growth of all BPBRs. Different alphabetical letters represent significant differences from a Bonferroni post-hoc test conducted following a significant ANOVA test.

light shielded suspended biomass. In other cultures, the suspended growth was the most active. However, these differences were not large. Hülsen et al. (2020b) are the sole researchers to our knowledge that have documented protein levels in both suspended growth and biofilm for a PNSB dominated system, noting significantly higher percentages (62.1–64.3 %) than those observed in the current study. This is likely attributed to the application of crude protein measurement methods. Nonetheless, they also concluded that protein content was comparable between suspended and biofilm growth.

The range of cellular protein content identified in this research aligns well with findings from Cao et al. (2021), who recorded protein levels between 35 and 55 %. Furthermore, investigations utilizing photosynthetic bacteria have reported even greater values, like the 62.7 % documented by Patthawaro and Saejung (2019). These significant variances can be ascribed to several factors, such as the type of substrate used, which in the case of the latter was nitrogen-rich chicken manure, and the techniques employed for protein extraction and analysis.

The glucose condition produced a protein content of 30 ± 0.2 % from suspended growth and 25.5 ± 0 % from biofilm. However, sucrose had around one-quarter of the protein in both suspended and fixed growth modes (8.5 ± 0 and 7.8 ± 0 %, respectively). Khan and Dahot (2010) compared different carbon sources and found biomass crude protein contents of 28 % and 30 % from cultures fed glucose and sucrose amended, 0.6 N H_2SO_4 pre-treated, rice husk. The protein content of glucose is in line with the findings of Khan and Dahot (2010) but sucrose findings are not. It is possible that the sucrose grown cells produced another component at high values, such as lipids or carbohydrates.

The protein mass of suspended and biofilm growth of all the BPBRs is shown in Fig. 5a. Protein productivity is a product of biomass growth and cellular protein content. It can be determined from the protein mass divided by the experiment time. The highest protein productivity from suspended growth of 33.4 ± 0.07 mg/d was obtained from FSW, followed by acetate, glucose and lastly sucrose which only achieved 6.5 ± 0.08 mg/d. For biofilm growth the highest protein productivity of 10 ± 0.0 mg-PN/day was obtained from FSW. This was followed by acetate, sucrose and glucose. The protein productivity of the glucose suspended growth was different ($p < 0.001$) from the suspended growth of all the other carbon sources, while for biofilm protein productivity only the FSW condition was different to the glucose condition ($p = 0.006$).

3.5.3. PHB

The mass of PHB associated with suspended and biofilm growth is shown in Fig. 5b for each BPBRs. In the suspended growth, the maximum PHB content of 18.1 ± 0.1 % was obtained from glucose, followed by FSW with 15.0 ± 0.3 %. A significant difference ($p < 0.001$) was observed between the PHB content of glucose and all other carbon sources. In the biofilm, a similar observation occurred, with glucose containing 12.9 ± 1.1 % PHB followed by FSW with 11.3 ± 0.5 %. However, no significant difference ($p = 0.65$) was observed between the PHB content of both carbon sources. The sucrose PHB content was low in both the suspended growth (0.55 ± 0.1 %) and the biofilm (3.85 ± 0.2 %) (Table 2).

Abdallah et al. (2020) studied PHA production using different carbon sources in a non-PNSB system. They found that glucose was the best carbon source, resulting in 35 % PHA. Yuan et al. (2015) obtained 42 % and 40 % of PHB from acetate and glucose, respectively using polyphosphate-accumulating organisms. Therefore, different organisms respond differently to these common substrates. A few studies reported PNSB-PHA production from acetate, glucose and sucrose, among other substrates (Lorrungruang et al., 2006; Touloupakis et al., 2021a). Papers report acetate and lactate as favourable substrates attaining 18–40 % PHB, though it appears also that the species of PNSB is an important factor. Acetate appears to be preferred by *Rhodopseudomonas* and *Rhodobacter* species and lactate by *Rhodovulum sulfidophilum* (Carlozzi and Touloupakis, 2021; Khatipov et al., 1998; Touloupakis et al., 2021a).

