



- (51) International Patent Classification:
C12P 7/62 (2006.01)
- (21) International Application Number:
PCT/US2012/040044
- (22) International Filing Date:
30 May 2012 (30.05.2012)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
61/491,484 31 May 2011 (31.05.2011) US
- (71) Applicant (for all designated States except US): **MI-CROMIDAS INC.** [US/US]; 930 Riverside Parkway, Suite 10, West Sacramento, CA 95605 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **DAVIS, Ryan** [US/US]; 930 Riverside Parkway, Suite 10, West Sacramento, CA 95605 (US). **ANDERSON, Dane** [US/US]; 930 Riverside Parkway, Suite 10, West Sacramento, CA 95605 (US). **CANNON, Doug** [US/US]; 930 Riverside Parkway, Suite 10, West Sacramento, CA 95605 (US).

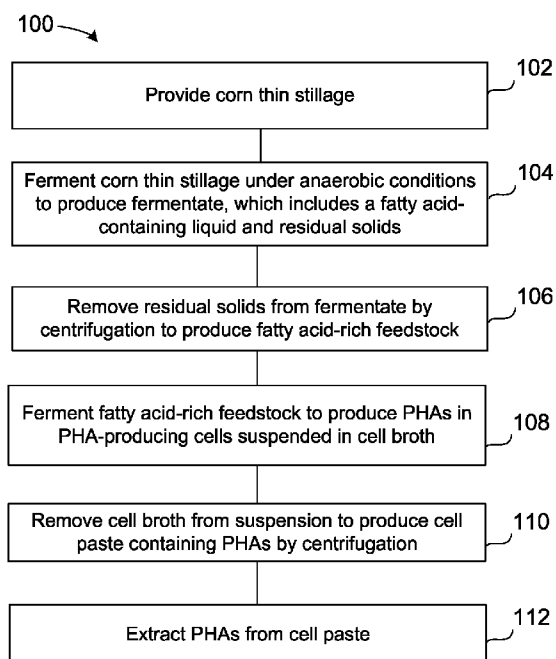
MCGRATH, Casey [US/US]; 930 Riverside Parkway, Suite 10, West Sacramento, CA 95605 (US). **BISSELL, John** [US/US]; 930 Riverside Parkway, Suite 10, West Sacramento, CA 95605 (US). **SMITH, Ryan, L.** [US/US]; 930 Riverside Parkway, Suite 10, West Sacramento, CA 95605 (US). **NIELSEN, Wade** [US/US]; 930 Riverside Parkway, Suite 10, West Sacramento, CA 95605 (US). **NIEDZWIEDZ, Philip** [US/US]; 930 Riverside Parkway, Suite 10, West Sacramento, CA 95605 (US). **INOUE, Mamie** [JP/US]; 930 Riverside Parkway, Suite 10, West Sacramento, CA 95605 (US).

(74) Agents: **WARD, Michael, R.** et al.; Morrison & Foerster LLP, 425 Market Street, San Francisco, CA 94105-2482 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD,

[Continued on next page]

(54) Title: METHOD TO PRODUCE POLYHYDROXYALKANOATES FROM WATER-BASED FEEDSTOCK STREAMS



(57) Abstract: The present disclosure provides methods to produce polyhydroxyalkanoates (PHAs) from water-based feedstock streams, which may be heterogeneous suspensions. The methods allow for handling of heterogeneous suspensions in very large flow rates, allowing for high volume processing, and resulting in more cost-effective and efficient process for PHA production.

FIG. 1



SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR,
TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, ML, MR, NE, SN, TD, TG).

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

METHOD TO PRODUCE POLYHYDROXYALKANOATES FROM WATER-BASED FEEDSTOCK STREAMS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 61/491,484 filed May 31, 2011, which is incorporated herein by reference in its entirety.

FIELD

[0002] The present disclosure generally relates to a process for producing polyhydroxyalkanoates (PHAs). In particular, the present disclosure relates to treating water-based feedstock streams, such as waste streams, to produce PHAs using a bacterial process.

BACKGROUND

[0003] Every day, millions of tons of petroleum-based plastic waste accumulate in the environment, resulting in growing non-biodegradable landfills and escalating waste disposal costs. A solution to this problem is to use biodegradable alternatives to plastics. One such alternative is polyhydroxyalkanoates (PHAs)—a family of high-performance, highly-marketable, biodegradable polymers possessing excellent physical properties suitable for a wide range of industrial applications.

[0004] PHAs are linear polyester macromolecules composed of hydroxyl fatty acid monomer subunits, produced by bacterial fermentation of sugar and lipids. The most common form of PHAs produced is a blend of polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV), which has properties very similar to polypropylene currently found in many containers, household items, and automotive parts. PHAs are UV-stable, resistant to a wide range of temperatures, and have attractive barrier properties. Unlike petroleum-based plastics that may take centuries to degrade, PHA-based plastics are completely biodegradable when placed in environments that foster decomposition, such as landfills, composting sites, or aquatic environments. Furthermore, PHA-based plastics can degrade quickly without any harmful effects on sea life or the greater ocean environment from chemical residues or other pollutants. In addition to these properties, PHAs are also biocompatible, gradually and harmlessly breaking down without inducing an inflammatory response in the body. As such, PHAs also have the potential to be useful for biomedical applications, such as medical sutures and tissue repair devices.

