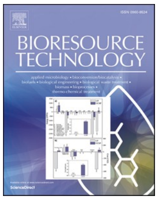
Bioresource Technology 333 (2021) 125194

Contents lists available at ScienceDirect

Bioresource Technology

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



Impact of wastewater cultivation on pollutant removal, biomass production, metabolite biosynthesis, and carbon dioxide fixation of newly isolated cyanobacteria in a multiproduct biorefinery paradigm

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HIGHLIGHTS

- Newly isolated cyanobacteria were evaluated for their biotechnological potential.
- Wastewater cultivation enhanced biomass and metabolite production.
- The biomass was subjected to process optimization for a cascading biorefinery.
- Wastewater cultivation and multiproduct processing was shown to be feasible.
- An integrated cascading biorefinery using wastewater as growth media is proposed.

ARTICLE INFO

Keywords:
Cyanobacteria
Low-cost cultivation
Pollutant removal
Integrated biorefinery
Environmental sustainability

ABSTRACT

The impact of wastewater cultivation was studied on pollutant removal, biomass production, and biosynthesis of high-value metabolites by newly isolated cyanobacteria namely *Acaryochloris marina* BERC03, *Oscillatoria* sp. BERC04, and *Pleurocapsa* sp. BERC06. During cultivation in urabn wastewater, its pH used to adjust from pH 8.0 to 11, offering contamination-free cultivation, and flotation-based easy harvesting. Besides, wastewater cultivation improved biomass production by 1.3-fold when compared to control along with 3.54–4.2 gL^{-1} of CO_2 fixation, concomitantly removing suspended organic matter, total nitrogen, and phosphorus by 100%, 53%, and 88%, respectively. Biomass accumulated 26–36% carbohydrates, 15–28% proteins, 38–43% lipids, and 6.3–9.5% phycobilins, where phycobilin yield was improved by 1.6-fold when compared to control. Lipids extracted from the pigment-free biomass were *trans*-esterified to biodiesel where pigment extraction showed no negative impact on quality of the biodiesel. These strains demonstrated the potential to become feedstock of an integrated biorefinery using urban wastewater as low-cost growth media.

1. Introduction

Increasing urbanization, intensive mobility, improving living standards, and upsurging industrialization have caused tremendous utilization of natural resources including land for agriculture, increasing consumption of freshwater for houses and industry, and excessive burning of fossil fuels to meet the needs. As a result, there are heavy emissions of greenhouse gases and huge production of urban wastewater while this problem is even worse in heavily populated countries including Bangladesh, China, India, Pakistan, Philippines, and

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https://doi.org/10.1016/j.biortech.2021.125194
Received 16 March 2021; Received in revised form 14 April 2021; Accepted 15 April 2021

Indonesia. This scenario demands establishing the processes keeping in view the nexus among mitigation of atmospheric carbon, wastewater treatment, and renewable energy production (Martinez-Hernandez & Samsatli, 2017). Cyanobacteria are believed to be the feedstock of future multiproduct biorefineries owing to their short cultivation period, notable metabolite composition, stress-tolerance mechanism, wastewater treatment efficiency, the highest CO_2 fixation rate, and accumulation of high-value metabolites of industrial, environmental, and pharmaceutical importance (Deviram et al., 2020). Besides, cyanobacteria have shown promising potential to become feedstock of choice to produce biofuels, polyhydroxyalkonates, pigments, pharmaceuticals, and biofertilizers (Koller, 2015). Despite their tremendous potential, they have not been completely explored commercially due to difficult cultivation and economic unfeasibility. In this regard, exploration of potential indigenous cyanobacteria is of prime importance to address the mentioned issues and to develop integrated wastewater-based sustainable biorefinery systems.

Water is the major requisite for cyanobacterial/algal biomass production which acts as a medium for thermal regulation and provides essential nutrients including macro (Ca, Mg, P, S, N, K etc.) and micronutrients (Fe, Ni, Na, etc) to support growth. However, the utilization of freshwater for cyabobacterial cultivation has been criticized due to the shortage of freshwater resources and higher production costs (Shahid et al., 2019). Alternatively, a growing population and rapid industrialization produce huge amounts of nutrient-rich wastewater which poses serious threats to the ecosystem. The utilization of wastewater as an alternative growth media for cyanobacterial growth has been in practice for the last 75 years in developed countries. It was found to be feasible due to its abundant availability, cost-effectiveness (50% reduction in cultivation cost), presence of ample nutrients (mainly nitrogen and phosphorus), environment-friendly biomass production, and bioproduct recovery (Salama et al., 2017). Many unicellular and filamentous strains of microalgae and cyanobacteria have been cultivated on wastewater to improve their biomass production and metabolite content. For instance, *Pseudanabaena mucicola* produced 0.55 gL^{-1} of biomass along with 237 mgg^{-1} of phycobilin in aquaculture wastewater (Khatoon et al., 2018). *Oscillatoria* sp. produced 2-folds higher biomass when cultured in diluted nitrate-rich wastewater when compared to the standard cultivation medium (Yeung et al., 2019). In a bioprospecting-based study, wastewater cultivation potential of 44 different cyanobacterial strains was evaluated, where *Synechococcus* sp. produced 0.36–2.0 gL^{-1} of biomass, while other cyanobacterial strains like *Anabaena*, *Nostoc,* and *Calothrix* produced 0.4–1.5 gL^{-1} of biomass in municipal wastewater (Aketo et al., 2020). Similarly, *Spirulina* (*Arthrospira*) *platensis* produced 2.2-fold higher biomass (0.62 gL^{-1}) on 50–100% of the palm-oil mill effluent in a two-stage cultivation setup. Additionally, this condition also favored phycocyanin (phycobilin) production (Nur et al., 2019). These studies indicated the viability of the wastewater as low-cost alternative cultivation medium for the higher biomass production with desired biochemical properties.

Although seems promising yet wastewater cultivation results in compromised biomass production due to contamination risks. Accordingly, wastewater often requires sterilization before culturing the algal strain. Many sterilization approaches and contamination control strategies have been reported but they have undesirable economic and environmental impacts (Molina et al., 2019; Pleissner et al., 2020). Hence, the most cost-effective and sustainable approach is the use of biopreservative strains which have the potential to outcompete the contaminating microbes by dominating the environment (due to their fast growth) when cultured in non-sterilized urban wastewater. For instance, the alkaliphilic strains have the potential to cope with the contamination risk via modulating the pH of the media, making it unsuitable for invasive microbes. The present study was aimed to evaluate the newly isolated cyanobacterial strains including *Acaryochloris marina* BERC03, *Oscillatoria* sp. BERC04, and *Pleurocapsa* sp. BERC06 for their potential to grow in non-sterilized urban wastewater followed by evaluating the impact of wastewater cultivation on the biosynthesis of high-value metabolites, content and quality of the biodiesel, for the very first time. Besides, biomass was evaluated for its potential utilization as feedstock in an integrated multiproduct biorefinery.

