Article Type: Review
J Name: Modern Phytomorphology
Short name: MP
ISSN: ISSN 2226-3063/ eISSN 2227-9555
Year: 2024
Volume: 18
Page numbers: 01-09
DOI: 10.5281/zenodo.200121
(10.5281/zenodo.2024-18)
Short Title: An investigative study of microalgae for an alternative of biofuel and PHA production

REVIEW ARTICLE

An investigative study of microalgae for an alternative of biofuel and PHA production

Chamundeshwari, Christy Chacko, Likitha G M, Muhammed Subin Siyas and Srividya Shivakumar*
Department of Microbiology, School of Sciences, Jain (Deemed-to-Be-University), Sudhama Nagar, Bangalore, India

***Corresponding author:** Srividya Shivakumar, Department of Microbiology, School of Sciences, Jain (Deemed-to-be-University), Sudhama Nagar, Bangalore
Email: sk.srivdya@jainuniversity.ac.in

Received: 29.10.2024**,** Manuscript No**:** mp-24-151360**| Editor Assigned:** 01.11.2024**,** Pre-QC No. mp-24-151360 (PQ) **| Reviewed:** 16.11.2024**,** QC No. mp-24-151360 (Q) **| Revised:** 22.11.2024**,** Manuscript No. mp-24-151360 (R) **| Accepted:** 28.11.2024**| Published:** 04.12.2024

Abstract

Similar to synthetic polymers, Polyhydroxyalkanoates are synthesized by microorganisms and are fully biodegradable. Polyhydroxyalkanoates or PHAs may be produced similarly to synthetic polymers and are considered as an alternative for petroleum-based plastics. Bacteria and significant volumes of organic carbon sources in the culture medium account for 50% of the entire cost of producing commercial PHAs. Studies on microalgae that promote PHA production are still in their infancy. Associating elements included 0.52 g/l PHB and 1.86 g/l lipids. This isolate was selected for genetic and morphological identification. *Oxenochlorella pyrenoidosa* was recognized as the isolate by phylogenetic study and molecular characterization. By enhancing the microalgal strain and maximizing growth conditions, the production cost will be reduced even further (such as light intensity, pH, and temperature). This study aims to explore the possibilities of petroleum-based plastics as a substitute of PHAs by composition and characteristics of biopolymers.

Keywords: Bioplastic, Bacteria, Microalgae, Polymers, Polyhydroxy butyrate (PHB).

Introduction

Plastic pollution is especially evident in underdeveloped Asian and African countries where garbage collection systems are occasionally ineffective or nonexistent as the world's capacity to cope with the rapidly rising demand for disposable plastic products along with the extensive search for alternative fuels like biodiesel overwhelms the globe's capacity to deal with them (Gao et al., 2011; Bomfim et al., 2022). However, the industrialized world has trouble adequately collecting used plastics, especially in nations with low recycling rates. As a result, plastic pollution has grown to be a major issue in both developing and wealthy nations. Recently, there has been a lot of focus on creating innovative, sustainable ways to make bioplastics like PHA and biodiesel from affordable raw materials (Dong et al., 2020).

Polyhydroxybutyrate (PHB):

Plastic has become an essential component of human civilization and plays a crucial function in the home, industry, or healthcare sectors. Plastic's inability to biodegrade causes serious environmental dangers. So, Polyhydroxy Butyrate (PHB), an environmentally benign bioplastic, has the potential to replace traditional plastics. Since fermentation

requires expensive sugar, PHB produced by bacteria is quite expensive. Cyanobacteria, which are genuine bacteria, create PHB intracellularly or store it as a source of carbon and energy utilizing sunlight but also carbon dioxide. When compared to an alternative or other prokaryotes, PHB from cyanobacteria is of higher quality. Under ideal conditions, a wide variety of naturally occurring and genetically altered cyanobacteria accumulate PHB on a lab scale, but only a small number of studies have documented large-scale PHB production (Arias et al., 2020; Winnacker et al., 2019).