[0005] These favorable properties of PHAs provide incentives to develop efficient ways of producing PHAs using biological systems. Despite the advantages of using PHA-based plastics, the high price of PHAs compared to the low price of petrochemical-based plastics has significantly limited its widespread use. Several factors, including substrate costs, fermentation time, and efficiency of downstream processing, need to be improved to develop cost-effective and efficient biological systems to commercially produce PHAs.

[0006] Early processes for commercial PHA production focus primarily on the use of pure substrates (*e.g.*, sugars and fatty acids) for microbial fermentation. The use of heterogeneous feedstock to produce PHAs presents several challenges. For example, municipal wastewater is typically heterogeneous in its composition, and contains varying amounts of lignocellulosic materials, sugars and fatty acids. While municipal wastewater shares common components, the relative amounts of these components vary significantly depending on the materials released into the sewage system. The heterogeneity of the feedstock may significantly impact the efficiency of the PHA production process. As such, there exists a need in the art for a commercially viable process that can handle the heterogeneous nature of feedstock used for PHA production.

BRIEF SUMMARY

[0007] The present disclosure addresses this need by providing processes for producing polyhydroxyalkanoates (PHAs) from water-based feedstock streams that may contain varying amounts of cellulose, hemicellulose, lignin, sugars, fats, fatty acids, proteins, humic materials, and inorganic debris.

[0008] In one aspect, provided is a process for producing one or more polyhydroxyalkanoates (PHAs), by: (a) providing a carbonaceous feedstock; (b) contacting the carbonaceous feedstock with fermentation bacteria under conditions suitable to produce a fermentate, wherein the fermentate is made up of a fatty acid-containing liquid and residual solids; (c) separating at least a portion of the residual solids from the fermentate to produce a fatty acid-rich feedstock; (d) contacting the fatty acid-rich feedstock with PHA-producing bacteria in a cell broth under conditions suitable to produce one or more PHAs, wherein the contacting of the fatty acid-rich feedstock with the PHA-producing bacteria in the cell broth forms a suspension; and (e) separating at least a portion of the cell broth from the suspension to produce a cell paste, wherein the cell paste is made up of the one or more PHAs.

[0009] In some embodiments, the cell paste has a PHA content per dry cell weight of at least 30%, at least 40% at least 50%, at least 60%, at least 70%, at least 80% or at least 90%. In other

embodiments, the cell paste has a PHA content per dry cell weight of between 30% and 90%, between 40% and 60%, between 50% and 80%, or between 60% and 90%.

[0010] In some embodiments, the process further includes extracting the one or more PHAs from the cell paste. In certain embodiments, the one or more extracted PHAs have a purity of at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90%. In other embodiments, the one or more extracted PHAs have a purity of between 40% and 95%, between 40% and 80%, between 40% and 70%, between 40% and 60%, between 50% and 90%, or between 70% and 90%. In some embodiments, the one or more PHAs are extracted from the cell paste using a polar organic solvent. In certain embodiments, the polar organic solvent may be chloroform, dichloromethane, ethyl acetate, isobutanol, ethanol, propanol, propylene carbonate, ethylene carbonate, isopropanol, or amyl alcohol, or a mixture thereof.

[0011] In some embodiments, the fermentation bacteria are acidogenic bacteria. In certain embodiments, the fermentation bacteria may include *Butyribacterium rettgeri*, *Pseudomonas aeruginosa*, *Clostridium acetobutylicum*, or *Acetobacter woodii*, or a combination thereof.

[0012] In some embodiments, the fatty acid-containing liquid has viable bacteria that may compete with the using the fatty acids and other nutrients, and the process further includes reducing the viable bacteria in the fatty acid-containing liquid before contacting the fatty acid-containing liquid with the PHA-producing bacteria. In certain embodiments, the viable bacteria are reduced by ozone, bleach, pasteurization, irradiation, heat, filtration, antibiotics, chlorination, sulfur dioxide, electroporation, pH, or peroxides, or a combination thereof. In other embodiments, the process further includes sterilizing and/or disinfecting the fermentate or the fatty acid-rich feedstock. The sterilization and/or disinfection can reduce the odor of the fatty acid-containing liquid in the fermentate or the fatty acid-rich feedstock. In certain embodiments, the sterilization and/or disinfection employs ozone, bleach, pasteurization, irradiation, heat, filtration, antibiotics, chlorination, sulfur dioxide, electroporation, pH, or peroxides, or a combination thereof.