2. Materials and methods

2.1. Sample collection and purification

Filamentous cyanobacteria dominating in the wastewater reservoirs of central Punjab, Pakistan were targeted for the sample collection (Table 1). Sample (10–15 g) was scooped from the water bodies in 100 mL plastic vials (Mutanda et al., 2011), and capped underwater. Collected and labeled samples were transferred to the lab within one day for further processing. Samples were passed through 20 μm mesh to remove collection media and washed thrice with distilled water to remove attached sediments or nutrients. Axenic cultures were obtained by employing repeated agar-plate cultivation as described previously (Santhakumaran et al., 2019), except BG11 media was used instead of BBM. The purity of the samples was ensured through routine microscopic analysis by compound microscope (Optika Microscope, Italy). All the axenic cultures were maintained in batches in standard media at 28–30 °C with 12:12 light (cool fluorescent light) and dark period.

2.2. Morphological and molecular identification

Morphological identification was performed based on cell morphology including cell size, shape, and appearance. Micrographs of the pre-purified and post-purified samples were taken by light microscope equipped with 5 megapixels digital camera (Optika Microscope, Italy). Micrographs of the axenic cultures were analyzed by comparing the characteristics with an online database (Santhakumaran et al., 2019). The genomic DNA of the strains was used as a template to amplify the 23S rRNA gene by following the procedure and conditions as described previously (Shahid et al., 2020). Recombinant plasmids (100 ng μL^{-1}) were sent to Macrogen Korea for sequencing. Phylogenetic analysis was performed against a non-redundant sequence database (NCBI) through FastTree software by adopting the maximum-likelihood method. Post scripting and visualization improvement were performed by iToL and CorelDraw, respectively (Letunic & Bork, 2019).

2.3. Wastewater cultivation to evaluate the pollutant removal potential

Municipal wastewater is a feasible source of nutrients for cyanobacterial cultivation. Untreated (non-sterilized) municipal wastewater (MWW) was collected from the local drainage site and was divided into two parts. One of which was left for the sedimentation of suspended particles, and later was decanted into a new glass-container where suspended organic solids were sedimented. Later, the decanted wastewater and the wastewater containing suspended particles (8.90 g L^{-1}), were separately used as cultivation media to culture all the strains. Synthetic wastewater (MBG) was devised as described earlier (Shahid et al., 2020) by manipulating the nutritional composition (nitrate, phosphate, and bicarbonate) of the BG11 media to attain more control on the culture conditions as the composition of municipal wastewater

Table 1
Details of the cyanobacteria samples collected from wastewater reservoirs.

Strain name	Strain morphology	Water type	Location coordinates	
			Longitude	Latitude
BERC03	Filamentous	Urban wastewater	30.895833	73.344167
BERC04	Filamentous	Urban wastewater	30.944444	73.326389
BERC06	Filamentous	Industrial wastewater	31.4089472	73.0759168

varies according to the collection site, seasons, and source. All three kinds of wastewaters, including synthetic wastewater (MBG), the wastewater without suspended solids, and the wastewater containing organic suspended solids, were inoculated with 0.05 g L^{-1} (dry weight) of each strain in a 10 L locally designed open-pond like glass photobioreactor (45 cm wide, 15 cm deep, 90 cm long) where the light was provided through cool-fluorescent LED bulbs (Philips, Japan) and photoperiod of 12 h:12 h day:night was set using an automatic timing-switch, while the temperature of the growth room was maintained between 28 and 30 °C. Cultures were naturally aerated, to simulate the open pond cultivation and to avoid consumption of any additional energy.

Strains were cultivated without pH control and pH variation was observed on daily basis to evaluate the low-cost production potential of the proposed setup. The strains were harvested from the wastewater containing suspended particles on the 5th day of cultivation. While the strains cultured in decanted wastewater and MBG were harvested on the 15th day of cultivation, and the spent media (urban wastewater only) was subjected to estimating total nitrogen and total phosphorus, where total phosphorus was determined by colorimetric method and total nitrogen by Kjeldahl method, as described previously (Gill et al., 2016). The suspended organic matter was measured in the wastewater before and after cultivation using gravimetric method.

2.4. Growth kinetics and CO_2 utilization efficiency

Growth kinetic analyses were performed at the end of each batch culture on a dry mass basis by following the gravimetric method adopted earlier (Gill et al., 2016) to study the impact of cultivation conditions on biomass production. A handheld pH meter (Model pH 450, Thermo Scientific Eutech, USA) was used to monitor the pH variation during cultivation. Harvested biomass was lyophilized (Alpha 2–4 LSCbasic, Christ, Germany) and was stored at −20 °C for further use. Biomass production (Eq. (1)), biomass productivities, specific growth rates, and doubling rates were estimated by standard formulae as described previously (Shahid et al., 2020).

$$P_{\text{biomass}}\left(\text{gL}^{-1}\right) = B_y - B_x/\text{Reaction volume} \tag{1}$$

P_{biomass} represents the biomass production, where B_y refers to final biomass and B_x represents initial biomass.

CO_2 utilization efficiency was measured based on the biomass productivity of the wastewater cultivated biomass. CO_2 utilization equation (Eq. (2)) was derived from algal molecular composition $CO_{0.48}$, $H_{1.83}$, $N_{0.11}$, $P_{0.01}$ (Chisti, 2007).

$$P_{CO2}\left(\text{gL}^{-1}\right) = 1.883 \times P_{\text{biomass}} \tag{2}$$

2.5. Biochemical analyses of the biomass

Lyophilized samples were analyzed for their biochemical constituents to evaluate their biotechnological potential. Accordingly, 20 mg of sample was subjected to acidic and hot-alkaline treatment for carbohydrate and protein extraction, respectively. Extracts were assessed for carbohydrate and protein content through standard phenol–sulfuric acid (Dubois et al., 1956) and Micro-biuret methods (Chen & Vaidyanathan, 2013), respectively. The absorbance of treated samples was measured at λ 490 nm and λ 310 nm, and absorbance values were compared with glucose and BSA (bovine serum albumin) standards curves for subsequent carbohydrate and protein estimation.

Chlorophyll content and secondary pigment (carotenoids and phycobilin) content represents the photosynthetic efficiency and the biotechnological potential of the biomass. Twenty (20) mg of the lyophilized sample was used for subsequent estimation of chlorophyll *a*, chlorophyll *b*, and carotenoid content. Briefily, the biomass sample was treated for 12 h at 4 °C in dark with absolute methanol (5 mL) to release pigments. The pigment concentration was calculated by previously described formulae (Arnon, 1949). For phycobilin (PBP) estimation, a 60 mg lyophilized sample of each strain was separately added in 3 mL phosphate buffer (0.1 M) overnight at room temperature in the dark. The concentrations of pigments were calculated by measuring the absorbances at specific wavelengths including λ 620 nm (for phycocyanin), λ 652 nm (for allophycocyanin), and λ 562 nm (for phycoerythrin) using a UV–VIS Spectrophotometer (UV–VIS 1200, A & E Labs, UK). These absorbance values were used to calculate the pigment concentration as described previously (Bennett & Bogorad, 1973; Shahid et al., 2020). The residual biomass after pigment extraction was stored for subsequent lipid extraction.

