

A METHOD FOR POLYHYDROXYALKANOATE PRODUCTION

Field of Invention

- 5 The current invention relates to the use of a bacterial population to generate substantial quantities of polyhydroxyalkanoates (PHAs).

Background

- 10 The listing or discussion of a prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

Purple phototrophic bacteria (PPB) have been investigated recently for wastewater
15 treatment and resource recovery. PPB can recover carbon (C), nitrogen (N), and phosphorus (P) by using endless and “free” light as the energy source (Kim, M. K. *et al.*, *Biotechnol. Lett.* **2004**, 26, 819–822). For example, Hülsen *et al.* (Hülsen, T. *et al.*, *Water Res.* **2016**, 100, 486–495) successfully enriched *Rhodopseudomonas* from primary settled domestic wastewater under infrared (IR) light and demonstrated that
20 with optimal COD:N:P ratios, removal efficiencies of 97%, 92%, and 94% can be obtained for total chemical oxygen demand (COD), N, and P, respectively.

Unlike carbon mineralization by heterotrophic microbes, PPB removes organics from wastewater through carbon assimilation (Khatipov, E. *et al.*, *FEMS Microbiol. Lett.*
25 **1998**, 162, 39–45). PPB’s biomass yields on simple substrates can reach 1.0 g of COD/g of COD_{removed} (Alloul, A. *et al.*, *Water Res.* **2019**, 152, 138–147). Meanwhile, P can be removed via inorganic polyphosphate formation (Hiraishi, A., Yanase, A. & Kitamura, H., *Bulletin of Japanese Society of Microbial Ecology* **1991**, 6, 25–32). In addition, polyhydroxyalkanoate (PHA) was also identified in the cells (Kranz, R. G. *et al.*, *Appl. Environ. Microbiol.* **1997**, 63, 3003–3009). A pure culture of
30 *Rhodopseudomonas palustris* (*R. palustris*) WP3-5 was used to evaluate PHA production using various carbon sources, and the results showed that hydrogen was also produced, with acetate and propionate as the carbon source (Wu, S. C., Liou, S. Z. & Lee, C. M. *Bioresour. Technol.* **2012**, 113, 44–50). Thus, poly- β -hydroxybutyrate

(poly-P), glycogen, hydrogen, and PHA have all been previously detected as metabolic products of PPB. The metabolism of PPB likely involves four key bioconversions: C, N, P, and light. However, it is unclear if all of the conversions would occur simultaneously and how PPB would arrange the preference of the conversions.

5 Although recent studies offered some insights into the possible biochemical reactions in PPB, the detailed metabolic mechanism and substrate bioconversion are still unclear, which limit the development, optimization, and application of the PPB process in other areas. Furthermore, the interaction among C, N, and P metabolic pathways in purple non-sulfur bacteria (PNSB) has been largely overlooked.

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Conventional sludge treatment employs an anaerobic digester to convert organic matter in sludge to biogas, which is further processed into electricity or heat. However, the efficiency of such energy conversion is relatively low.

15 Therefore, there is a need to discover new methods that use the PPB process for wastewater treatment and resource recovery.

Summary of Invention

20 Aspects and embodiments of the invention will now be referred to by the following numbered clauses.

1. A method for producing a polyhydroxyalkanoate, the method comprising the steps of:

- 25 (a) providing a mixture comprising a bacterial population that has undergone an initialisation process;
- (b) adding a feed comprising a carbon source to the mixture to generate a feast phase for the bacterial population in the mixture;
- (c) subsequently subjecting the bacterial population to a famine phase by allowing
- 30 the carbon source to become depleted and not adding any further carbon source to the mixture for a period of time; and
- (d) repeating steps (b) and (c) in an alternating pattern, wherein:

the method further comprises periodically removing a portion of the mixture and harvesting a polyhydroxyalkanoate from the bacterial population in the removed portion of the mixture;

each periodical removal of a portion of the mixture is conducted during a feast
5 phase before the carbon source is entirely depleted from the mixture;

steps (b) and (c) are conducted in the presence of a light source that has a wavelength greater than 715 nm; and

the bacterial population is substantially formed from purple phototropic bacteria.

10 2. The method for producing a polyhydroxyalkanoate according to Clause 1, wherein each periodical removal of a portion of the mixture is conducted during the feast phase before the carbon source is reduced to a value of from 34 to 50 mg/L carbon, such as before the carbon source is reduced to a value of from 34.5 to 40 mg/L carbon, such as before the carbon source is reduced to a value of about 34.8
15 mg/L carbon.

3. The method for producing a polyhydroxyalkanoate according to Clause 1 or Clause 2, wherein each periodical removal of a portion of the mixture is conducted during from day 3 to day 4 of the feast phase.
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4. The method for producing a polyhydroxyalkanoate according to any one of the preceding clauses, wherein the carbon source is a volatile fatty acid.

5. The method for producing a polyhydroxyalkanoate according to Clause 4,
25 wherein the volatile fatty acid is selected from one or more of the group consisting of acetic acid, butyric acid, propionic acid, caproic acid, valeric acid, iso-butyric acid, iso-caproic acid, iso-valeric acid, and salts thereof, optionally wherein the volatile fatty acid is acetic acid or a salt thereof.

30 6. The method for producing a polyhydroxyalkanoate according to any one of the preceding clauses, wherein the feed comprises from 0.5 to 2 g/L of a carbon source, from 2.50 to 8.0 g/L of KH_2PO_4 , from 2.0 to 5.0 g/L of K_2HPO_4 , from 0.7 to 2.0 g/L of NH_4Cl , from 0.4 to 1.5 g/L of KCl , from 0.1 to 0.3 g/L of MgCl_2 , from 0.005 to 0.02 g/L of CaCl_2 , and from 0.25 to 0.75 g/L of NaCl , optionally wherein the feed comprises 1.0

g/L of a carbon source (e.g. acetic acid or a salt thereof), 4.4 g/L of KH_2PO_4 , 3.4 g/L of K_2HPO_4 , 1.3 g/L of NH_4Cl , 0.78 g/L of KCl , 0.2 g/L of MgCl_2 , 0.0146 g/L of CaCl_2 , and 0.5 g/L of NaCl .

5 7. The method for producing a polyhydroxyalkanoate according to any one of the preceding clauses, wherein one or more of the following conditions are applied and maintained in the method:

(ai) a pH of from 7.2 to 7.5;

(aii) a hydraulic and solids retention time of 4 - 7 days; and

10 (aiii) a biomass concentration of from 1 to 1.2 g volatile suspended solids/L.

8. The method for producing a polyhydroxyalkanoate according to any one of the preceding clauses, wherein each feast phase uses an uptake ratio of chemical oxygen demand:N:P of 100:(from 5 to 10):1 (e.g. 100:(from 5 to 7):1), such as 1 g/L of a carbon
15 source, 5 mM of phosphate buffer and 60 mg/L of NH_4^+ .