[0013] In some embodiments, the carbonaceous feedstock is chemically treated before fermentation to produce the fermentate. In some embodiments, the carbonaceous feedstock is fermented under anaerobic conditions. In other embodiments, the carbonaceous feedstock is fermented at a pH between 4.0 and 8.0. In certain embodiments, the carbonaceous feedstock is fermented at a pH between 5.5 and 7.0, or between 6.0 and 7.0. In one embodiment, the carbonaceous feedstock is fermented at a pH of about 5.5.

[0014] In some embodiments, the fatty acid-containing liquid includes short-chain fatty acids, medium-chain fatty acids, or a combination thereof. In certain embodiments, the fatty acid-containing liquid includes one or more fatty acids selected from butyrate, propionate, acetate, caproic acid, caprylic acid, capric acid, and lauric acid.

[0015] In some embodiments, the one or more PHAs are selected from polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV), polyhydroxybutyratevalerate (PHBV), and polyhydroxyhexanoate (PHH).

[0016] In some embodiments, the carbonaceous feedstock includes organic solid materials, inorganic solid materials, or a combination thereof. Examples of organic solid materials may include cellulose, hemicellulose, lignin, fats, fatty acids, saccharides, proteins, or humic materials, or a combination thereof. In other embodiments, the carbonaceous feedstock is dispersed in a water-based liquid. In yet other embodiments, at least 40% by weight of the carbonaceous feedstock is water. In certain embodiments, the carbonaceous feedstock is wastewater, animal manure, pulp waste, food processing plant waste, restaurant waste, yard waste, forest waste, biodiesel transesterification waste products, ethanol process waste, or a combination thereof. In certain embodiments, the carbonaceous feedstock is a heterogeneous carbonaceous feedstock.

[0017] In some embodiments, the fatty acid-rich feedstock is contacted with the PHA-producing bacteria in one or more continuous reactors. One example of a continuous reactor is a continuous stirred tank reactor. In some embodiments, the fatty acid-rich feedstock is contacted with the PHA-producing bacteria in two or more continuous reactors. In one embodiment, one of the two or more continuous reactors may be a growth reactor, and one or more of the two or more continuous reactors may be production reactors. The fatty acid-rich feedstock may be contacted with the PHA-producing bacteria in the growth reactor to produce an inoculated suspension under conditions suitable for growing the PHA-producing bacteria, and at least a portion of the inoculated suspension is transferred into the one or more production reactors, wherein the conditions in the one or more production reactors are suitable for PHA accumulation in the PHA-producing bacteria.

[0018] In certain embodiments, one or more of the following conditions are present in the growth reactor: (i) the temperature in the growth reactor is between 22°C and 35°C; (ii) the dissolved oxygen content in the growth reactor is between 10% and 50%; and (iii) the pH in the growth reactor is between 5 and 9, or between 6.5 and 9. In certain embodiments, the dilution

rate in the growth reactor is between 0.1 h^{-1} and 0.3 h^{-1} . In other embodiments, one or more of the following conditions are independently present in each of the one or more production reactors: (i) the temperature in each of the one or more production reactors is independently between 20°C and 32°C ; (ii) the dissolved oxygen content in each of the one or more production reactors is independently between 0.2% and 30%; and (iii) the pH in each of the one or more production reactors is independently between 6.5 and 9. In certain embodiments, the dilution rate in each of the one or more production reactors is independently between 0.04 h^{-1} and 0.2 h^{-1} . In some embodiments, the dilution rate in the growth reactor is greater than the dilution rate in production reactor(s). In other embodiments, the dilution rate in the growth reactor is less than the dilution rate in the production reactor(s). In yet other embodiments, the dilution rate in the growth reactor is about the same as the dilution rate in the production reactor(s).

[0019] In some embodiments, the process further includes producing one or more plastics from the cell paste. In yet other embodiments, the process further includes converting the one or more extracted PHAs into one or more plastics. In yet other embodiments, the process further includes removing any remaining cell broth from the cell paste to form dried PHA-containing cells. In yet other embodiments, the cell paste further includes residual cells. In one embodiment, the residual cells may contain nitrogen, phosphate, or a combination thereof.

[0020] In another aspect, provided are the dried cells produced by the process described above. In some embodiments, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% by weight of the dried cells is one or more PHAs. In other embodiments, between 15% and 99%, between 20% and 80%, between 30% and 70%, between 50% and 99%, or between 60% to 90% by weight of the dried cells is the one or more PHAs.

[0021] In yet another aspect, provided are the residual cells in the cell paste, produced by the process described above. The residual cells may be rich in nitrogen, phosphate, or a combination thereof.