2.6. Fatty acid and biodiesel compositional analysis

Lipid estimation provides the basis for the biodiesel production potential of the biomass. Here, fifty (50) mg of the pigment-free residual powdered biomass of each strain was processed by the modified Bligh and Dyer method (Ranjith Kumar et al., 2015) for lipid estimation. The supernatant of the overnight treated samples (with 2:1 chloroform: methanol mixture) was processed with 1% NaCl (w/v) for improved lipid recovery and removal of non-lipid constituents. The obtained organic phase was evaporated at 50 °C (until constant weight) to obtain pure lipids. Following formulae of lipid content (LC) and production were used for measurement

$$\text{LC}(\%) = (L_{wy} - L_{wx})/W_s \times 100 \tag{3}$$

Where L_{wy} and L_{wx} respectively represent the final weight and initial weight, and W_s is the weight of the sample taken in mg.

$$P_{\text{lipid}}(\text{mg L}^{-1}) = (\text{biomass} \times \text{LC})/100 \tag{4}$$

Furthermore, the qualitative and quantitative fatty acid compositional analysis was performed on 25 mg of transesterified lipids and analyzed by autosampler equipped gas chromatograph (Agilent 7000A Triple quadrupole, USA) coupled with a mass spectrometer (Agilent 7890, USA) as described previously (Musharraf et al., 2012). MRM mode of the GC was used with 30 eV collision energy, 5 min solvent delay, and 6.5 cycles s^{-1} of scan rate for FAME quantification. Data processing was performed through the Mass Hunter software of Agilent. Critical biodiesel parameters including saponification value, cetane number, high heating values, cloud point, pour point, cold filter plugging point, viscosity, and density were calculated based on the relative composition of FAMEs, position of double bonds, and carbon chain length by employing the equations as described previously (Arguelles & Martinez-Goss, 2021).

3. Results and discussion

3.1. Morphological and molecular identification

Among several hundred thousand existing algal strains only 3000 are identified, and less than 100 strains are well-studied. Due to various problems related to difficult cultivation, contamination, and expensive harvesting, it is required to explore new strains having the potential of low-cost contamination-free cultivation and easy harvesting. In the bioprospecting of algal strains, microscopic assessment of the collected sample is the initial and crucial step for strain identification. Under the microscope, the filaments of all samples were shown to be unbranched, sheathed motile chains of cylindrical cells with high length to width ratio where akinetes were absent. Morphologically, the strains were shown to be related to the *Oscillatoria* group of cyanobacteria (Komárek, 2018). However, the identification of samples through the polyphasic approach is not viable due to the polyphyletic and heterogeneous nature of these organisms which results in unstable morphological features in response to environmental conditions. Strain identification through both morphological and molecular features is advantageous as it provides more details. Here, phylogenetic analysis, based on the gene sequence of 23S rRNA, showed that all three strains belonged to different species. Where BERC03 and BERC06 strains respectively showed 89% and 72% resemblance with *Acaryochloris marina* and *Pleurocapsa* sp. Although BERC04 did not exhibit exact resemblance with any strain present in the databases, however, the results signified its relatedness to the cyanobacteria (Fig. 1). The reason for such results could be the conserved nature of genetic marker that makes it difficult to identify the inter and intra-species differences among members of the same genus (Cegłowska et al., 2020). Further identification based on cyanobacterium-specific markers has been suggested for more precise identification.

3.2. Impact of wastewater cultivation on pollutant removal, biomass production, and floatation-based easy harvesting

All the strains exhibited a promising potential to utilize wastewater as low-cost media for biomass production. Interestingly, all strains removed 100% of the suspended organic solids (8.9 gL^{-1}) from the urban wastewater in 4–5 days, during both indoor and outdoor cultivation. Accordingly, the appearance of the urban wastewater was changed from blackish to almost clear water. Besides, microscopic observation showed that suspended solids were attached with the surface of the cells (data not shown), which indicated that cells removed these suspended particles through adsorption. Previously, several studies have employed cyanobacteria to remove pollutants, but specifically the cyanobacterium *Synechococcus elongatus* was used to develop textile-based bio-composites which showed promising potential for a range of phyco-remediation applications (Hart et al., 2021). While in this study, microscopy of the strains cultured in the wastewater containing suspended solids showed that all strains removed the organic solids through bio-adsorption. Besides, BERC03, BERC04, and BERC06 removed total nitrogen by 53–54% and removed total phosphorus by 54%, 88%, and 63%, respectively. Wastewater cultivation improved the biomass production by 1.30-fold, 1.31-fold, and 1.24-fold when compared to control which respectively corresponded to the final biomass production of 1.88 g L^{-1} for BERC03, and 2.23 g L^{-1} for BEERC04 and BERC06 (Fig. 2A). The strains BERC03, BERC04, and BERC06 fixed 3.54 g L^{-1}, and 4.2 g L^{-1} of CO_2 when cultured in synthetic wastewater (Fig. 2B). Although biomass production was a bit compromised in urban wastewater when compared to synthetic wastewater yet BERC06 performed comparatively better than other cyanobacterial strains. It could be possibly due to the nutrient composition of the wastewater. The results indicated the suitability of the wastewater as low-cost cultivation for cyanobacterial production with additional environmental benefits. The findings were comparable to previously studied cyanobacteria where 1.68 g L^{-1} of *Scytonema hyalinum*, 2.58 g L^{-1} of *Tetraselmis*, 2.03 g L^{-1} of *Parachlorella*, and 2.06 g L^{-1} of *Synechococcus* was produced in wastewater (Aketo et al., 2020; Wu et al., 2020). Whereas, hydrogen carbonate supplementation or the efficient nutrient uptake could be the possible reasons for the higher biomass production in the present case.

Another interesting feature of the under-study cyanobacteria was the tendency to increase the pH of cultivation media to 10.2–10.7 on the final cultivation day (15th) which was a sharp increase from the initial pH of 8.0. This rise in pH helped to control the contamination of the invasive microbes (fungi and bacteria), and no contamination was observed during the batch cultivation under a microscope (data not shown). Besides, increasing pH helped easier floatation-based harvesting of the strains. This shift in pH from neutral to alkalinity could be attributed to the photosynthetic dissociation of hydrogen carbonate into CO_2 and OH^- in the carboxysome of cyanobacteria through the carbon-concentration mechanism (CCM) (Kishi et al., 2020). Such alkaliphilic cyanobacterial strains (especially filamentous) offer the benefits of contamination control (Shahid et al., 2019), effectual CO_2 utilization (Kishi et al., 2020), easy harvesting which may reduce capital cost, and require low-energy for harvesting (Canon-Rubio et al., 2016).