9. The method for producing a polyhydroxyalkanoate according to any one of the preceding clauses, wherein each famine phase lasts from 1 to 7 days, such as from 1 to 3 days or from 4 to 5 days.

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10. The method for producing a polyhydroxyalkanoate according to any one of the preceding clauses, wherein the periodical removal occurs in each feast phase.

11. The method for producing a polyhydroxyalkanoate according to any one of the preceding clauses, wherein purple phototropic bacteria forms from 68 to 100%, such
25 as from 70 to 95%, such as at least (or greater than or equal to) 93% of the bacterial population.

12. The method for producing a polyhydroxyalkanoate according to any one of the preceding clauses, wherein the purple phototropic bacteria comprises
30 *Rhodopseudomonas palustris*, optionally wherein the dominant species within the purple phototropic bacteria is *Rhodopseudomonas palustris*.

13. The method for producing a polyhydroxyalkanoate according to any one of the preceding clauses, wherein the mixture comprising a bacterial population that has undergone an initialisation process is a mixture comprising a bacterial population substantially formed from purple phototropic bacteria that has undergone a first feast
5 phase and a first famine phase.

Drawings

- FIG. 1** depicts the profile of (A) COD, (B) ammonium, (C) OD₆₆₀ and (D) light
10 absorbance under different wavelengths; and (E) color change in the serum bottle as a function of enrichment time for 12 days of PNSB enrichment period.

- FIG. 2** depicts the distribution of bacteria at the species level. Top 25 taxa for effluent,
light, and dark conditions.

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- FIG. 3** depicts the cyclic changes of (A) acetate, $\text{NH}_4^+\text{-N}$, $\text{PO}_4^{3-}\text{-P}$, and biomass (based
on COD); and (B) intracellular phosphorus, carbohydrate, and PHB under light
conditions.

- 20 **FIG. 4** depicts the liquid chromatography-organic carbon detection (LC-OCD)
chromatogram of reactor fed with fermented liquor (FL) during one cycle.

- FIG. 5** depicts the confocal fluorescence micrographs of PNSB. (A) Nile red
fluorescence; (B) 4',6-diamidino-2-phenylindole (DAPI) fluorescence; and (C) merged
25 image of Nile red and DAPI.

- FIG. 6** depicts the cyclic changes of (A) acetate, $\text{NH}_4^+\text{-N}$, $\text{PO}_4^{3-}\text{-P}$ and biomass (based
on COD); and (B) intracellular phosphorus, carbohydrate and PHB in Stage III under
dark condition.

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- FIG. 7** depicts the cyclic changes of (A) total organic carbon (TOC), acetate, and
valeric acid; and (B) $\text{NH}_4^+\text{-N}$, $\text{PO}_4^{3-}\text{-P}$, and biomass (based on COD) using FL as the
substrate under light conditions.

FIG. 8 depicts the profile of PHB in the biomass from FL.

FIG. 9 depicts the variations in concentrations of phosphorus, ammonium and acetate in (upper panel) feast operation; and (lower panel) feast-famine operation.

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FIG. 10 depicts the variations in concentrations of intracellular PHB, glycogen and Poly-P in (upper panel) feast operation; and (lower panel) feast-famine operation.

Description

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It has been surprisingly found that it is possible to generate a bacterial population/community that is predominantly formed from purple phototropic bacteria that can be used to generate substantial quantities of polyhydroxyalkanoates.

15 Thus in a first aspect of the invention, there is provided a method for producing a polyhydroxyalkanoate, the method comprising the steps of:

(a) providing a mixture comprising a bacterial population that has undergone an initialisation process;

20 (b) adding a feed comprising a carbon source to the mixture to generate a feast phase for the bacterial population in the mixture;

(c) subsequently subjecting the bacterial population to a famine phase by allowing the carbon source to become depleted and not adding any further carbon source to the mixture for a period of time; and

(d) repeating steps (b) and (c) in an alternating pattern, wherein:

25 the method further comprises periodically removing a portion of the mixture and harvesting a polyhydroxyalkanoate from the bacterial population in the removed portion of the mixture;

each periodical removal of a portion of the mixture is conducted during a feast phase before the carbon source is entirely depleted from the mixture;

30 steps (b) and (c) are conducted in the presence of a light source that has a wavelength greater than 715 nm; and

the bacterial population is substantially formed by purple phototropic bacteria.

In embodiments herein, the word “comprising” may be interpreted as requiring the features mentioned, but not limiting the presence of other features. Alternatively, the word “comprising” may also relate to the situation where only the components/features listed are intended to be present (e.g. the word “comprising” may be replaced by the phrases “consists of” or “consists essentially of”). It is explicitly contemplated that both the broader and narrower interpretations can be applied to all aspects and embodiments of the present invention. In other words, the word “comprising” and synonyms thereof may be replaced by the phrase “consisting of” or the phrase “consists essentially of” or synonyms thereof and *vice versa*.

The phrase, “consists essentially of” and its pseudonyms may be interpreted herein to refer to a material where minor impurities may be present. For example, the material may be greater than or equal to 90% pure, such as greater than 95% pure, such as greater than 97% pure, such as greater than 99% pure, such as greater than 99.9% pure, such as greater than 99.99% pure, such as greater than 99.999% pure, such as 100% pure.

When used herein, the term polyhydroxyalkanoate may be referred to herein as PHA. When used herein, the term polyhydroxyalkanoate may refer to a singular material or a plurality of materials. A PHA is a biodegradable aliphatic copolyester and more than 100 different PHA structures have been identified. PHA structures can vary in a number of ways. For example, PHAs can vary according to the structure of the pendant groups, which are typically attached to a carbon atom having (*D*)-stereochemistry. The pendant groups form the side chain of the hydroxyalkanoic acid and do not contribute to the PHA’s carbon backbone. Additionally, PHAs can vary according to the number and types of their repeating units. For example, PHAs can be homopolymers, copolymers, or terpolymers. These variations in PHA structure can cause variations in their physical characteristics, which makes each type of PHA a valuable resource for the production of a range of products that are commercially marketable.

Bacterial PHAs that may be mentioned herein include, but are not limited, to poly (3-hydroxybutyrate) (PHB), poly (3-hydroxybutyrate-co-valerate) (PHBV), poly-β-hydroxy-2-methylvalerate (PH2MV) and polyhydroxyoctanoates (PHOs).

In the method above, it will be appreciated that the periodic removal of a portion of the mixture comprising a bacterial population (referred to herein as a harvesting step) may be conducted as often as desired, provided that it takes place during a feast phase. For example, the harvesting step may be conducted during every feast phase, every other feast phase, every third feast phase etc. For example, the harvesting step may be conducted using the following patterns:

- harvest every feast/famine cycle (e.g. feast, harvest, famine, feast, harvest, famine, etc.);
- harvest every other feast/famine cycle (e.g. feast, famine, feast, harvest, famine, feast, famine, feast, harvest, famine, etc.);
- harvest every third feast/famine cycle (e.g. Feast, famine, feast, famine, feast, harvest, famine, feast, famine, feast, famine, feast, famine, feast, harvest, famine, etc.).