[0022] In yet another aspect, provided is a process for producing polyhydroxyalkanoates (PHAs), by: (a) providing a carbonaceous feedstock; (b) contacting the carbonaceous feedstock with fermentation bacteria under conditions suitable to produce a fermentate, wherein the fermentate is made up of a fatty acid-containing liquid and residual solids; (c) separating at least a portion of the residual solids from the fermentate to produce a fatty acid-rich feedstock; (d) transferring at least a portion of the fatty acid-rich feedstock into a growth reactor; (e) contacting

the fatty acid-rich feedstock in the growth reactor with PHA-producing bacteria in a cell broth under conditions suitable for growing the PHA-producing bacteria, wherein the contacting of the fatty acid-rich feedstock with the PHA-producing bacteria in the cell broth forms a suspension in the growth reactor; (f) transferring at least a portion of the suspension from the growth reactor into one or more production reactors, wherein the conditions in the one or more production reactors are suitable for PHA accumulation in the PHA-producing bacteria; and (g) separating at least a portion of the cell broth from the suspension in the one or more production reactors to produce a cell paste, wherein the cell paste is made up of the one or more PHAs.

[0023] In some embodiments, one or more of the following conditions are present in the growth reactor: (i) the temperature in the growth reactor is between 22°C and 35°C; (ii) the dissolved oxygen content in the growth reactor is between 10% and 50%; and (iii) the pH in the growth reactor is between 5 and 9, or between 6.5 and 9. In certain embodiments, the dilution rate in the growth reactor is between 0.1 h⁻¹ and 0.3 h⁻¹.

[0024] In other embodiments, one or more of the following conditions are independently present in each of the one or more production reactors: (i) the temperature in each of the one or more production reactors is independently between 20°C and 32°C; (ii) the dissolved oxygen content in each of the one or more production reactors is independently between 0.2% and 30%; and (iii) the pH in each of the one or more production reactors is independently between 6.5 and 9. In certain embodiments, the dilution rate in each of the one or more production reactors is independently between 0.04 h⁻¹ and 0.2 h⁻¹. In some embodiments, the dilution rate in the growth reactor is greater than the dilution rate in production reactor(s). In other embodiments, the dilution rate in the growth reactor is less than the dilution rate in the production reactor(s). In yet other embodiments, the dilution rate in the growth reactor is about the same as the dilution rate in the production reactor(s).

[0025] In some embodiments, the growth reactor is a continuous reactor. In other embodiments, the one or more production reactors are each a continuous reactor.

[0026] In some embodiments, the process further includes transferring at least a portion of the fatty acid-rich feedstock into the one or more production reactors; and producing one or more PHAs from the fatty acid-rich feedstock in the one or more production reactors.

[0027] In some embodiments, the cell paste has a PHA content per dry cell weight of at least 30%, at least 40% at least 50%, at least 60%, at least 70%, at least 80% or at least 90%. In other

embodiments, the cell paste has a PHA content per dry cell weight of between 30% and 90%, between 40% and 60%, between 50% and 80%, or between 60% and 90%.

[0028] In some embodiments, the process further includes extracting the one or more PHAs from the cell paste. In certain embodiments, the one or more extracted PHAs have a purity of at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90%. In other embodiments, the one or more extracted PHAs have a purity of between 40% and 95%, between 40% and 80%, between 40% and 70%, between 40% and 60%, between 50% and 90%, or between 70% and 90%. In some embodiments, the one or more PHAs are extracted from the cell paste using a polar organic solvent. In certain embodiments, the polar organic solvent may be chloroform, dichloromethane, ethyl acetate, isobutanol, ethanol, propanol, propylene carbonate, ethylene carbonate, isopropanol, or amyl alcohol, or a mixture thereof.

[0029] In some embodiments, the carbonaceous feedstock is fermented by acidogenic fermentation bacteria. In certain embodiments, the fermentation bacteria may include *Butyribacterium rettgeri*, *Pseudomonas aeruginosa*, *Clostridium acetobutylicum*, or *Acetobacter woodii*, or a combination thereof.

[0030] In other embodiments, the fatty acid-containing liquid further has viable bacteria that may compete with the using the fatty acids and other nutrients, and the process further includes reducing the viable bacteria in the fatty acid-containing liquid before contacting the fatty acid-containing liquid with the PHA-producing bacteria. In certain embodiments, the viable bacteria is reduced by ozone, bleach, pasteurization, irradiation, heat, filtration, antibiotics, chlorination, sulfur dioxide, electroporation, pH, or peroxides, or a combination thereof. In other embodiments, the process further includes sterilizing and/or disinfecting the fermentate or the fatty acid-rich feedstock. The sterilization and/or disinfection can reduce the odor of the fatty acid-containing liquid in the fermentate or the fatty acid-rich feedstock. In certain embodiments, the sterilization and/or disinfection employs ozone, bleach, pasteurization, irradiation, heat, filtration, antibiotics, chlorination, sulfur dioxide, electroporation, pH, or peroxides, or a combination thereof.

[0031] In some embodiments, the carbonaceous feedstock is chemically treated before fermentation to produce the fermentate. In some embodiments, the carbonaceous feedstock is fermented under anaerobic conditions. In other embodiments, the carbonaceous feedstock is fermented at a pH between 4.0 and 8.0. In certain embodiments, the carbonaceous feedstock is

fermented at a pH between 5.5 and 7.0, or between 6.0 and 7.0. In one embodiment, the carbonaceous feedstock is fermented at a pH of about 5.5.