3.3. Impact of wastewater cultivation on biochemical composition of the biomass

The economic feasibility of algal biodiesel could be achieved by including some coproducts in the processing pipeline. Biomass characterization through biochemical analysis provides the catalog of primary and secondary metabolites. The biochemical component analysis of the understudy cyanobacteria indicated a tremendous increasing trend in the lipid biosynthesis of all strains when cultivated in wastewater. The

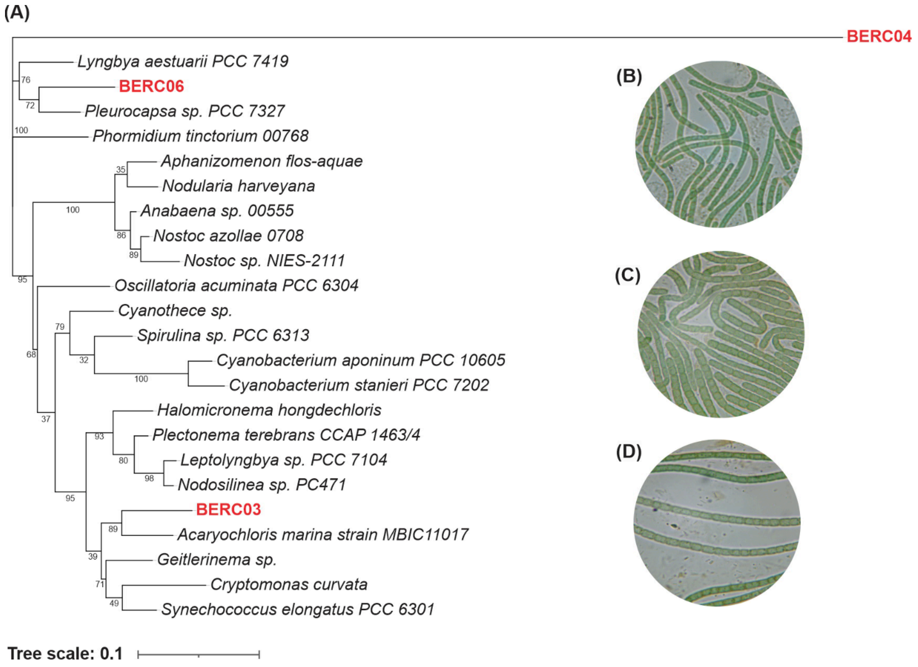

Fig. 1. The maximum-likelihood method based phylogenetic analysis of the newly-isolated strains namely (A) BERC03 (B), BERC04 (C), and BERC06 (D) based on the sequence of 23S rRNA genes.

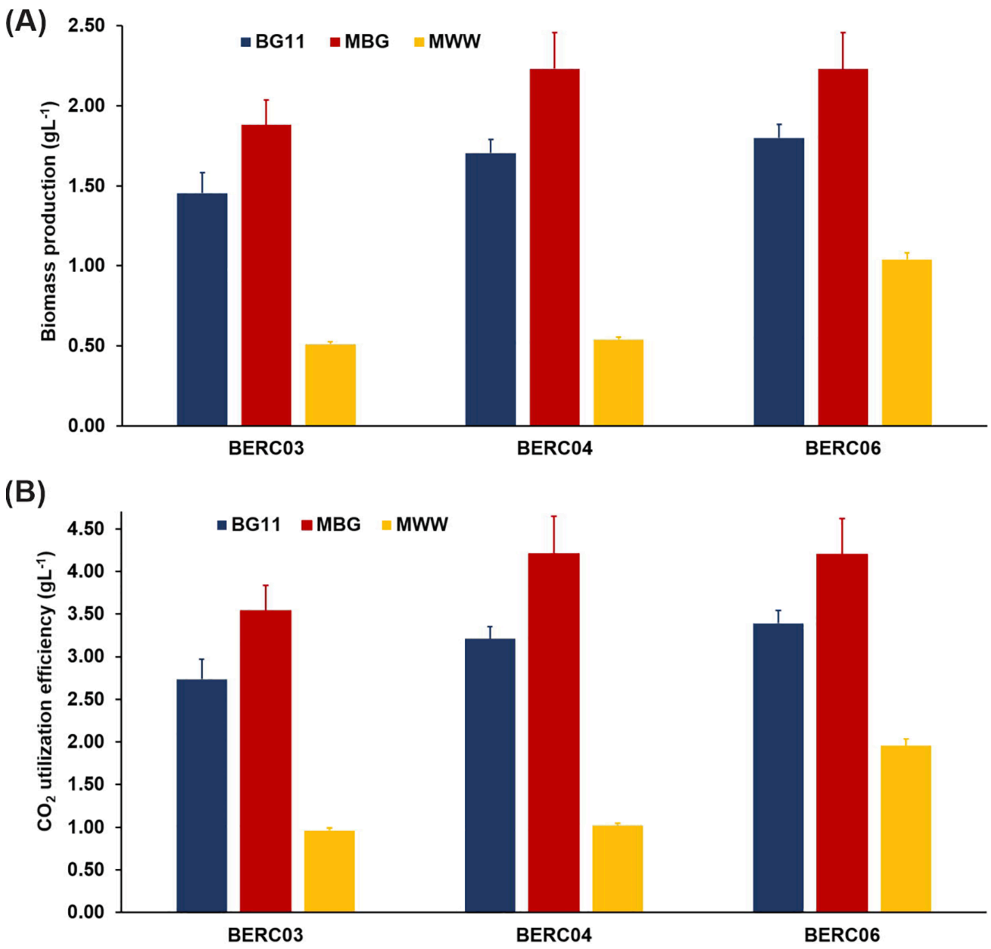

Fig. 2. Impact of wastewater cultivation (MBG; synthetic wastewater and MWW; Urban wastewater) on the (A) biomass production and (B) CO_2 utilization efficiency of the BERC03, BERC04, and BERC06. Each value is the mean of three replicates and error bar indicates standard deviation among them.

carbohydrate and protein content of all the strains was shown to be ranging between 26 and 36% and 15–28%, respectively, in response to various growth media (Fig. 3). However, the highest protein and carbohydrate production of 0.62 g L^{-1} and 0.63 g L^{-1} was reported in synthetic wastewater-cultivated by BERC06. These results indicated that nitrogen limiting conditions (control) favored the carbohydrate accumulation which was also observed for *Leptolyngbya* and *Synechococcus* that accumulated 43% and 54% of carbohydrate in nitrogen limiting condition (de Farias Silva et al., 2020; Papadopoulos et al., 2020). Impaired protein and carbohydrate content in wastewater is believed to be associated with the redirection of nitrogen assimilation towards lipid biosynthesis (Papadopoulos et al., 2020).