Polyhydroxy Butyrate (PHB), a hydrophobic chemical, is identified by the presence of an alkyl substitution group for methyl. PHB is a biocompatible polymer, thus it is not rejected by the body. Furthermore, its absorption results in residues that are safe. It is frequently produced chemically or biologically to create this biodegradable polymer. Numerous bacteria, cyanobacteria, or microalgae may prefer to intracellularly generate PHB as a source of energy (Jiang et al., 2016; Riaz et al., 2021). PHB may be a delicate substance, which reduces the range of applications for it. Making blends of synthetic biodegradable polymers, non-biodegradable polymers, as well as components from natural sources is crucial to improving the physical, mechanical, or thermal characteristics of such materials (Arias et al., 2020; Di Caprio et al., 2022).

Recent developments in PHB-based composites also offer fresh insights on their potential uses in bioremediation, food packaging, agriculture, and health. In this fashion, the paper discusses the chemical, physical, and biological properties of PHB as well as the primary chemical and biologic syntheses that are used to produce this polymer. The uses of these materials in many fields are also addressed in this paper, as well as methods for using PHB in composites or blends. Due to the usage of expensive sugar in the fermentation process, PHB from bacteria is highly expensive; thus, there is a need for a more beneficial and affordable alternative source, which may come from microalgae.

Literature Review

Grubisic et al. studied diverse bioactive chemicals that may be found in marine microalgae and cyanobacteria, which have potential biotechnological uses in the food, pharmaceutical, feed, cosmetic, nutraceutical, or biofuel sectors. Neutral lipids made up a significant portion of *Nanofrustulumchili* D1's biomass (68.36%) while glycolipids but also phospholipids made up the majority of *Picochlorumspecies*. D3, *Tetraselmis sp*. Z3, *Tetraselmis sp.* C6, or *Euhalothece sp.* C1's biomass (75%). The lipids of all the microalgae being studied include a significant amount of unsaturated fatty acids. Lutein, neoxanthin, and fucoxanthin were the pigments with the highest quantities in Chlorophyta members or *Bacillariophyta strains*, respectively. The majority of the pigments were carotenoids. In comparison to Gram-positive *S. aureus* and Gram-negative *E. coli* or *S. typhimurium*, all microalgal extracts shown antibacterial and antioxidant activity (Grubisic et al., 2022).

Eleni Koutra et al. studied Development and research on renewable, innovative, and sustainable sources that have become necessary as a result of the recent rise in demand for food, energy, or precious chemicals. Microalgae provide a viable alternative for producing a range of goods with ecologically favorable uses. However, several obstacles must be removed to lower manufacturing costs. The quality and efficiency of the biomass as well as the particular goal products, as well as any additional steps or cost-effectiveness, must all be taken into account. Further study will be necessary to completely use the produced biomass and properly scale up the procedure (Koutra et al., 2018).

S. Magdouli et al. studied Co-culture to produce lipids. Co-cultures have undergone extensive research to solve individual strain constraints in substrate utilization for other bioproduct syntheses. As a result, there are numerous questions concerning how this method may affect lipid production. The co-culture technique has only been thoroughly studied in algal species, even though oleaginous microorganisms have been the subject of substantial study, and the majority of the original research has focused on the various nutritional development modes (e.g. heterotrophic or mixotrophic). Furthermore, the available literature shows a lack of knowledge on methods for increasing lipid synthesis using species other than microalgae. This study will emphasize co-culture systems that are currently used for increased lipid and biomass production, among other species, from a systematic approach (Magdouli et al., 2016).