[0032] In some embodiments, the fatty acid-containing liquid includes short-chain fatty acids, medium-chain fatty acids, or a combination thereof. In certain embodiments, the fatty acid-containing liquid includes one or more fatty acids selected from butyrate, propionate, acetate, caproic acid, caprylic acid, capric acid, and lauric acid.

[0033] In some embodiments, the one or more PHAs are selected from polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV), polyhydroxybutyratevalerate (PHBV), or polyhydroxyhexanoate (PHH).

[0034] In some embodiments, the carbonaceous feedstock includes organic solid materials, inorganic solid materials, or a combination thereof. Examples of organic solid materials may include cellulose, hemicellulose, lignin, fats, fatty acids, saccharides, proteins, or humic materials, or a combination thereof. In other embodiments, the carbonaceous feedstock is dispersed in a water-based liquid. In yet other embodiments, at least 40% by weight of the carbonaceous feedstock is water. In certain embodiments, the carbonaceous feedstock is wastewater, animal manure, pulp waste, food processing plant waste, restaurant waste, yard waste, forest waste, biodiesel transesterification waste products, ethanol process waste, or a combination thereof. In certain embodiments, the carbonaceous feedstock is a heterogeneous carbonaceous feedstock.

[0035] In some embodiments, the process further includes producing one or more plastics from the cell paste. In yet other embodiments, the process further includes converting the one or more extracted PHAs into one or more plastics. In yet other embodiments, the process further includes removing any remaining cell broth from the cell paste to form dried PHA-containing cells. In yet other embodiments, the cell paste further includes residual cells. In one embodiment, the residual cells may contain nitrogen, phosphate, or a combination thereof.

[0036] In another aspect, provided are the dried cells produced by the process described above. In some embodiments, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% by weight of the dried cells is one or more PHAs. In other embodiments, between 15% and 99%, between 20% and 80%, between 30% and 70%, between 50% and 99%, or between 60% to 90% by weight of the dried cells is the one or more PHAs.

[0037] In yet another aspect, provided are the residual cells in the cell paste, produced by the process described above. The residual cells may be rich in nitrogen, phosphate, or a combination thereof.

[0038] Another aspect of the present disclosure provides a process for producing polyhydroxyalkanoates (PHAs) by: (a) providing a carbonaceous feedstock; (b) contacting the carbonaceous feedstock with fermentation bacteria to produce a fermented feedstock, in which the fermented feedstock includes a fatty acid-containing liquid and residual solids; (c) separating the fermented feedstock to remove at least a portion of the residual solids; (d) contacting the fatty acid-containing liquid with PHA-producing bacteria to produce a suspension that includes PHA-producing cells suspended in a cell broth; (e) separating the suspension to form one or more PHA-containing solids, in which the one or more PHA-containing solids includes PHAs; and (f) extracting the PHAs from the one or more PHA-containing solids.

[0039] In some embodiments, the extracting of the PHAs from the one or more PHA-containing solids includes: forming a cell paste from the one or more PHA-containing solids, in which the cell paste includes the one or more PHA-containing solids suspended in a liquid; and extracting the PHAs from the cell paste using a polar organic solvent. In other embodiments that may be combined with any of the preceding embodiments, the extracting of the PHAs from the cell paste includes: removing at least a portion of the liquid from the cell paste to form dried PHA-containing cells prior to extracting the PHAs. In some embodiments that may be combined with any of the preceding embodiments, the process further includes converting the PHAs into one or more plastics.

[0040] In some embodiments that may be combined with any of the preceding embodiments, the separating of the fermented feedstock to remove at least a portion of the residual solids employs flocculation. In one embodiment, the flocculation of the fermented feedstock employs a flocculant. In a specific embodiment, the flocculant includes a cationic polymer. In another embodiment, the flocculation of the fermented feedstock is performed by increasing the pH of the fermented feedstock.

[0041] In some embodiments that may be combined with any of the preceding embodiments, the separating of the suspension to form the one or more PHA-containing solids employs flocculation, and the one or more PHA-containing solids are one or more flocs. In one embodiment, the flocculation of the suspension to form the one or more flocs is performed by increasing the pH of the cell broth to a first pH. In one embodiment, the first pH is between 10.0

and 13.5. In another embodiment, the first pH is between 11.0 and 12.5. In yet another embodiment, the process further includes increasing the size of the one or more flocs by decreasing the pH of the cell broth to a second pH. In one embodiment, the second pH is between 6.0 and 11.0. In another embodiment, the second pH is between 10.0 and 11.5.