The maximum lipid content of 43% was produced by BERC04 followed by BERC06 and BERC03 who produced 41% and 39% lipids, respectively, in wastewater-cultivated biomass when compared to 35%, 37%, and 35% of the respective lipid content of control samples. Overall, the lipid content of the wastewater-cultivated strains BERC03, BERC04, and BERC06 was shown to be increased by 3%, 5%, and 4.5% respectively, and strains produced 0.73 g L^{-1}, 0.96 g L^{-1}, and 0.91 g L^{-1} of lipids, respectively. Higher lipid accumulation in wastewater may be attributed to the high carbon availability (0.6 g L^{-1} bicarbonate) in MBG and nitrogen limitation (due to its consumption during cultivation) which could have triggered Rubisco activity and activated pentose phosphate pathway which in turn promoted triglyceride biosynthesis (Patel et al., 2020). Similar propensity was observed in the case of *Chlamydomonas*, *Scenedesmus*, and *Spirulina* where correspondingly nitrogen limitation, bicarbonate supplementation, and wastewater cultivation improved the lipid accumulation (Mata et al., 2020; Pancha et al., 2015; Yang et al., 2018).

3.4. Impact of wastewater cultivation on the content and quality of biodiesel

In the perspective of bioenergy production, in addition to enhancement in lipid content, it is important to find biodiesel quality upgrading conditions. Evaluation of cultivation conditions on the biodiesel (FAME) composition is necessary to elucidate its bioenergy potential. The present study indicated the feasibility of wastewater to improve lipid content without affecting biodiesel quality. It was shown that wastewater-cultivation had no negative impact on the FAME composition of BERC03 and BERC04. While slight increment in PUFA (polyunsaturated fatty acids) from 15% to 19% was observed in wastewater cultivated by BERC06 (Fig. 4). Balanced FAME composition in biodiesel is of importance as unsaturated FAME-rich biodiesel can be easily oxidized while saturated FAME-rich biodiesel can be readily solidified (Nzayisenga et al., 2020). All the strains under study produced short-chain fatty acid (SCFA) comparable to the FME composition of *Chlorella vulgaris* between the range of C_{14}-C_{18}, which are the most preferred FA for biodiesel production (Church et al., 2017). FAME analysis showed the predominance of palmitic acid (C_{16}) followed by oleic acid (C_{18}) and myristic acid (C_{14}) as major FA (Fig. 4).

Wastewater cultivation had a positive impact on the FAME yield of the BERC03 while showed a slightly negative impact on the FAME yield of the BERC04 and BERC06. The highest palmitic acid yield of 34 mg g^{-1} was produced in BERC06 followed by 31.7 mg g^{-1} in BERC04 and 30 mg g^{-1} in BERC03. Similarly, wastewater cultivated *Neochloris aquatica* and

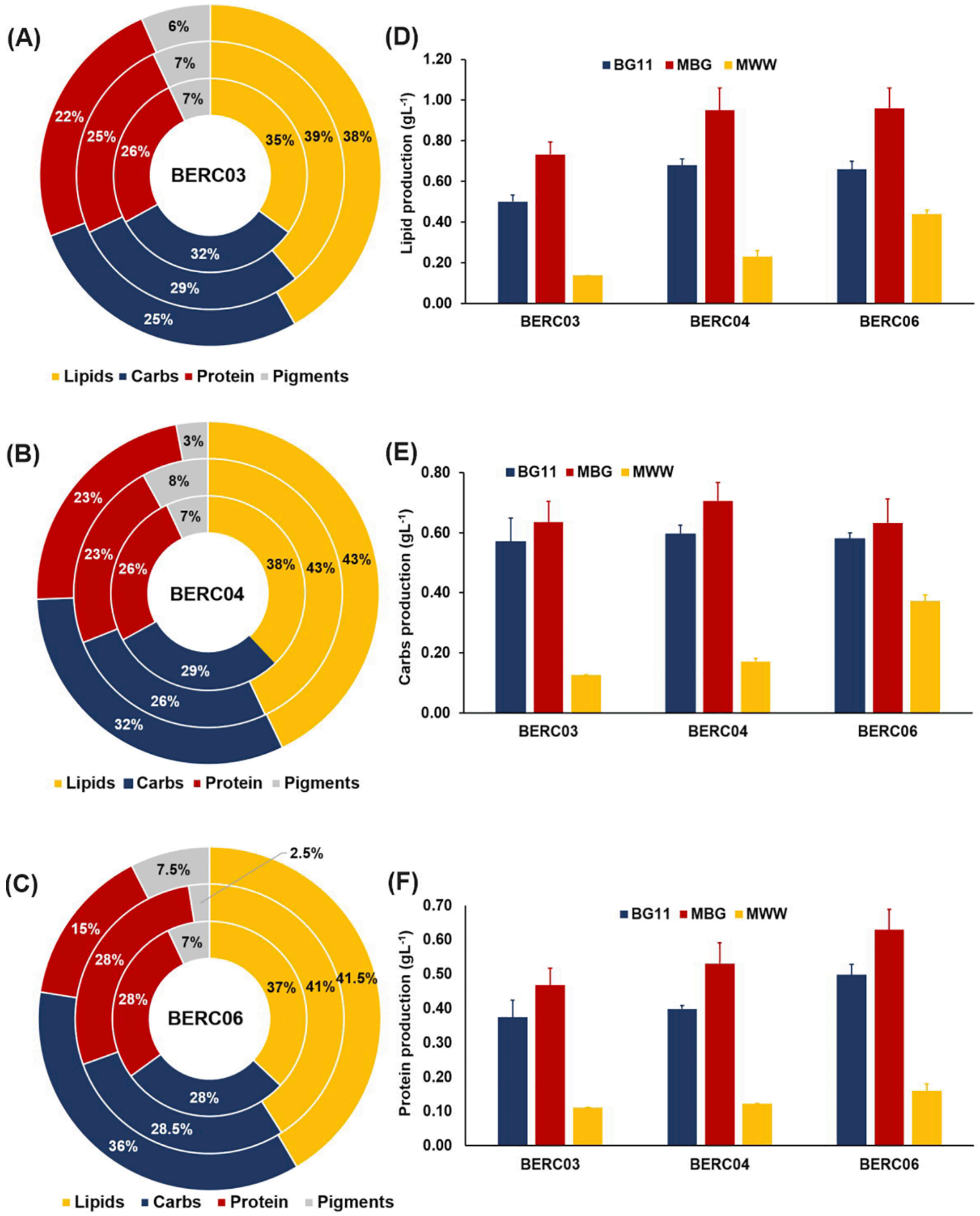

Fig. 3. Impact of wastewater cultivation (control, synthetic wastewater, and urban wastewater inner to outer circle) on the biochemical composition (A-C), and metabolite production in gL^{-1} (D-F) of the cyanobacterial strains. Each value is the mean of three replicates and error bar indicates standard deviation among them.