Martin Koller et al. studied Microalgae as adaptable cellular producers of valuable goods. Many microalgal species have great nutritional value in addition to providing ecological benefits, and they also produce valuable bio-products: The potential for effective market penetration includes pigments, lipids, bioactive substances, specific polysaccharides, bio-hydrogen, and even polyesters with plastic-like qualities. For light absorption and CO_2 fixation, three significant pigment groups—chlorophylls, carotenoids, and phycobilins are required. In cosmetics, aquaculture, “functional food”, medicines, or food technology, such pigments will probably achieve fast commercial success (Koller et al., 2014).

Methodology

Design

This study is designed for the production of microalgae for biofuel and PHA production. For this study, the author performs various tests, including the isolation and screening of microalgae from freshwater, lipid estimation, and screening for PHB via Sudan black. Biodiesel and PHA are seen as potential substitutes for conventional fuels and conventional plastics, respectively, in response to the growing global demand for bio-based goods. These bio-based goods reduce environmental pollution and are sustainable and biodegradable. The main barrier to the development of bio-based goods is the higher production cost.

Sample

The microalgae samples were collected from various freshwater sources in plastic tubes and labeled as S1, S3, S4, S7, S8, S9a, S9b, and S15, as shown in tab. 1 and 2. The samples were stored in refrigerated conditions till further use as shown in fig. 1.

Table 1. **Sample collection details.**

Sample	Collection Site	Sampling Period
S1	Garden pond	December
S3	Near the paddy field	December
S4	Waterfall	December
S7	Nursery ground	December
S8	Public water tank	December
S9 a & b	Lotus pond	December
S15	Pond	December

Figure 1. **Sample collection sites. a) Garden pond, b) near the paddy field, c) Waterfall, d) Nursery pond, e) Public water tank, f) Lotus pond, g) Pond.**

Enrichment of samples: The collected algal samples were enriched using BG 11 medium. 1 ml of the sample was inoculated in 100 ml of sterile BG 11 broth and incubated for 14 days at room temperature under the photoperiod of light and dark.

Table 2. Illustrate the Composition of BG11 media.

Constituents	Weight (in g)
$NaNO_3$	1.5
$CaCl_2.2H_2O$	0.036
$MgSO_4.7H_2O$	0.075
K_2HPO_4	0.04
Na_2CO_3	0.02
Ferric ammonium citrate	0.006
Citric acid	0.006
Na_2CO_3	0.02
Agar	10
Trace metal mix	1ml

Data collection

Isolation of microalgal strains: Serial dilution was used to extract the algal cultures, which were then plated using the spread plate method on BG 11 agar. The isolated colonies were picked and sub-cultured on BG 11 agar medium through quadrant streaking and incubated for 14 days at room temperature.

Culture conditions: 1ml of each pure sample was inoculated into 100 ml of BG 11 broth and exposed to natural light (1000 lux) and incubated for 14 days at room temperature in triplicates.

Screening for PHB-producing algal cultures: Algal samples were placed on a clean glass slide. Smear was prepared and air-dried and heat-fixed. The slides were dipped in Sudan Black B stain for 10 mins-15 mins at room temperature. Later the slides were destained with xylene. The smear was then destaining and counter-stained with safranin for a minute. The slides were dried by air after being cleaned with distilled water. Then, slides were examined while immersed in oil (1000X).

Data analysis

Evaluation of growth and biomass: Dry cell Weight: A gravimetric analysis was used to assess the microalgal sample's biomass content. A 10 ml sample of an algal culture was centrifuged at 10,000 rpm for 15 minutes. The cell pellets were further dried at 50°C in a hot air oven overnight after the supernatant was removed. The final weight was calculated based on the weight of the dried algal samples. The initial weight was the measured weight of the empty Eppendorf tube. The total dry cell weight is calculated from the difference between the beginning and final cell weights.
Measurement of algal growth: An algae culture of 14 days was used to measure the growth by subjecting the samples to 680 nm and the OD was recorded. The control was used as a blank.