As will be appreciated, it may be convenient that the periodic removal occurs in each feast phase.

As will be appreciated, the method disclosed herein helps one to maximize the content of valuable PHA in the biomass at each point of harvest. Additionally, the recovery of these potentially valuable bacterial PHAs can combine discharge savings with substantial revenue streams. In the conventional biological wastewater treatment process, a significant quantity of activated sludge (i.e. biomass) is discharged that requires further treatment at a high cost. For the process disclosed herein, the collected biomass contains a high content of PHA that can be harvested for further use. Thus, in the current process, the cost associated with this discharge is either alleviated or may become even profitable due to the recovery of the valuable bacterial PHAs.

In embodiments of the invention, each periodical removal of a portion of the mixture may be conducted during the feast phase before the carbon source is reduced to a value of from 34 to 50 mg/L carbon, such as before the carbon source is reduced to a value of from 34.5 to 40 mg/L carbon, such as before the carbon source is reduced to a value of about 34.8 mg/L carbon.

In embodiments of the invention, each periodical removal of a portion of the mixture may be conducted during from day 3 to day 4 of the feast phase.

5 It will be appreciated that the portion of the mixture comprising a bacterial population removed in the periodic removal (or harvesting) step will be of a size that allows for the bacterial population to approximately return to (or exceed) its original concentration in the mixture before the harvesting step is repeated.

10 It is believed that the concentration of biomass needs to be maintained at at least 1-1.2 g/L to maintain reactor activity. With this in mind, the harvesting phase should always look to retain this concentration within the remaining biomass in the reactor. For PPB, the biomass yield is about 90% $\text{COD}_{\text{biomass}}/\text{COD}_{\text{removed}}$. This means that 90% of COD consumed will be converted to biomass, meaning that the amount of biomass collected will depend on the COD availability (external carbon) supplied to the system.

15 For example, if the total volume of the reaction (i.e. the biomass, medium and feed) is 1,000 mL (with an initial biomass concentration of from 1.15 g/L), and if the COD is about 1,000 mg/L in the total volume of the reaction, then 900 mg/L of COD is removed (i.e. 90% of the COD supplied to the system). Based on the biomass yield, there will be an increase of 810 mg/mL COD biomass generated (so there will be an overall

20 increase of biomass in the region of 1.15 g/L of biomass, meaning that one can harvest 500 mL of the reaction volume.

The duration of the feast phase may be linked to the duration of the hydraulic retention time (HRT) of the reactor. For example, if the reactor has a HRT of 7 days, then the

25 feast phase needs to be kept within 7 days. This means before the external carbon is totally consumed, fresh feed needs to be added to support significant carbon concentration in order to maintain the feast phase for the desired length of time. For example, in a sequencing batch reactor set-up, the HRT may be about 7 days and the external carbon source (e.g. sodium acetate) may be provided in an amount of 1 g/L;

30 the external carbon source would not be expected to be fully consumed before 7 days, so the fresh nutrients was added at the end of 7 days to keep the bacteria always incubated under significant carbon concentration. It will be appreciated that the actual length of the feast phase may be determined by the person skilled in the art based on

their knowledge and skill and may vary according to the change of variables and the reaction system used.

5 In embodiments of the invention, each famine phase may last from 1 to 7 days, such as from 1 to 3 days or from 4 to 5 days. For the avoidance of doubt, it is explicitly contemplated that where a number of numerical ranges related to the same feature are cited herein, the end points for each range are intended to be combined in any order to provide further contemplated (and implicitly disclosed) ranges. For example, for the famine phases mentioned above, each famine phase may last:

10 from 1 to 3 days, from 1 to 4 days, from 1 to 5 days, from 1 to 7 days;
from 3 to 4 days, from 3 to 5 days, from 3 to 7 days;
from 4 to 5 days, from 4 to 7 days; or
from 5 to 7 days.

15 Any combination of the total duration for each feast phase and famine phase noted above is contemplated herein.

When used herein, the bacteria that has undergone an initialisation process may refer to a mixture that has undergone a first feast phase and a first famine phase, where no
20 harvesting step takes place in this initialisation phase. As will be appreciated, the bacterial population used in this initialisation phase may be a bacterial population substantially formed from purple phototropic bacteria.

When used herein, the term "substantially formed from purple phototropic bacteria" in
25 relation to the bacterial population may refer to a bacterial population that comprises from 55 to 100% of purple phototropic bacteria, such as from 60 to 100%, such as from 68 to 100%, such as from 70 to 95%, such as greater than or equal to 93% of the bacterial population in the mixture.

30 Any suitable purple phototropic bacteria may be used in embodiments of the invention. For example, the purple phototropic bacteria may include any of those mentioned in the experimental section below. In particular embodiments, the purple phototropic bacteria may comprise *Rhodopseudomonas palustris*. In yet more particular embodiments of the invention, the dominant species within the purple phototropic

bacteria may be *Rhodopseudomonas palustris*. In embodiments where *Rhodopseudomonas palustris* is the dominant species of purple phototropic bacteria, it may form from 68 to 100%, such as from 70 to 95%, such as at least 93% of the total purple phototropic bacterial population. As will be appreciated, in embodiments where
5 *Rhodopseudomonas palustris* is the dominant species of purple phototropic bacteria, excellent carbon and nutrient removal performance are demonstrated.

The source of the bacterial population may be any suitable source, such as the effluent from a bioelectrochemical system (e.g. from a microbial fuel cell) or any other suitable
10 wastewater source that includes a bacterial population. In order to generate a bacterial population that is substantially formed from purple phototropic bacteria from the selected source, the source of the bacterial population may be subjected to any suitable selection pressure. For example, the source of the bacterial population may be incubated at room temperature under an *infra-red* (IR) light source (e.g. a light
15 source having a wavelength of greater than or equal to 680 nm, such as greater than or equal to 715 nm) for a period of time to provide a bacterial population that is substantially formed from purple phototropic bacteria. As will be appreciated, solar light can potentially be used as the IR light source, which may substantially reduce operational costs. Any suitable wavelength of IR light (up to the upper limit of 1 mm)
20 may be used. It is noted that wavelengths of from 850 to 920 nm have been tested and work in the process described herein.

The carbon source used in the feed may be any suitable carbon source (or mixture thereof) that can be used to generate PHAs (e.g. PHB). A suitable carbon source that
25 can be used in the current method may be a volatile fatty acid. Suitable volatile fatty acids that may be mentioned in embodiments disclosed herein may be selected from one or more of the group consisting of acetic acid, butyric acid, propionic acid, caproic acid, valeric acid, iso-butyric acid, iso-caproic acid, iso-valeric acid, and salts thereof. For example, the volatile fatty acid may be acetic acid or a salt thereof.