[0042] In another embodiment that may be combined with any of the preceding embodiments, the fatty acid-containing liquid further includes viable bacteria, and the process further includes reducing the viable bacteria in the fatty acid-containing liquid before contacting the fatty acid-containing liquid with the PHA-producing bacteria. For example, the viable bacteria may be reduced by ozone, bleach, pasteurization, irradiation, heat, filtration, antibiotics, chlorination, sulfur dioxide, electroporation, pH, or peroxides, or a combination thereof. In other embodiments, the process further includes sterilizing and/or disinfecting the fatty acid-containing liquid. For example, the sterilization and/or disinfection may employ ozone, bleach, pasteurization, irradiation, heat, filtration, antibiotics, chlorination, sulfur dioxide, electroporation, pH, or peroxides, or a combination thereof. In certain embodiments, the sterilization and/or disinfection reduces the odor of the fatty acid-containing liquid.

[0043] In yet other embodiments that may be combined with any of the preceding embodiments, the contacting of the carbonaceous feedstock with fermentation bacteria occurs under anaerobic conditions. In some embodiments that may be combined with any of the preceding embodiments, the contacting of the carbonaceous feedstock with fermentation bacteria occurs at a pH between 4.0 and 8.0. In one embodiment, the contacting of the carbonaceous feedstock with fermentation bacteria occurs at a pH between 5.5 and 7.0. In another embodiment, the contacting of the carbonaceous feedstock with fermentation bacteria occurs at a pH of about 5.5.

[0044] In some embodiments that may be combined with any of the preceding embodiments, the fatty acid-containing liquid includes short-chain fatty acids, medium-chain fatty acids, or combinations of two. In other embodiments that may be combined with any of the preceding embodiments, the PHAs may include polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV), polyhydroxyhexanoate, (PHH), polyhydroxyoctanoate, polyhydroxydecanoate, PHA copolymers (*e.g.*, polyhydroxybutyratevalerate (PHBV)), or combinations of these products. In one embodiment, the PHAs include polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV), polyhydroxybutyratevalerate (PHBV), or combinations of these products. In another embodiment, the PHAs include polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV), or combinations of these products.

[0045] In other embodiments that may be combined with any of the preceding embodiments, the process further includes contacting the removed cell broth with second PHA-producing bacteria to produce additional PHA-producing cells. In yet other embodiments that may be combined with any of the preceding embodiments, the polar organic solvent used for extracting the PHAs may include chloroform, dichloromethane, ethyl acetate, isobutanol, ethanol, propanol, propylene carbonate, ethylene carbonate, isopropanol, amyl alcohol, or mixtures of these solvents.

[0046] In yet other embodiments that may be combined with any of the preceding embodiments, the carbonaceous feedstock may include organic solid materials, inorganic solid materials, or a combination of both solid materials. The organic solid materials may include cellulose, hemicellulose, lignin, fats, fatty acids, proteins, saccharides, or humic materials, or a combination of these materials. In yet other embodiments, the carbonaceous feedstock is dispersed in a water-based liquid. In some embodiments, the carbonaceous feedstock is wastewater, animal manure, pulp waste, food processing plant waste (*e.g.*, tomato paste production waste), restaurant waste, yard waste, forest waste, biodiesel transesterification waste products (*e.g.*, glycerol), or combinations thereof. In other embodiments that may be combined with any of the preceding embodiments, the carbonaceous feedstock is heterogeneous in nature.

[0047] Another aspect of the present disclosure provides dried cells produced by the process described above. In some embodiments, the dried cells may contain at least 10% of PHAs by weight. In other embodiments, the dried cells may contain at least 15% of PHAs by weight. In another embodiment, the dried cells may contain 10-30% PHAs by weight. In yet another embodiment, the dried cells may contain 15-20% PHAs by weight.

[0048] Another aspect of the present disclosure provides a process for producing polyhydroxyalkanoates (PHAs) by: (a) providing wastewater; (b) contacting the wastewater with fermentation bacteria to produce fermented wastewater, in which the fermented wastewater includes a fatty acid-containing liquid and residual solids; (c) flocculating the fermented wastewater to remove at least a portion of the residual solids; (d) contacting the fatty-containing liquid with PHA-producing bacteria to produce a suspension, which includes PHA-producing cells suspended in a cell broth; (e) flocculating the suspension to form one or more flocs, in which the one or more flocs includes PHAs; (f) forming a cell paste from the one or more flocs, in which the cell paste includes the one or more flocs suspended in a liquid; (g) removing at least a portion of the liquid from the cell paste to form dried PHA-containing cells before extracting the PHAs; and (h) extracting the PHAs from the dried PHA-containing cells using a polar

organic solvent. In some embodiments, the fatty acid-containing liquid further includes viable bacteria, and the process further includes reducing the viable bacteria in the fatty acid-containing liquid before contacting the fatty acid-containing liquid with the PHA-producing bacteria. In other embodiments that may be combined with any of the preceding embodiments, the wastewater is heterogeneous in nature.