Lipid extraction: An algal culture in 10 ml was centrifuged at 8000 rpm for 10 minutes. Following this, the cell pellets were centrifuged at 8000 rpm for 10 minutes with 10 ml of ice-cold 0.2 perchloric acid. After that, the supernatant was discarded. The pellet was treated with a 2:1 solution of chloroform and methanol, followed by a 10-minute centrifugation at 8000 rpm with the supernatant removed. After adding 2 cc of distilled water, the supernatant underwent another centrifugation at 8000 rpm for 10 minutes. The lower organic phase was removed and dried by evaporation. After the plates had evaporated, they were weighed.

Standardization of PHB: Commercial PHB weighing 0.01g was added to 10ml of chloroform, which was then heated in a water bath at 65°C–70°C until it became translucent. A stock solution containing 1 mg/ml PHB is the outcome. A glass stopper was positioned on top of the tube during heating. A brand-new tube was filled with the 1 mg/ml stock and 9 ml of chloroform before being heated to between 65°C to 70°C and vortexed. The tubes were secured with glass stoppers, and a

10ml solution of sulfuric acid was then added. The tubes were then heated in a water bath for 20 minutes at a temperature between 94°C and 96°C to completely convert PHB to crotonic acid. The PHB was found by measuring the absorbance at 235 nm with a spectrophotometer.

PHB extraction: A 100 cc culture sample was centrifuged for 15 minutes at 10,000 rpm. 1 cc of methanol was added and left overnight at 4°C after the supernatant was removed. The material was centrifuged for 15 minutes at 10,000 rpm. The pellets were then given 10ml of a 4% sodium hypochlorite solution, which was then incubated for two hours at 30°C after the supernatant was discarded. The material was centrifuged for 15 minutes at 5000 rpm after incubation. After discarding the supernatant, the pellets were centrifuged after being cleaned with distilled water and methanol. After being dissolved in 5 cc of boiling chloroform, the pellets were left to evaporate. Sulfuric acid (5 ml) was added and allowed to boil in a water bath. As a control, sulfuric acid was utilized. At 235 nm, the absorbance was measured.

Identification of algal cultures: Morphology: The morphology was studied using a light microscope. A drop of algal culture was placed on the clean grease-free slide and covered with the coverslip and it was observed under oil immersion.
Gene sequencing: The 18S rDNA sequence was used to further identify the microalgae isolates, and genomic DNAs were extracted from different microalgae strains using the DNA separation technique. The genomic DNA of green algae was amplified using standard 18srDNA primers. Using MEGA software, PCR amplification or phylogenetic analysis were carried out.

Results and Discussion

Isolation and screening of microalgae from freshwater

Isolation: A total of eight samples collected from different sources were isolated with serial dilution followed by the spread plate technique on BG-11 media. Further, the obtained colonies were sub-cultured and incubated for 14 days at room temperature to obtain pure cultures. Each of the isolates showed robust growth under natural phototrophic conditions of temperature (20°C-25°C), in the laboratory. The isolated small colonies started appearing on the fifth day of incubation. A proper algal colony was visible on the 14th day of incubation (Battista et l., 2020; de Paula et al., 2017).

The composition of the media is a major factor that influences the production of high biomass and lipid content in algae. For the isolation and cultivation of microalgae, various media with varying compositions have been developed. BG-11 media is a universal medium for microalgae cultivation and maintenance that does not significantly reduce biomass and lipid productivity. BG-11 was the most cost-effective and efficient medium for microalgae cultivation, as shown in fig.2 and 3. Different microalgae isolates were isolated from the samples and were subjected to preliminary staining. An isolate with the highest lipid content was selected for identification and molecular characterization.

Figure 2. Microalgae slant cultures.