Asterarcys quadricellulare produced palmitic acid and oleic acid, as dominant FA (Odjadjare et al., 2018). Likewise, wastewater cultivated *Plectonema terebrans* produced 13.07 mg g^{-1} and 2.61 mg g^{-1} of palmitic acid and oleic acid, respectively (Shahid et al., 2020). The higher content of palmitic acid in biodiesel signifies the higher cetane number, high oxidative stability, and lower NOx emission while, the presence of oleic acid in biodiesel imparts balanced fuel properties like viscosity, combustion heat, lubricity, ignition quality, and oxidative stability. These properties indicate the suitability of biomass for high-quality biodiesel production (Arif et al., 2021).

3.5. *Impact of wastewater cultivation on biodiesel properties*

It is also important to study the impact of fatty acid composition and content on the fuel properties of biodiesel. Biodiesel compositional analysis was performed based on the predominant biodiesel fatty acids (Table 2). Ideally, biodiesel should contain myristic acid (C14:0), palmitoleic acid (C16:1), and oleic acid (C18:1) in a 1:5:4 ratio (Schenk et al., 2008) for a balanced composition. However, in our study, these fatty acids were in 1:6:10 ratio, respectively. The results are in accordance with the fact that algal biodiesel is rich in oleic acid, however, it signifies the need for biodiesel blending or alteration in fatty acid composition for perfect proportion (Loh et al., 2021).

Biodiesel compositional analysis showed that wastewater cultivation does not have any negative impact on the fuel quality of the obtained algal biodiesel from the three cyanobacterial strains (Table 2). Results showed that the quality of the biodiesel followed the set limits of American (D6751ASTM), and European (EN14214) biodiesel. Iodine value is an important fuel parameter to determine the unsaturation of biodiesel while cetane number is used to access the ignition quality measured by combustion performance. Wastewater cultivation slightly increased the iodine value, however, all the strains had iodine value within the set limit ($\leq$120 g I_2). Similarly, the cetane value was estimated to be within the range of 50.3–60.1 which is shown to be near the European (>51) and American (>47) biodiesel quality limits. Biodiesel with a higher cetane number is good for blending with traditional

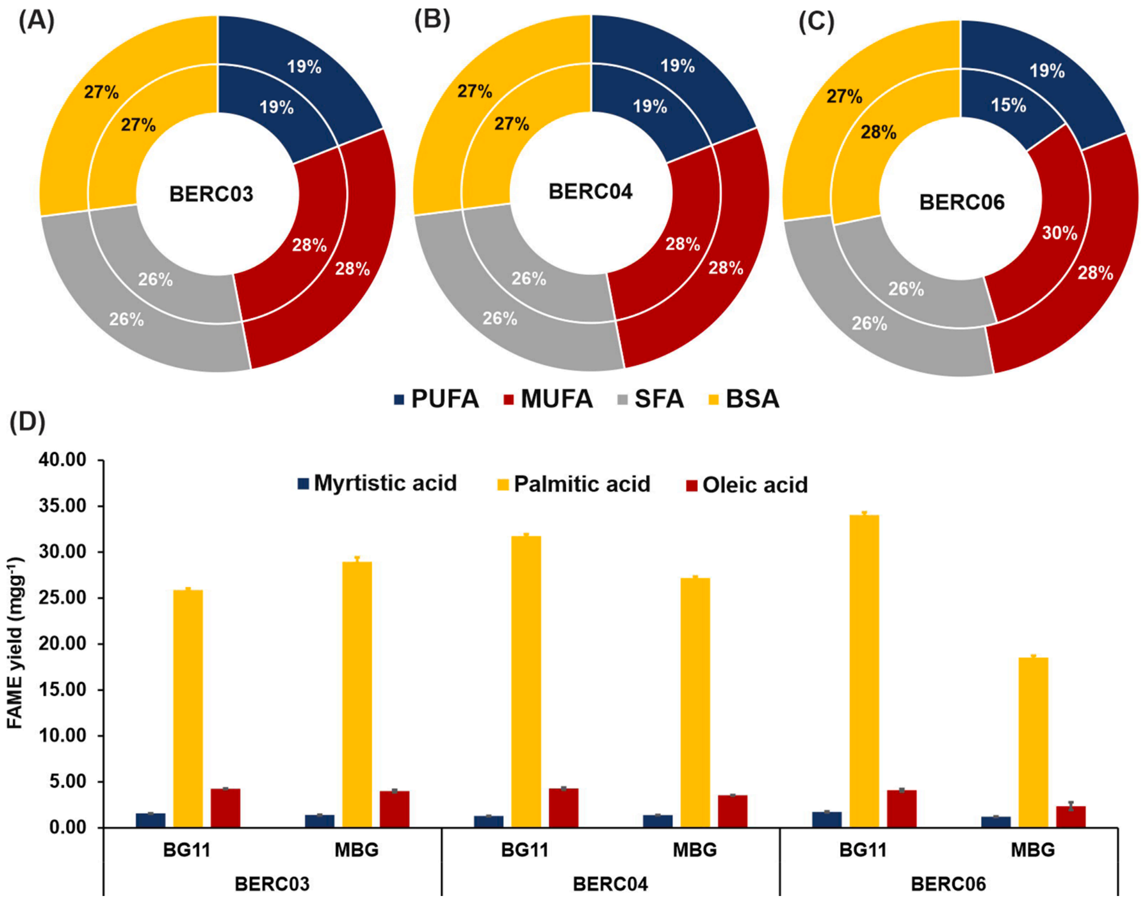

Fig. 4. FAME compositional analysis of control (the inner circle) and wastewater cultivated cyanobacteria (the outer circle) based on quality (A-C) and quantity (D) of the biodiesel. Each value is the mean of three replicates and error bar indicates standard deviation among them.

Table 2
Fuel properties of the biodiesel produced from the lipids obtained from the wastewater-cultivated cyanobacteria.