30 Any suitable feed may be used in the methods disclosed herein. For example, the feed may be a fermented wastewater or any waste water containing a high concentration of volatile fatty acids suitable for conversion to PHAs. In particular embodiments of the invention, the feed may be one that comprises from 0.5 to 2 g/L of a carbon source,

from 2.50 to 8.0 g/L of KH_2PO_4 , from 2.0 to 5.0 g/L of K_2HPO_4 , from 0.7 to 2.0 g/L of NH_4Cl , from 0.4 to 1.5 g/L of KCl , from 0.1 to 0.3 g/L of MgCl_2 , from 0.005 to 0.02 g/L of CaCl_2 , and from 0.25 to 0.75 g/L of NaCl , optionally wherein the feed comprises 1.0 g/L of a carbon source (e.g. acetic acid or a salt thereof), 4.4 g/L of KH_2PO_4 , 3.4 g/L of K_2HPO_4 , 1.3 g/L of NH_4Cl , 0.78 g/L of KCl , 0.2 g/L of MgCl_2 , 0.0146 g/L of CaCl_2 , and 0.5 g/L of NaCl .

In the method described herein, any suitable operating conditions may be used. For example, one or more of the following operating conditions may be applied and maintained in the method:

- (ai) a pH of from 7.2 to 7.5;
- (aii) a hydraulic and solids retention time of 4 - 7 days; and
- (aiii) a biomass concentration of from 1 to 1.2 g volatile suspended solids/L.

As noted above, the concentration of biomass needs to be maintained at at least 1-1.2 g/L to keep the activity of the system. For PPB, the biomass yield is about 90% $\text{COD}_{\text{biomass}}/\text{COD}_{\text{removed}}$. It means, the 90% of COD consumed will convert to biomass. Hence, how much biomass that can be collected depends on the COD (external carbon).

As an example, if one starts with a concentration of 1.2 g VSS in 1 L, after several days' operation (in feast phase) the biomass will increase to 2.3 g VSS in 1L. If calculated based on the volume, one can collect a maximum 500 mL of total volume to harvest the biomass – to the remaining amount one will top up with 500 mL of fresh medium, thereby reducing the concentration back to 1.15 g VSS in 1L. More generally, for a 1 L reaction volume, one would look to collect a maximum of about 1.1 g VSS (that is, somewhat less than 500 mL) and then top up/refill with fresh medium to provide the original reaction volume for the next feast-famine cycle. As will be appreciated, in the famine phase, the biomass will grow at a much slower rate (if at all). As will be appreciated, the harvested volume is tied to the amount of external carbon source supplied to the biomass and will vary proportionately to this feed amount.

- In embodiments of the invention, any suitable uptake ratio of chemical oxygen demand:N:P may be used in each feast phase. For example, each feast phase may use an uptake ratio of chemical oxygen demand:N:P of 100:(from 5 to 10): 1, such as an uptake ratio of chemical oxygen demand:N:P of 100:(from 5 to 7): 1. Such a ratio may be obtained from 1 g/L of a carbon source, 5 mM of a phosphate buffer and 60 mg/L of NH_4^+ . For example, if one makes use of acetate as the carbon source, the chemical oxygen demand (COD) of 1 g/L of acetate is about 780 mg/L, an ammonia concentration of 60 mg/L (so a concentration of 46.67 mg/L of N, giving a COD:N ratio of about 100:6) and one may make use of a phosphate source to supply at least 7.8 mg/L (e.g. one may make use of a phosphate buffer having a concentration of 5 mM). As will be appreciated, the above uptake ratios may refer to the minimum amounts of the nitrogen and phosphorous in the system in order for bacterial growth in the feast phase. For example, when operating the system on a large scale, one may need to monitor and add phosphorous as required to maintain the minimum ratio. However, when operating the system on a small scale under laboratory conditions, where the pH may be prone to more variation than in a larger scale, one may make use of a phosphate buffer to control the pH of the system, and also to supply the desired phosphorous to the system too.
- Following each periodic removal of a portion of the mixture comprising the bacterial population, the PHA may be extracted therefrom. Any suitable method of extraction may be used to obtain the PHA. For example, the following protocol may be used.
1. The portion may be centrifuged at 4000 x g for 15 mins to settle the biomass.
 2. Suspend the biomass in H_2SO_4 (3%, V/V) and chloroform (1:1 H_2SO_4 and CHCl_3).
 3. Heat the sample to 100 °C for 6 hours.
 4. Add deionised/distilled water to the sample and agitate vigorously.
 5. Separate the aqueous phase and remove the water to obtain the PHA.

Further methods for the extraction of PHAs that may be used (or adapted) for the PHAs obtained herein include those disclosed in WO 2006/004814, WO 2006/092033, WO 2013/016558 and many others.

- 5 Other advantages of the method disclosed herein include pollution reduction, resource capture, energy conservation, and industrial-scale application, which ultimately brings economic and environmental benefits.

Further aspects and embodiments of the invention will now be discussed by reference
10 to the following non-limiting examples.

Examples

Materials

- 15 All chemicals used were purchased from Sigma-Aldrich Pte Ltd Singapore, unless otherwise stated.

Analytical techniques

Determination of total chemical oxygen demand (COD) and soluble COD (sCOD)

- 20 TCOD and sCOD were determined by COD kits (8000 DR800 HR, Hach Co., Loveland, CO).

Determination of the concentration of dissolved ions

- 25 Dissolved $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$, $\text{NO}_2^-\text{-N}$, and $\text{PO}_4^{3-}\text{-P}$ were determined by standard methods.

1. For $\text{NH}_4^+\text{-N}$, standard Nessler method (Hach method 8038) was used to quantify the concentration. All chemicals used were purchased from Hach.
2. $\text{NO}_3^-\text{-N}$ and $\text{NO}_2^-\text{-N}$ were determined by Hach Nitrate Test Kit NI-11 (146803) and
30 Hach Nitrite Test Kit NI-6 (224000), respectively. All kits used were purchased from Hach.
3. $\text{PO}_4^{3-}\text{-P}$ was determined with the vanadomolybdophosphoric acid colorimetric method (4500-P C) (APHA, 2001)

Determination of total nitrogen (TN) and TOC

TN and TOC were measured by a TOC analyzer (TOC-5000A, Shimadzu) using the combustion-infrared method.

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Gas chromatography

VFAs were analysed by a gas chromatograph (Agilent Technologies 7890A GC System) equipped with a flame ionization detector (GC/FID) and a polar capillary column (DB-FFAP). The gas composition in headspace was determined on an Agilent
10 GC 7890A gas chromatograph equipped with a thermal conductivity detector (TCD) detector.