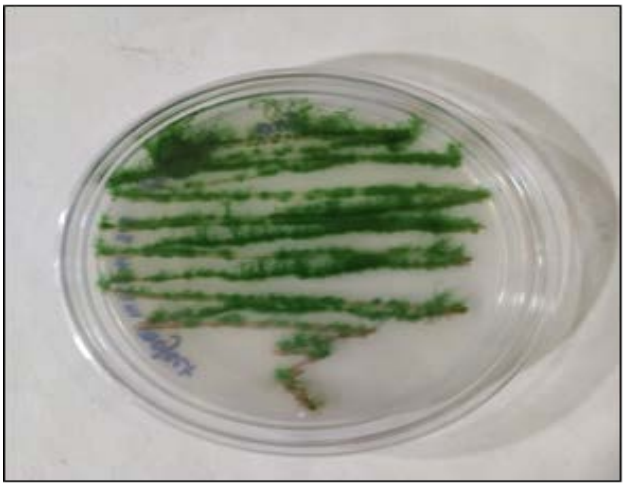

Figure 3. Microalgae growth in the Petri plate.

Screening for PHB via Sudan Black: The screening of the isolates was done with Sudan black staining techniques for the production of PHB. Sudan dyes are lysochrome pigments that may dissolve in lipids and lipid-based solvents. Lysochrome dyes that are soluble in fats and lipids as well as lipid solvents are classified as Sudan groups of dyes. They are diazo dyes structurally speaking, which are dyes used commercially to colour fabrics, clothing, and plastic (Hierro-Iglesias et al., 2021; Kim et al., 2021).

Sudan Black B is a slightly basic dye that stains the phospholipids, lipoproteins, or triglycerides present in the staining material by reacting with the acidic groups in lipid molecules. The PHB granules were identified due to their affinity for the dye Sudan Black, which is a presumptive test for PHB presence. The stained preparations were examined under a compound microscope with an oil immersion lens to determine cellular PHB accumulation. The PHB granules were black, while the cells were pink. 2018 (Robert and Iyer). All the isolated isolates showed the presence of PHB hence, all the isolates were subjected to various growth parameters analyses like cell density, dry biomass production, PHB, and lipid content.

Biomass concentration: On the 14th day of incubation, the growth of the algal cells in the medium was monitored at absorbance (680 nm). The S9b sample had the largest biomass, with an OD value of 0.76, while the S9a sample had the lowest biomass, with an OD value of 0.31. Microalgae species can develop in a photoautotrophic, light-dependent, heterotrophic, or mixotrophic, both inorganic and organic, manner in the presence of light, organic carbon, or even both. Carbon-based manner. One method to produce high cell-density biomass with a high lipid content for high-quality biodiesel feedstock is to grow algae in a heterotrophic mode.

Table 3. Illustrate the biomass concentration at 680 nm.

SAMPLES	OD AT 700 nm
S1	0.54
S3	0.68
S15	0.74
S8	0.5
S9b	0.76
S9a	0.31
S7	0.45
S4	0.6

In comparison with the other isolates, the S8 isolate showed high PHB content. It showed around 0.52 g/L of PHB which is highest when compared to the other isolates, followed by S9b and S9a which showed 0.47 g/L and 0.4 g/L of PHB content respectively, as shown in tab. 3. The least PHB content was observed in S1 (0.04 g/L), as tabulated in fig. 4.

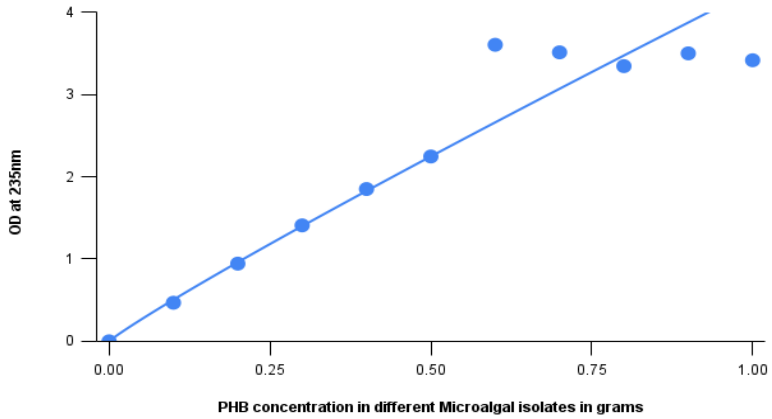

Figure 4. Standard graph of PHB.