[0049] Another aspect of the present disclosure provides a process for producing polyhydroxyalkanoates (PHAs) by: (a) providing a carbonaceous feedstock, in which the carbonaceous feedstock includes one or more organic materials dissolved or partially dissolved in water; (b) contacting the fermented feedstock with PHA-producing bacteria to produce a suspension, which includes PHA-producing cells suspended in a cell broth; and (c) isolating PHAs produced from the PHA-producing cells in the suspension. In one embodiment, the one or more organic materials may include glucose, fructose, or a combination of these materials. In another embodiment, the one or more organic materials may include oils and fats. In yet other embodiments that may be combined with any of the preceding embodiments, the isolating of PHAs includes: flocculating the suspension by increasing the pH of the cell broth to a first pH to form one or more flocs, in which the one or more flocs include PHAs; removing at least a portion of the cell broth from the flocculated suspension to isolate the one or more flocs; forming a cell paste from the one or more flocs, in which the cell paste includes the one or more flocs suspended in a liquid; removing at least a portion of the liquid from the cell paste to form dried PHA-containing cells; and extracting the PHAs from the dried PHA-containing cells using a polar organic solvent.

[0050] Another aspect of the present disclosure provides a process of producing dried PHA-containing cells by: (a) providing a carbonaceous feedstock; (b) contacting the carbonaceous feedstock with fermentation bacteria to produce a fermented feedstock, in which the fermented feedstock includes a fatty acid-containing liquid and residual solids; (c) separating the fermented feedstock to remove at least a portion of the residual solids; (d) contacting the fatty acid-containing liquid with PHA-producing bacteria to produce a suspension that includes PHA-producing cells suspended in a cell broth; (e) separating the suspension to form one or more PHA-containing solids, in which the one or more PHA-containing solids includes PHAs; and (f) extracting the PHAs from the one or more PHA-containing solids. In some embodiments that may be combined with the preceding embodiment, the producing of dried PHA-containing cells from the one or more PHA-containing solids includes: forming a cell paste from the one or more PHA-containing solids, in which the cell paste includes the one or more PHA-containing solids

suspended in a liquid; and removing at least a portion of the liquid from the cell paste to produce dried PHA-containing cells. In other embodiments that may be combined with the preceding embodiments, the separating of the fermented feedstock to remove at least a portion of the residual solids employs flocculation, in which the separating of the suspension to form the one or more PHA-containing solids employs flocculation, and the one or more PHA-containing solids are one or more flocs. In some embodiments that may be combined with any of the preceding embodiments, the carbonaceous feedstock is heterogeneous in nature.

[0051] Another aspect of the present disclosure provides a process for producing one or more bioproducts, the process comprising: (a) providing a heterogeneous wastestream; (b) contacting the heterogeneous wastestream with fermentation bacteria to produce a fermented wastestream, in which the fermented wastestream includes a volatile fatty acid-containing liquid and residual solids; (c) separating the fermented wastestream to remove at least a portion of the residual solids; (d) contacting the volatile fatty acid-containing liquid with bioproduct-producing bacteria to produce a suspension that includes bioproduct-producing cells suspended in a cell broth; (e) separating the suspension to form one or more bioproduct-containing solids, in which the bioproduct-containing solids include one or more bioproducts; and (f) extracting the one or more bioproducts from the bioproduct-containing solids. In some embodiments, the contacting of the heterogeneous wastestream with fermentation bacteria occurs under anaerobic conditions. In yet other embodiments that may be combined with any of the preceding embodiments, the bioproduct may include polyhydroxyalkanoates (PHAs).

DESCRIPTION OF THE FIGURES

[0052] The present application can be understood by reference to the following description taken in conjunction with the accompanying drawing figures, in which like parts may be referred to by like numerals:

[0053] FIGS. 1 and 2 depict two exemplary processes for producing polyhydroxyalkanoates (PHAs) from a carbonaceous feedstock involving a first separation to remove residual solids from the carbonaceous feedstock after anaerobic fermentation, and a second separation downstream in the PHA production process; and

[0054] FIG. 3 depicts an exemplary process scheme for producing PHAs from fatty acid-rich feedstock using continuous reactors.

DETAILED DESCRIPTION

[0055] The following description sets forth numerous exemplary configurations, processes, parameters, and the like. It should be recognized, however, that such description is not intended as a limitation on the scope of the present disclosure, but is instead provided as a description of exemplary embodiments.

[0056] The following description relates to a process for producing polyhydroxyalkanoates (PHAs). The production of PHAs from water-based carbonaceous feedstock streams, such as waste streams, typically involves three basic steps: (1) anaerobic fermentation of the feedstock stream to generate fatty acids (*e.g.*, volatile fatty acids), which in turn is used as feedstock for PHA-producing bacteria; (2) production of PHAs by bacteria in an aerobic reactor; and (3) extraction and purification of the PHAs.