Biodiesel properties (Unit of the quality parameter)	*Acaryochloris marina* BERC03		*Oscillatoria* sp.BERC04		*Pleurocapsa* sp.BERC06		Algal biodiesel	European 14,214	ASTM D6751
	Control	Wastewater	Control	Wastewater	Control	Wastewater			
Saponification value(mg KOH/g)	217.7	218.4	192.2	213.9	178.2	192.3	–	–	–
Iodine value (gI$_2$)	91.5	93.3	82.4	90.9	74.6	79.4	–	≤120	–
Cetane number	50.8	50.3	56.1	51.3	60.1	56.8	71.2	>51	>47
Higher heating value (MJ/kg)	41.1	41.2	36.2	40.3	33.4	35.9	24–40	–	–
Cloud point (°C)	−16.5	−16.5	−16.5	−16.5	−16.5	−16.5	−15 to 2	–	3
Pour point (°C)	−4.99	−4.99	−4.99	−4.99	−4.99	−4.99	−14	3.0–15.0	–
Cold filter plugging point (°C)	−12.2	−12.2	−12.2	−12.2	−12.2	−12.2	−8 to −10	–	–
Viscosity (mm/s)	3.91	3.90	3.29	3.75	2.98	3.22	3.83	3.5–5.0	1.9–6.0
Density (g/cm^3)	0.92	0.92	0.81	0.90	0.74	0.80	0.80	0.86–0.9	n.a

biodiesel as it provides a smooth run with good cold start properties (Sinha et al., 2016). In terms of fuel quality, cold flow properties including cold-filter plugging point (CFPP), pour point (PP), and cold point (CP) are of importance to evaluate the feasibility of the biodiesel to be used in cold countries. Though the European standard does not mention the minimum temperature in its list of specifications, it suggested evaluating biodiesel in terms of CFPP values. The result indicated that wastewater does not influence any of the cold-flow properties and the obtained values are below 0 °C as recommended by the standards.

According to the European biodiesel standard, the content of FA (containing more than four double bonds) should be less than 1% while, linoleic acid (C18:3) should be less than 12%. Both conditions were met in the present study as all the detected FA contain a maximum of two double bonds while linolic acid was not detected at all in any of these samples. Fuel properties of the biodiesel produced from the lipids of wastewater-cultivated by BERC03, BERC04, and BERC06 were found to be better when compared to the wastewater-cultivated *Scenedesmus* sp. (Álvarez-Díaz et al., 2015) and nitrogen-starved cultures of *Chlorella* and *Chlorolobion* sp. (Arguelles & Martinez-Goss, 2021) which is possibly due to better FAME profile of the wastewater-cultivated cyanobacteria. However, further improvement in the fuel properties is recommended as in current form the produced biodiesel does not seems suitable as standalone biodiesel.

3.6. *Impact of wastewater cultivation on the biosynthesis of high-value bioproducts*

Cyanobacteria produce phycobilin protein (PBP) in addition to the photosynthetic pigments as light-harvesting molecules to enhance the photosynthetic efficiency of the cyanobacteria. In the present study, it was shown that in all instances chlorophyll *a* was produced as major pigment followed by chlorophyll *b* and carotenoids (Fig. 5A). In the case of BERC04 and BERC06, wastewater cultivation improved the pigment yield while an opposite trend was observed for BERC03. Similarly, the bicarbonate supplemented *Tetraselmis* (White et al., 2013) and wastewater cultivated *Scenedesmus* (Fan et al., 2020) produced more chlorophyll as compared to their counterparts. An increase in the pigment content might be due to hydrogen carbonate supplementation and additional nutrients that provided the basic material for the energy-rich molecules (ATP and NADPH) that improved the photosynthetic efficiency and biomass production. In wastewater-cultivated by BERC03, photosystem II degradation might have happened which resulted in a decreased pigment yield (Wang et al., 2020) which indicated a lower stress-tolerance threshold of this strain when compared to other strains.

Phycobilin proteins (PBPs) are colored pigments of great commercial importance due to their antioxidant, nutraceutical, and color-inducing properties. Furthermore, these compounds aid in the photosynthesis process to fill in the photosynthetic gaps of the chlorophyll. Interestingly, wastewater cultivation improved the PBP yield of BERC06 because the highest PBP yield of 102 mg g^{-1} was shown in the urban wastewater-cultivated sample which was 1.07-fold higher than control and 1.63-fold higher than the synthetic wastewater cultivated sample. However, in the case of BERC03, and BERC04 urban wastewater had a negative effect on the phycobilin yield while in response to synthetic wastewater the PBP yield of BERC03 was improved from 65 mg g^{-1} to 90 mg g^{-1} and BERC04 from 65.5 mg g^{-1} to 89 mg g^{-1}. Phycocyanin was produced as a major PBP component with 39–79 mg g^{-1} of phycocyanin (PC) yield was observed for BERC03, BERC04, and BERC06 in response to wastewater cultivation (Fig. 5B) which is higher than 13–18 mg g^{-1} PC yield of cyanobacterial consortia (Arashiro et al., 2020), and 45 mg g^{-1} of PC yield by *Euhalothece* sp. (Mogany et al., 2018) in response to wastewater cultivation. PBP degradation to fulfill the nutritional requirement (Li et al., 2020) for high biomass production could be the possible reason for the reduced PBP yield of wastewater grown BERC03 and BERC04.

3.7. A cascading biorefinery integrated with wastewater treatment plants

Cyanobacteria-based biorefineries integrated with the wastewater treatment plants offer promising potential to enhance the profitability and sustainability of the water-energy-environment nexus. Future biorefineries are supposed to employ bioengineered cyanobacteria for the biological fixation of atmospheric carbon into food/feed, biofuels, and a range of high-value bioproducts (Jaiswal et al., 2020). Although cyanobacteria have a remarkable potential to become the key players of the integrated biorefineries yet there is a huge research gap in the exploration, evaluation, and effective utilization of this valuable biological resource keeping in view the geographical diversity in climate, local resources/needs, and varying policies. Hence, evaluating novel cyanobacterial strains is frowningly important to explore and harness their potential for practical applications at the large scale. The findings of the current study have shown that three cyanobacterial strains namely *Acaryochloris marina* BERC03, *Oscillatoria* sp. BERC04, and *Pleurocapsa* sp. BERC06 have promising potential to grow in the untreated urban wastewater, where all strains removed 100% of the suspended organic solids via biosorption. Besides, the two kinds of biomasses were obtained here, one which contained the bioadsorbed-organic matter, and the other which was pure biomass. The pure biomass showed to accumulate valuable metabolites including pigments, lipids, protiens, and carbohydrates. Hence, we propose a bioprocessing pipeline to establish an integrated biorefinery using these cyanobacteria (Fig. 6). It is proposed that, at first step, strains should be cultivated in the non-treated (unsterilized) urban wastewater for 4–5 days to remove the suspended solids, and biomass obtained (which also contains organic solids) can be mixed with the agricultural soils to improve soil fertility. The spent media can now either be sent to a wastewater treatment plant or can be used to culture the same cyanobacteria to produce more biomass. The data demonstrated that cultivation of the cyanobacterial strain removed