Lipid estimation: The highest lipid content was generated by S8 (1.86 g/L), and the lowest lipid was produced by S9b (1.00 g/l) as mentioned in fig. 5 and tab. 4.

Table 4. Lipid weight of the samples.

Samples	Lipid Weight g/L
S1	1.09
S3	1.17
S4	1.2
S7	1.17
S8	1.86
S9a	1.19
S9b	1
S15	1.7

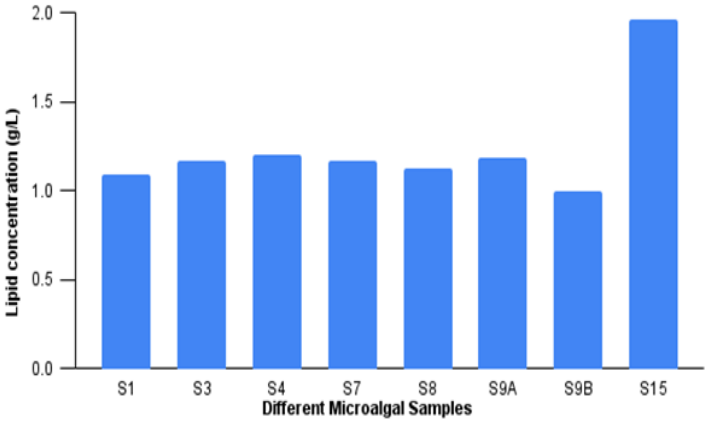

Figure 6. Lipid concentrations in different microalgae isolates.

Identification of the potential strain: The selection of strains based on rapid growth and lipid production is critical for any algal mass culture, particularly for biodiesel. The ability to produce maximum biomass, have a high lipid content, and be less susceptible to contamination are essential parameters for mass algal cultivation for biofuel. Even if a micro-algal species has a high lipid content, it cannot be used for biodiesel production if it does not grow sufficiently. Based on these characteristics, isolate S8 with robust growth and high PHB and lipid content was chosen as a potential isolate for biodiesel and PHB production. The selected isolate was identified morphologically using a microscope. The isolate was further identified by molecular characterization (Maheshwari et al., 2022).

Morphological identification: The Microscopic examination revealed that all of the isolates were chlorophytes, with the majority of them being unicellular. 18s sequencing methods were used to confirm the identification. Cells were found to be green in color, spherical and oval. According to F.E Fritsch's classification, the isolated isolate was morphologically identified as chlorophytes. Chlorophytes are green algae that, as the names indicate, are green due to the presence of chlorophyll in the thylakoid. They also contain pigments such as xanthophyll and beta-carotene. They can be unicellular or multicellular, depending on the species.

Conclusions

Chlorella sp. in particular has been studied as a potential source of microalgae for the creation of bio-based goods. The increased interest in microalgae is due to the rising need for biopolymers like PHB and biodiesel. The goal of this study was to evaluate the effectiveness of biomass yield, PHB production, or high lipid-producing microalgae production. All of the isolates demonstrated PHB production in an initial screening. The isolates underwent further testing to examine the lipid content, PHB concentration, dry cell weight, and biomass concentration.

The isolate S8 sample produced greater PHB and lipid content throughout the study, along with good biomass production. 1.86 g/l of lipid and 0.52 g/l of PHB were determined to be the respective contents. For morphological and molecular identification, this isolate was chosen. It was determined by phylogenetic analysis and molecular characterization that the isolate was *Auxenochlorella pyrenoidosa*. The enormous demand for environmentally friendly goods has fueled interest in novel bio-based resources. A cost-effective option for producing biodiesel and bioplastics, microalgae have a high specific growth rate, the capacity to store large quantities of intracellular lipids, and the ability to grow in wastewater. The high lipid content of the microalgae offers new opportunities in bio-based product application, further studies should be done to make use of these strains for large-scale mass cultivation.