Example 1. Microbial community composition

15 *Inoculum and reactor configuration*

R. palustris was found to be highly abundant in a microbial fuel cell (MFC) that recovers resources from sludge-fermented liquor (Wu, D. *et al.*, *Chem. Eng. J.* **2019**, 361, 1207–1214). Thus, the effluent of the MFC was collected to enrich PNSB in serum bottles, with an IR light source. Two reactors, each with a working volume of 2
20 L, were operated for the long-term enrichment of PPB cultures. The effluent was centrifuged, and the biomass was collected and transferred to four serum bottles containing 150 mL of fresh feed. The fresh feed consisted of 1.0 g/L sodium acetate (sCOD = 780 – 800 mg/L), 4.4 g/L KH₂PO₄, 3.4 g/L K₂HPO₄, 1.3 g/L NH₄Cl, 0.78 g/L KCl, 0.2 g/L MgCl₂, 0.0146 g/L CaCl₂, 0.5 g/L NaCl, and trace vitamins and minerals
25 (Wu, D. *et al.*, *Chem. Eng. J.* **2019**, 361, 1207–1214). The culture medium was flushed with argon for 5 min before the bottles were sealed. The culture was incubated at room temperature under an IR light source (Wega Optical Co.), which transmitted only $\lambda > 680$ nm light to select phototrophic bacteria against oxygenic phototrophs (Badalamenti, J. P., Torres, C. I. & Krajmalnik-Brown, R., *Biotechnol. Bioeng.* **2013**,
30 110, 1020–1027). The operating condition of hydraulic retention time (HRT) was 7 days. During enrichment, samples were collected from each bottle daily to analyze the concentration of COD and N, and the optical density (OD₆₆₀). Once the OD₆₆₀ was stable, the samples were scanned from 450 to 1100 nm to identify the absorbance and confirm the enrichment (Hülsen, T., Batstone, D. J. & Keller, J., *Water Res.* **2014**,

50, 18–26). The enriched culture was combined into a 1 L reactor, further incubated with the same medium, and employed as the parent culture for batch experiments. The incubation medium of the parent reactor was replaced with fresh feed weekly. The reactor was operated under the optimal COD:N:P uptake ratios of around 100:(5–10):1 (e.g. 1 g/L sodium acetate, 5 mM phosphate buffer solution, 60 mg/L $\text{NH}_4^+\text{-N}$, and trace vitamins and minerals), with an IR light source.

Microbial community analysis

DNA of the samples was collected from the effluent of MFC under light and dark conditions (end of the cycle) for DNA extraction. The DNA was extracted using the fast DNA extraction kit for soil (MP Biomedicals, Singapore), and stored at $-20\text{ }^\circ\text{C}$ before being sequenced. Primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') were used (Cai, J. *et al.*, *Chemosphere* **2021**, 263, 127922) for high-throughput sequencing targeting V3 and V4 regions of rRNA genes. The low-quality sequences and artificial replicate sequences were removed by quality control (QC) assessment (NovogeneAIT Genomics Singapore Pte Ltd.). The QC-passed sequences were clustered into operational taxonomic units (OTUs) at 97% similarity (Ji, B. *et al.*, *Water Res.* **2020**, 179, 115884).

Results and discussion

After acclimating for 12 days and being enriched under IR, PNSB were consistently enriched, and an obvious biomass color change was observed in the serum bottles (FIG. 1). The OD_{660} increased from 0.2 to 0.6 in 6 days and remained stable during the remaining incubation period. Samples taken on day 10 displayed light adsorption peaks at 805 and 866 nm separately (FIG. 2), demonstrating the enrichment of phototrophic bacteria (Hülse, T., Batstone, D. J. & Keller, J., *Water Res.* **2014**, 50, 18–26). This enriched culture was used as the parent culture for the batch experiments that were performed under either light or dark conditions.

FIG. 2 describes the microbial community of the seed biomass under light and dark conditions at the end of the enrichment cycle. Under light conditions, the relative abundance of *R. palustris* was increased to 93% from the value of 53% of the seed. However, under dark conditions (without an IR light source), its relative abundance was only 60%. This result indicates that the light source is crucial for maintaining a

high abundance of *R. palustris*. At the same time, other exoelectrogenic bacteria such as *Rhodocyclaceae* (2%), were found under dark conditions. Species related to complex organic degradation such as *Pseudomonas caeni* (2%), *Escherichia coli* (1%), and *Lactobacillus pentosus* (1%), were also present under dark conditions (FIG. 2).
5 Compared to a previous study, PPB selected from wastewater had an abundance of 43%, comprising *Rhodopseudomonas*, *Rhodobacter*, *Allochromatium*, and *Blastochloris* (Hülse, T. et al., *Bioresour. Technol.* **2018**, 254, 214–223). A high abundance of *R. palustris* was achieved under light conditions, suggesting the feasibility of enriching PNSB from the bioelectrochemical system (BES).

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Example 2. C, N, and P transformation in PNSD

Batch experiments for C/N/P biotransformation

50 mL of the parent culture (prepared in Example 1) was collected and centrifuged at
15 4000 g for 15 min at 20 °C. The supernatant was discarded, and the culture pellet was resuspended in 50 mL of phosphate-buffered saline (PBS). The washing step was repeated twice to remove any potential residual substrate. The pelletized biomass was finally resuspended in 60 mL of PBS for the experiments described below.

20 Six serum bottles were filled with 140 mL of fresh feed and 10 mL of the enriched phototrophic biomass mentioned above. PBS (5 mM) was used to maintain a stable neutral pH. Three bottles were covered with aluminum foil and used as the dark control. The other three bottles were illuminated under an IR light source. All six bottles were continuously shaken at 150 rpm and sampled daily for dissolved and/or intracellular C,
25 N, and P analysis.

To explore the biotransformation of C, N, and P, the test was repeated three times (in three batch tests) with identical operating conditions in the reactor (described in Example 1) so as to confirm the culture performance with the dissolved C, N, and P
30 and accumulate sufficient biomass for analysis of solid samples. In batch I, only liquid samples were taken for dissolved carbon and nutrient concentration analysis. Samples were collected from each batch reactor every 24 h and centrifuged at 12000 g for 10 min. The supernatant was filtered through a 0.45 µm membrane filter for organic matter and nutrient analysis. In batch II, 5 mL of sample was collected daily, and the

biomass pellet was vacuum-dried to determine the biomass concentration change and intracellular compounds (i.e., PHA, total carbohydrate, total N, and P). In batch III, gas samples in the headspace were taken every 24 h for CO₂, H₂, O₂, CH₄, and N₂ analysis. In each batch experiment, triplicate runs were performed for each light/dark condition. Additionally, 15 mL of samples were collected at the end of the cycle (both light and dark conditions) for microbial community analysis by following the protocol in Example 1.

Nile red and DAPI double staining

To localize the polyP and PHA within the same cells, DAPI and Nile red were applied sequentially to double stain the biomass (Pinzon, N. M. *et al.*, *mBio* **2011**, 2, 00109-11). The stained biomass was visualized using a Nikon A1R confocal laser scanning microscope and analyzed with NIS Elements version 4.10 by thresholding (Mukherjee, C., Chowdhury, R. & Ray, K., *Front. Microbiol.* **2015**, 6, 1421). PHA quantification was performed using the method described in a previous study (Shen, N. & Zhou, Y. *Appl. Microbiol. Biotechnol.* **2016**, 100, 4735–47450) to determine the levels of PHB, poly-β-hydroxyvalerate (PHV), and poly-β-hydroxy-2-methylvalerate (PH2MV) with an Agilent 7890 A GC system.