In the authors previous study with PNSB treating FSW, a PHB content of no >12 % was achieved in suspended nitrogen deficient biomass (Shaikh et al., 2023a). The results from this study show a small but notable improvement, attributed to the abundance of the carbon source. In comparison to the previous study which lasted 19 days and had a COD removal rate of 72.5 %, the current study was conducted over 15 days with a COD removal rate of 37.4 %. The lower COD removal rate observed in this study, in contrast to our previous findings, does not necessarily indicate a less effective treatment process as the initial COD concentrations were slightly lower than the previous study and were conducted with different batches of FSW.

In both this and the previous study (Shaikh et al., 2023a) higher PHA values were observed in the suspended biomass. High light intensities have been demonstrated as an important factor for PHB production in PNSB (Fradinho et al., 2013). It is therefore hypothesized that the suspended biomass has higher PHB due to its small size, meaning all cells in the flocs are exposed to high light intensity, vs the significant light shielding that occurs for biomass not at the surface of the biofilm.

The total PHB mass of the PBBR was greatest for the FSW condition, due to its high growth and relatively high PHA content. This was followed by acetate, glucose, and sucrose. It should be noted that the difference in total PHB between FSW and other carbon substrates was statistically significant ($p < 0.006$). This indicates that FSW is a potentially favourable substrate for PHB production if using a PNSB-based system, particularly as the wastewater is naturally nitrogen deficient. The effectiveness of FSW as a substrate for PHB production in PNSB could be attributed to its high and mixed VFAs content. VFAs are excellent PHB precursors and the variety present in FSW could allow for more efficient PHB synthesis by multiple species (Szacherska et al., 2021), compared to simpler substrates like acetate. This makes FSW a promising option for PHB production in PNSB-based systems, leveraging wastewater for biotechnological applications. The maximum PHB yield of 0.237 ± 0.05 mg-PHB/mg-COD was obtained from glucose, followed by acetate, FSW, and sucrose. No significant difference ($p > 0.38$) was

Table 3
The total PHB (suspended + biofilm) production, and PHB yield by photosynthetic bacteria cultivated on various carbon substrates. Different alphabetical letters represent significant differences from a Bonferroni post-hoc test conducted following a significant ANOVA test.

Carbon source	Total PHB (mg)	$Y_{PHB/S}$
FSW	253.16 ± 1.4^{a}	0.069 ± 0.0^{b}
Acetate	149.78 ± 9.2^{b}	0.073 ± 0.0^{b}
Glucose	104.59 ± 3.5^{c}	0.237 ± 0.05^{a}
Sucrose	74.72 ± 7.4^{c}	0.020 ± 0.0^{b}

observed between the PHB yield of the different carbon sources except glucose ($p < 0.001$), which differed from all others by an order of magnitude as illustrated in Table 3. This was most likely due to the excess carbon and light compared to biomass, but may also be associated with uncertainty caused by the very low biomass concentration used in the calculation. However, it should be noted that PHB yield of acetate and FSW obtained in this study falls centrally within the range of yields of 0.027–0.67 mg-PHB/mg-COD reported in the literature for PNSB (Fradinho et al., 2016; Policastro et al., 2020; Touloupakis et al., 2021a, 2021b).

4. Conclusions

This study evaluated the influence of various carbon sources on PNSB biofilm formation and bio-product production in a biofilm photobioreactor. Results show that an enriched PNSB culture was suitable for FSW treatment and formed biofilm more readily than with other substrates. FSW had highest overall productivity for most bio-products due to high biomass growth, but glucose produced the highest PHB content and acetate gave the greatest protein content. This indicates wastewater composition should be considered carefully when evaluating specific resource recovery targets from PNSB systems.

CRediT authorship contribution statement

Sultan Shaikh: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mirna N.A. Abdelnabi:** Investigation, Formal analysis. **Annette Shoba Vincent:** Writing – review & editing, Supervision, Resources. **Gordon McKay:** Writing – review & editing, Supervision. **Hamish Robert Mackey:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Additional data supporting the findings of this study, including detailed specifications of materials used, microscope images evidencing biomass characteristics, FSW characteristics, and analyses of suspended and biofilm growth biomass samples under various conditions, are available in the electronic Supplementary materials. Supplementary data to this article can be found online at https://doi.org/10.1016/j.biteb.2024.101945.

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