References

Gao X, Chen J-C, Wu Q, Chen G-Q. (2011). Polyhydroxyalkanoates as a source of chemicals, polymers, and biofuels. *Curr Opin Biotechnol.* **22**:768-774.

Bomfim A, Oliveira D, Voorwald H, Benini K, Dumont M-J, Rodrigue D. (2022). Valorization of Spent Coffee Grounds as Precursors for Biopolymers and Composite Production. *Polymers (Basel).* **14**:437.

Dong T, Wang B, Xiong W, Sweeney N, Pienkos PT, Yu J. (2020). System-Level Optimization to Improve Biofuel Potential via Genetic Engineering and Hydrothermal Liquefaction. *ACS Sustain Chem Eng.* **8**:2753-2762.

Arias DM, García J, Uggetti E. (2020). Production of polymers by cyanobacteria grown in wastewater: Current status, challenges and future perspectives. *N Biotechnol.* **55**:46-57.

Winnacker M. (2019). Polyhydroxyalkanoates: Recent Advances in Their Synthesis and Applications. *Eur J Lipid Sci Technol.* **121**:1900101.

Jiang G. (2016). Carbon Sources for Polyhydroxyalkanoates and an Integrated Biorefinery. *Int J Mol Sci.* **17**:1157.

Riaz S, Rhee KY, Park SJ. (2021). Polyhydroxyalkanoates (PHAs): Biopolymers for Biofuel and Biorefineries. *Polymers (Basel).* **13**:253.

Di Caprio F. (2022). A fattening factor to quantify the accumulation ability of microorganisms under N-starvation. *N Biotechnol.* **66**:70-78.

Grubišić M. (2022). Bioprospecting of Microalgae Isolated from the Adriatic Sea: Characterization of Biomass, Pigment, Lipid and Fatty Acid Composition, and Antioxidant and Antimicrobial Activity. *Molecules.* **27**:1248.

Koutra E, Economou CN, Tsafrakidou P, Kornaros M. (2018). Bio-Based Products from Microalgae Cultivated in Digestates. *Trends Biotechnol.* **36**:819-833.

Magdouli S, Brar SK, Blais JF. (2016). Co-culture for lipid production: Advances and challenges. *Biomass Bioenergy.* **92**:20-30.

Koller M, Muhr A, Braunegg G. (2014). Microalgae as versatile cellular factories for valued products. *Algal Res.* **6**:52-63.

Battista F. (2020). Food wastes and sewage sludge as feedstock for an urban biorefinery producing biofuels and added-value bioproducts. *J Chem Technol Biotechnol.* **95**:328-338.

de Paula FC, Kakazu S, de Paula CBC, Gomez JGC, Contiero J. (2017). Polyhydroxyalkanoate production from crude glycerol by newly isolated Pandoraea sp. *J King Saud Univ - Sci.* **29**:166-173.

Hierro-Iglesias C, Masó-Martínez M, Dulai J, Chong KJ, Blanco-Sanchez PH, Fernández-Castané A. (2021). Magnetotactic Bacteria-Based Biorefinery: Potential for Generating Multiple Products from a Single Fermentation. *ACS Sustain Chem Eng.* **9**:10537-10546.

Kim BS, Sawant SS, Tran TK. (2021). Production of Polyhydroxyalkanotes from Complex Polysaccharides. *Mol Sci Appl.* **1**:13-15.

Maheshwari N, Thakur IS, Srivastava S. (2022). Role of carbon-dioxide sequestering bacteria for clean air environment and prospective production of biomaterials: a sustainable approach. *Environ Sci Pollut Res.* **29**:38950-38971.