Results and discussion

To study the C, N, and P transformation, a batch test was repeated three times in detailed cyclic studies under both light and dark conditions. Variations of acetate, biomass, intracellular N and P, total carbohydrate, and PHA are shown in FIG. 3 (light conditions). Acetate was rapidly reduced from the start of the cycle and was almost depleted around 120 h (FIG. 3A), and this was accompanied by the consumption of NH₄⁺-N and PO₄³⁻-P. The biomass increased from 0.94 ± 0.06 to 1.41 ± 0.01 g of volatile suspended solids per litre (VSS/L) at the end of the cycle (FIG. 3A). The biomass yield was estimated to be 0.85 g of COD_{biomass}/g of COD_{removed}. It should be noted that the only organic substrate in the feed was acetate that was completely consumed within the cycle. However, 34.7 mg of TOC/L remained in the end. According to LC-OCD results, some low-molecular weight acids were detected at the end of the cycle. However, these acids were not picked up by GC-FID, thus indicating they were not VFAs (FIG. 4). Low-molecular weight acids, such as succinate and citrate, were likely produced and released due to the metabolic activities (Yin, X. *et al.*,

Biotechnol. Adv. **2015**, 33, 830–841). In addition, high-molecular weight organic compounds, if any, that cannot be detected by LC-OCD may also exist.

TN in the biomass increased with the reduction of $\text{NH}_4^+\text{-N}$ and acetate during the entirety of stage III, indicating the gradual accumulation of protein (FIG. 3B). Intracellular carbon and P transformation did not follow such a consistent trend. According to the PHA profile, the reaction cycle can be separated into three phases. PHB was the only PHA compound detected given acetate was the sole carbon source in the feed. During phase I (0–72 h), stored PHB in the cells rapidly degraded. In the meantime, 25% of the P was removed and converted into poly-P (FIG. 3B). Minor carbohydrate (5.34 mg of carbohydrate/g of biomass) also formed in the cells. The degraded PHB was likely utilized to produce energy for P uptake and cell growth during phase I. It was noted that 0.43 g of acetate/L (equivalent to 0.466 g of COD/L) was taken up by the biomass in phase I, while the biomass growth was around 0.342 g of VSS/L (equivalent to 0.48 g of COD/L). Assuming biomass mainly consists of PHA, poly-P, total carbohydrate, and active biomass, this result suggests that consumed acetate was almost completely converted to active biomass with a biomass yield of 1.03 g of $\text{COD}_{\text{biomass}}$ /g of $\text{COD}_{\text{acetate}}$. At this stage, it is not clear if acetate was diverted for growth or went through PHA polymerization and the biomass was then synthesized from PHA degradation. Notwithstanding, the net PHA content was reduced. It is likely that acetate was utilized for growth and/or the PHA production rate was slower than its consumption rate. It also means that apparently, photophosphorylation was not able to support 100% energy requirement.

Interestingly, in phase II (72–120 h), acetate and $\text{NH}_4^+\text{-N}$ were continually taken up while P was released. PHB sharply accumulated despite both $\text{NH}_4^+\text{-N}$ and $\text{PO}_4^{3-}\text{-P}$ still being abundant (FIG. 3). At 120 h, the PHB content peaked at 0.56 mg/mg of biomass, which is almost 4.3-fold higher than that in previous report (Guerra-Blanco, P. *et al.*, *Eur. Polym. J.* **2018**, 98, 94–104). In the meantime, the culture released 109 ± 6 mg of P/L of P from 72 to 96 h with 90 ± 1 mg/L taken back at the highest PHB level (FIG. 3B). The observation in this study is different from a previous conclusion that PPB biomass growth and PHA accumulation can be regulated by the level of nutrient availability (Morgan-Sagastume, F. *et al.*, *Water Res.* **2010**, 44, 5196–5211). Continuous biomass growth and both consumption and production of PHA were all

observed in the presence of high N and P concentrations. It is known that higher organic availability would favor growth while low organic availability (but not too low to limit the substrate uptake rate) may instead stimulate polymer accumulation (Morgan-Sagastume, F. *et al.*, *Water Res.* **2010**, 44, 5196–5211). Hence, PHA accumulation in this study was likely regulated by the availability of acetate rather than nutrient levels. Biomass growth requires very high ATP demand, while PHA formation does not involve ATP consumption (Fradinho, J., Reis, M. & Oehmen, A., *Water Res.* **2016**, 105, 421–428). To support biomass growth in phase II, energy can be supplied from photophosphorylation and/or poly-P hydrolysis. According to the PO_4^{3-} -P concentration in the bulk liquid and intracellular TP profile (FIG. 3), poly-P hydrolysis may play a major role in energy supply at the beginning of PHA formation while photophosphorylation continued the ATP supply at a later stage.

In phase III, when the external carbon source was exhausted, NH_4^+ -N consumption and biomass growth continued, regardless. Meanwhile, the accumulated PHB was rapidly consumed and P experienced release and uptake turnover again (FIG. 3B). Both energy and the carbon source are essential for biomass growth. According to the PHA and P profiles, it is highly possible that PHA was utilized as the carbon and energy source accompanied by a supply of energy from poly-P hydrolysis and/or photophosphorylation. When the level of PHA is low, the culture started to accumulate poly-P for energy storage. Additionally, hydrogen was detected only after 120 h. This confirms that PPB would require an alternative pathway to eliminate the reducing power produced from biomass growth when there is no external carbon source for PHA accumulation (Fülöp, A. *et al.*, *Int. J. Hydrogen Energy* **2012**, 37, 4915–4924).

To confirm the location of PHB and poly-P in PNSB cells, Nile red and DAPI double staining was performed. As shown in FIG. 5, PHB and poly-P granules were found within the same cells (FIG. 5A-B). According to the results, the enriched PNSB culture could capture COD, N, and P simultaneously under strictly anaerobic and IR irradiation conditions. Despite C and P experiencing various turnovers, the carbon source was eventually converted to biomass and/or PHA (depending on the reaction period), and P was removed and accumulated in the form of poly-P.