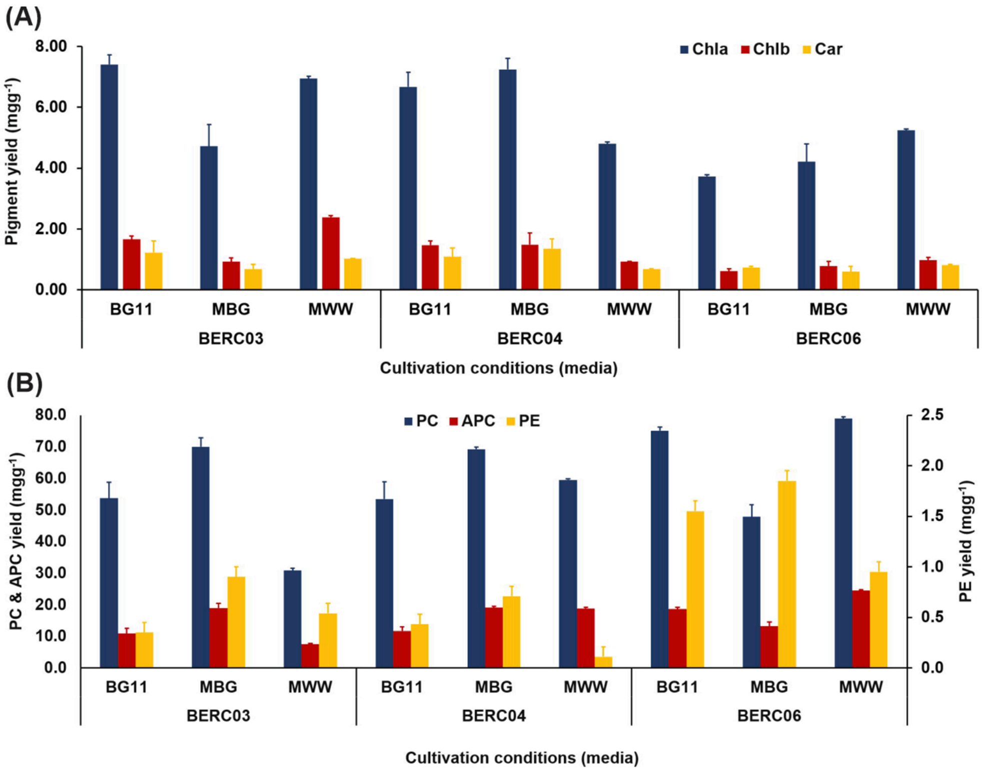

Fig. 5. Impact of wastewater cultivation on the (A) pigment (chlorophyll *a*, chlorophyll *b*, and carotenoid) yield and (B) phycobilin (phycocyanin, allophycocyanin, and phycoerythrin) yield of the filamentous cyanobacteria. Each value is the mean of three replicates and error bar indicates standard deviation among them.

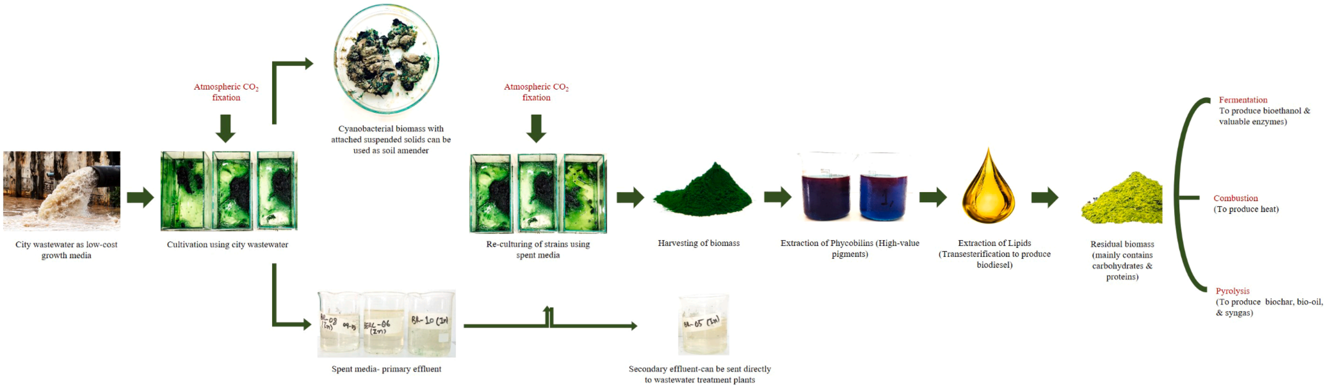

Fig. 6. A proposed scheme to establishing a multiproduct cascading biorefinery integrated with wastewater treatment plant.

53–54% of total nitrogen and 63–88% of total phosphorus while producing 1.88–2.23 g L^{-1} of biomass and fixing 3.54–4.20 g L^{-1} of the atmospheric carbon, which seems more sustainable when compared to the direct treatment of the wastewater in a treatment plant. In the third step, the biomass should be subjected to extraction of phycobilins (mainly phycocyanin) which are high-value pigments and suitable for applications in the food and pharmaceutical industry. These pigments are extracted by using low-strength sodium phosphate buffer and have no negative interference with the subsequent utilization of the biomass in the downstream processing. In the fourth step, the pigment-extracted biomass should be subjected to lipid extraction, the data showed that pigment-extraction had no negative impact on the quality or quantity of the lipids and biodiesel. Finally, the residual biomass which mainly contained proteins and carbohydrates can be employed for myriads of applications including biological or thermochemical conversion to bioethanol, bio-oil, biochar, syngas, or can be used as animal-feed supplement, however, it needs further detailed studies to make the right choice. This approach may help to enhance the environmental and economic sustainability of the biorefinery and wastewater treatment plants. However, further pilot-scale studies are required in the future for process optimization and fine-tuning.

4. Conclusions

Developing integrated biorefineries require exploring new cyanobacterial strains capable of promising growth potential in wastewater while accumulating a spectrum of bioproducts to achieve cost-effectiveness. Here, newly isolated cyanobacteria including *Acaryochloris marina* BERC03, *Oscillatoria* sp. BERC04, and *Pleurocapsa* sp. BERC06 showed contamination-free considerable biomass production, along with wastewater treatment and CO_2 fixation. Interestingly, wastewater cultivation significantly improved the yield (>40%) and quality of the biodiesel along with 63–90 mg g^{-1} of high-value phycobilins (~48–70 mg g^{-1} of phycocyanin). Based on the data, a cascading biorefinery approached to produce high-value pigments, high-quality biodiesel, and biological/thermochemical conversion of residual biomass to bioenergy is proposed.

CRediT authorship contribution statement

Ayesha Shahid: Investigation, Methodology, Writing - original draft. **Muhammad Usman:** Data curation, Methodology. **Zahida Atta:** Methodology. **Syed Ghulam Musharraf:** Software, Writing - review & editing. **Sana Malik:** Data curation, Methodology. **Ali Elkamel:** Writing - review & editing. **Muhammad Shahid:** Formal analysis. **Nuha Abdulhamid Alkhattabi:** Data curation. **Munazza Gull:** Formal analysis. **Muhammad Aamer Mehmood:** Conceptualization, Supervision, Resources, Writing - review & editing.

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.

Acknowledgments

Authors are obliged to Higher Education Commission, Pakistan for providing funds to Ms. Ayesha Shahid under Indigenous Ph.D. Fellowship program.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2021.125194.

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