In contrast, all of the metabolic activities were slowed under dark conditions (FIG. 6). The amount of acetate was slowly reduced from 0.71 ± 0.01 g/L to 0.57 ± 0.42 mg/L within a cycle. Instead of constant consumption, $\text{NH}_4^+\text{-N}$ displayed a reduction and release trend. The biomass slightly increased from 0.94 ± 0.06 to 1.01 ± 0.01 g of VSS/L at the end of the cycle. Interestingly, PHA experienced multiple consumption and production patterns within one cycle, with the content increasing from 0.09 ± 0.01 to 0.48 mg/mg of biomass, which was different from that under light conditions. According to the variation of $\text{PO}_4^{3-}\text{-P}$ in the bulk liquid, poly-P may also play certain role in energy supply and conservation. However, the changes were quite minor compared to light conditions. In the meantime, H_2 was not detected during the whole cycle (Table 1). Under dark conditions, PPB may have to switch its pathway to chemotrophy, which may be less favorable for bacterial growth (Kimble, L. K., Stevenson, A. K., & Madigan, M. T., *FEMS Microbiol. Lett.* **1994**, *115*, 51–55; and Biebl, H. & Wagner-Döbler, I., *Process Biochem.* **2006**, *41*, 2153–2159). The ability of such an adaptation is evidently significant for the survival of this microorganism in the absence of light under anaerobic conditions (Kimble, L. K., Stevenson, A. K., & Madigan, M. T., *FEMS Microbiol. Lett.* **1994**, *115*, 51–55). Given that the abundance of *R. palustris* was only 33% at the end of stage III, the obtained performance may not be fully attributed to *R. palustris*. Hence, the detailed substrate transformation and pathways will not be discussed further.

Table 1. The gas composition in headspace during one cycle.

Time (h)	Ar (mL)	N ₂ (mL)	CO ₂ (mL)	H ₂ (μL)
24	8.66	0.788	-	-
48	7.12	1.96	-	-
72	7.12	2.84	0.133	-
96	8.24	1.78	0.09	-
120	8.24	0.98	0.07	0.532
144	7.74	1.24	0.08	0.787
168	6.24	2.68	0.118	1.4

Example 3. Nutrient Recovery from FL.

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The enriched culture prepared in Example 2 was then exposed to FL to investigate nutrient recovery.

Sludge fermentation

The feed sludge was a mixed primary and secondary sludge [1:1 (w/w)] from a local water reclamation plant. The fermentation of sludge was conducted in a 7 L fermenter with a 3 L working volume at 55 °C and pH 9.9 aiming for VFA production. The detailed operation condition can be found in a previous study (Chen, Y. *et al.*, *Water Res.* **2017**, 112, 261–268). The characteristics of the FL are listed in Table 2.

Table 2. Characteristics of FL.

parameter	units	FL value
pH		7.0 ± 0.2
total organic carbon (TOC)	g/L	2.43 ± 8.77
ammonium	mg/L	479.01 ± 69.79
phosphate	mg/L	465.61 ± 5.17
acetic acid	mM	17.85 ± 0.63
valeric acid	mM	1.65 ± 0.25

Nutrient recovery batch experiment

The nutrient recovery batch experiment was performed by following the protocol in Example 1. The feed can be replaced with other types of VFAs and/or real wastewater. Prior to feeding PNSB, the FL was diluted 10 times and centrifuged at 12000 g for 10 min to remove the solids, and the pH was adjusted to 7.0 ± 0.2 with 1 M HCl. During the 7 day operation, samples were collected daily and centrifuged at 12000 g for 10 min to separate the biomass and supernatant. The supernatant was filtered through a 0.45 µm membrane filter for COD, VFA, and dissolved N and P analysis. The biomass (10 mL, 1–1.2 g VSS/L) was collected and freeze-dried for intracellular compound analysis.

Results and discussion

The total dissolved COD was around 5340 mg/L, with 30.7% in the form of VFAs. The performance of the fermenter and the characteristics of the FL were relatively stable during the operation period. As shown in Table 2, FL consisted of VFA and other organic matter. For a typical cycle, variations of acetate, valeric acid, TOC, and dissolved N and P in FL are shown in FIG. 7 (light conditions). Acetate was rapidly reduced and preferably utilized by PNSB from the start of the cycle and almost depleted around 5 days (FIG. 7A). 13.43 mg of valeric acid/g of VSS was consumed

from day 5 when acetate was depleted. Within 7 days, 53.4% of TOC was removed by PNSB, of which 72% was contributed by acetate and valeric acid consumption. Other organic matter was also gradually transformed and/or consumed by the culture, especially low-molecular weight neutral compounds. Carbon removal was accompanied by the consumption of $\text{NH}_4^+\text{-N}$ and $\text{PO}_4^{3-}\text{-P}$. 43.4% of $\text{NH}_4^+\text{-N}$ and 36% of $\text{PO}_4^{3-}\text{-P}$ were converted despite release, and the uptake pattern was also observed in this test. The biomass increased from 0.72 ± 0.01 to 1.14 ± 0.21 g of VSS/L at the end of the cycle (FIG. 7B). The biomass yield was estimated to be 0.89 g of $\text{COD}_{\text{biomass}}/\text{g}$ of $\text{COD}_{\text{removed}}$.

Approximately 0.24–0.3 mg of PHB/mg of biomass was also observed to accumulate in PNSB fed with FL (FIG. 8). Other types of PHAs were not detected. The PHB also experienced rapid consumption and production, while P demonstrated release and uptake turnover (FIG. 7B). This further confirms that PHA was utilized as the carbon and energy source accompanied by energy supply from poly-P hydrolysis and/or photophosphorylation.

Example 4. Feast-famine operation

PPB has the capability to quickly store substrates and consume stored substrate in a more balanced way, which has a strong competitive advantage over organisms without the capacity of substrate storage. The PHA production process with mixed microbial communities commonly requires the selection of a PHA accumulating culture by applying a feast and famine (FF) strategy regarding carbon availability. To increase the yield of PHA, a short-term starvation period can be provided. During the starvation stage, the internal polymers like glycogen and PHA is consumed to maintain the metabolisms and growth of PPB. A short famine phase (1-3 days) can then allow PPB exhibit rapidly uptake of substrates. Thus, the feast-famine operation is an optimized condition for PHB generation by PPB. For PHB production, the biomass should be harvested during the feast phase according to the remaining carbon source availability.

Feast-famine operation

The FF strategy was based on intermittent feeding of the substrate, where the external carbon is taken up and accumulated intracellularly as PHA (feast phase), followed by

phases without substrate addition that favors cell growth on storage products (famine phase), thus creating a selection pressure for organisms capable of storing PHA.

Six serum bottles were filled with 140 mL of fresh feed and 10 mL of the enriched phototrophic biomass mentioned above. 5 mM PBS was used to maintain the stable neutral pH. Three bottles were illuminated under IR light source. All six bottles were continuously shaken at 150 rpm and sampled daily for dissolved and/or intracellular C/N/P analysis. The six batch reactors were operated for 6 cycles in different phase. Each cycle lasted 7 days. Batch 1-3 were conducted to confirm the performance of enriched PPB under feast conditions (Table 3). For batch 4-6, the culture was only fed with 5 mM PBS to create starvation conditions after feast. Feed was resumed after famine and the culture was exposed to the same feed as feast. The detailed feed composition in different stages is shown in Table 3. The feast-famine-feast operation was to compare the performance difference between feast and famine conditions with the focus on the dissolved C/N/P removal.

Table 3. Different feed composition under different stages.

Stages	Feed composition
Feast	1 g/L sodium acetate, 5 mM PBS, 80 mg/L $\text{NH}_4^+\text{-N}$, trace vitamins and minerals
Famine	5 mM PBS (Starvation)

The carbon source consumption rate was around 4.46 mg COD/L·h. The harvest time of PPB was around 3–4 days into feast phase. The biomass concentration varied from 1.1 g VSS/L to 2.3 g VSS/L. Thus, the consumption rates varied from 632.5 mg/g biomass to 220.4 mg/g biomass. Alternatively, the COD level in the reactor can be monitored. The biomass should be harvested before the carbon level reduced to 34.8 mg/L C (≈ 14 mg/L COD). The effluent was collected and centrifuged at 4000 g for 15 min to settle the biomass. After centrifugation, the supernatant was discharged, and the biomass was used for further extraction of PHB.

Extraction method for PHB:

- a) Suspend the biomass in 2 mL H_2SO_4 (3%, V/V) and 2 mL of chloroform.
- b) Screw the tube and heat the sample to 100 °C for 6 h.

- c) Add 1 mL of DI water and shake the tube vigorously.
- d) Dissolve PHB in the DI water phase.

Results and discussion

- 5 The maximum PHA attained here was 0.56 g/g biomass. A maximum PHA accumulation value of 77% per cell dry weight (cdw) with wastewater from paper mill effluents has been achieved thus far with aerobic mixed microbial cultures (Jiang, Y. *et al.*, *Water Res.* **2012**, 46, 5517-5530). FIG. 9 depicts the concentrations of P, ammonium and acetate while FIG. 10 depicts the concentrations of intracellular PHB, glycogen and Poly-P. In order to have maximum PHA and biomass growth, the organic substrate should be reduced to a relatively low level but it should not be completely depleted to avoid internal PHA consumption. On the whole, selective pressure created by the FF mode played a dominant role for the selection of PHA accumulating cultures.
- 15 69 ± 2.3 % of TOC and 30% ± 0.93 % of NH₄⁺-N were removed at the end of each cycle under light conditions. The results were repeatable for continuous feast feed of every cycle. These results indicate that PPB utilized light as the main energy source for catabolic activities. However, the PHA content was 0.25 g/g biomass. The storage polymers are used for growth when the external substrate is depleted. In this manner,
- 20 the organisms are capable to balance their growth when substrate is depleted.

Claims

1. A method for producing a polyhydroxyalkanoate, the method comprising the steps of:
 - (a) providing a mixture comprising a bacterial population that has undergone an initialisation process;
 - (b) adding a feed comprising a carbon source to the mixture to generate a feast phase for the bacterial population in the mixture;
 - (c) subsequently subjecting the bacterial population to a famine phase by allowing the carbon source to become depleted and not adding any further carbon source to the mixture for a period of time; and
 - (d) repeating steps (b) and (c) in an alternating pattern, wherein:
 - the method further comprises periodically removing a portion of the mixture and harvesting a polyhydroxyalkanoate from the bacterial population in the removed portion of the mixture;
 - each periodical removal of a portion of the mixture is conducted during a feast phase before the carbon source is entirely depleted from the mixture;
 - steps (b) and (c) are conducted in the presence of a light source that has a wavelength greater than 715 nm; and
 - the bacterial population is substantially formed from purple phototropic bacteria.
2. The method for producing a polyhydroxyalkanoate according to Claim 1, wherein each periodical removal of a portion of the mixture is conducted during the feast phase before the carbon source is reduced to a value of from 34 to 50 mg/L carbon, such as before the carbon source is reduced to a value of from 34.5 to 40 mg/L carbon, such as before the carbon source is reduced to a value of about 34.8 mg/L carbon.
3. The method for producing a polyhydroxyalkanoate according to Claim 1 or Claim 2, wherein each periodical removal of a portion of the mixture is conducted during from day 3 to day 4 of the feast phase.
4. The method for producing a polyhydroxyalkanoate according to any one of the preceding claims, wherein the carbon source is a volatile fatty acid.

5. The method for producing a polyhydroxyalkanoate according to Claim 4, wherein the volatile fatty acid is selected from one or more of the group consisting of acetic acid, butyric acid, propionic acid, caproic acid, valeric acid, iso-butyric acid, iso-caproic acid, iso-valeric acid, and salts thereof, optionally wherein the volatile fatty acid is acetic acid or a salt thereof.

6. The method for producing a polyhydroxyalkanoate according to any one of the preceding claims, wherein the feed comprises from 0.5 to 2 g/L of a carbon source, from 2.50 to 8.0 g/L of KH_2PO_4 , from 2.0 to 5.0 g/L of K_2HPO_4 , from 0.7 to 2.0 g/L of NH_4Cl , from 0.4 to 1.5 g/L of KCl , from 0.1 to 0.3 g/L of MgCl_2 , from 0.005 to 0.02 g/L of CaCl_2 , and from 0.25 to 0.75 g/L of NaCl , optionally wherein the feed comprises 1.0 g/L of a carbon source (e.g. acetic acid or a salt thereof), 4.4 g/L of KH_2PO_4 , 3.4 g/L of K_2HPO_4 , 1.3 g/L of NH_4Cl , 0.78 g/L of KCl , 0.2 g/L of MgCl_2 , 0.0146 g/L of CaCl_2 , and 0.5 g/L of NaCl .

7. The method for producing a polyhydroxyalkanoate according to any one of the preceding claims, wherein one or more of the following conditions are applied and maintained in the method:

- (ai) a pH of from 7.2 to 7.5;
- (aii) a hydraulic and solids retention time of 4 - 7 days; and
- (aiii) a biomass concentration of from 1 to 1.2 g volatile suspended solids/L.

8. The method for producing a polyhydroxyalkanoate according to any one of the preceding claims, wherein each feast phase uses an uptake ratio of chemical oxygen demand:N:P of 100:(from 5 to 10):1 (e.g. 100:(from 5 to 7):1, such as 1 g/L of a carbon source, 5 mM of phosphate buffer and 60 mg/L of NH_4^+).

9. The method for producing a polyhydroxyalkanoate according to any one of the preceding claims, wherein each famine phase lasts from 1 to 7 days, such as from 1 to 3 days or from 4 to 5 days.

10. The method for producing a polyhydroxyalkanoate according to any one of the preceding claims, wherein the periodical removal occurs in each feast phase.

11. The method for producing a polyhydroxyalkanoate according to any one of the preceding claims, wherein purple phototropic bacteria forms from 68 to 100%, such as from 70 to 95%, such as at least 93% of the bacterial population.
12. The method for producing a polyhydroxyalkanoate according to any one of the preceding claims, wherein the purple phototropic bacteria comprises *Rhodopseudomonas palustris*, optionally wherein the dominant species within the purple phototropic bacteria is *Rhodopseudomonas palustris*.
13. The method for producing a polyhydroxyalkanoate according to any one of the preceding claims, wherein the mixture comprising a bacterial population that has undergone an initialisation process is a mixture comprising a bacterial population substantially formed from purple phototropic bacteria that has undergone a first feast phase and a first famine phase.