[0057] The present disclosure provides a process for producing PHAs that is capable of processing carbonaceous feedstock with varying heterogeneity. The process removes residual solids from the feedstock stream after anaerobic fermentation. Additionally, the process separates out PHA-containing solids downstream after fermentation by PHA-producing bacteria. In some embodiments, the PHA-containing solids may be obtained in the form of a cell paste containing PHAs and other cellular materials. In other embodiments, the second separation step may produce PHA-containing solids that can be made into a PHA-containing cell paste. This cell paste can be dried to form dried PHA-containing cells, which can be easily stored and/or transported before further processing to isolate the PHAs. Storing dried cells allows for aggregation of crude PHA-containing materials from various sources and/or locations for batch processing to extract the PHAs. Moreover, transporting the dried cells allows for extraction to obtain PHAs at a different plant or site from the PHA production, as well as centralized processing by aggregating dried cells from various PHA-producing plants or sites.

[0058] With reference to FIG. 1, process **100** is an exemplary embodiment for producing PHAs from corn thin stillage involving two centrifugation steps. In step **102**, corn thin stillage is provided from a corn ethanol producer. In step **104**, the corn thin stillage is contacted with fermentation bacteria under anaerobic conditions suitable for producing a fermentate containing a fatty acid-containing liquid and residual solids. The fatty acid-containing liquid is made up of short- and medium-chain fatty acids, such as butyrate, propionate, and acetate. The residual solids may include any lignin and cellulose that may have been present in the corn thin stillage.

The fermentation bacteria may be intrinsic to the feedstock, or extrinsic bacteria may be added to the feedstock.

[0059] In step 106, these solid materials in the fermentate are removed by centrifugation, resulting in a fatty acid-rich feedstock used for the subsequent PHA production. It should be understood that in other exemplary embodiments, other separation methods may include, for example, filtration. A combination of separation methods may also be used.

[0060] In step 108, the fatty acid-rich feedstock is inoculated with PHA-producing bacteria in a cell broth, and the PHA-producing cells are suspended in the cell broth. Since the PHA-producing bacteria may produce PHAs *in vivo*, PHAs may be released when the wall or membrane of the PHA-producing cells is made permeable.

[0061] After PHAs have been produced by the PHA-producing bacteria, at least a portion of the cell broth is removed from the suspension by centrifugation to produce a cell paste in step 110. While centrifugation is used in both steps 106 and 110 of exemplary process 100, it should be understood that the separation method used in step 106 may be, in some embodiments, the same or, in other embodiments, different from step 110. In step 112, the PHAs are extracted from the cell paste. Various methods may be used to extract the PHAs, including the use of solvents.

[0062] With reference to FIG. 2, process 200 is another exemplary embodiment for producing PHAs from a different carbonaceous feedstock, municipal wastewater, involving two flocculation steps. The wastewater provided in step 202 is a water-based feedstock, containing carbonaceous materials such as one or more of cellulose, hemicellulose, lignin, sugars, fats, fatty acids, proteins, humic materials, and/or inorganic debris. In step 204, the wastewater is contacted with fermentation bacteria, and undergoes anaerobic fermentation to produce fermented wastewater containing short- and medium-chain fatty acids. In particular, the sugars, fats, fatty acids, and some of the hemicellulose in the wastewater are converted into short- and medium-chain fatty acids, such as butyrate, propionate, and acetate. This fermented wastewater also contains solid materials, such as lignin and cellulose.

[0063] In step 206, these solid materials are removed by flocculation. The addition of a cationic polymer to the fermented wastewater causes most of the residual solids to flocculate, which can then be filtered off. In step 208, ozonation is applied to reduce the amount of viable bacteria in the fatty acid-containing liquid (*i.e.*, to sterilize and/or disinfect the fatty acid-

containing liquid). Moreover, ozonation can reduce some of the liquid's undesirable odors. It should be understood, however, that other sterilization and/or disinfection techniques may be employed, including the use of peroxides (*e.g.*, hydrogen peroxide, peracetic acid).

[0064] In step **210**, the sterilized/disinfected fatty acid-containing liquid is inoculated with PHA-producing bacteria in a cell broth, forming a suspension of PHA-producing cells in the cell broth. Since the PHA-producing bacteria may produce PHAs *in vivo*, PHAs may be released when the wall or membrane of the PHA-producing cells is made permeable. As depicted in step **212**, in this exemplary embodiment, the pH of the cell broth is increased to lyse the PHA-producing cells, which also causes flocculation of cellular debris and PHA granules to form flocs. While flocculation is used in both steps **206** and **212**, it should be understood that the separation method used in step **206** may be, in some embodiments, the same or, in other embodiments, different from step **212**.

[0065] In step **214**, the PHA-containing flocs are isolated from the cell broth by removing the supernatant. In step **216**, these isolated flocs are re-suspended in water, and the suspension is ground to form a PHA-containing cell paste. In step **118**, the PHA-containing cell paste is dried to form dried PHA-containing cells. In step **220**, a polar organic solvent (*e.g.*, chloroform or dichloromethane) is added to the dried PHA-containing cells to extract the PHAs.

[0066] Further, it should be noted that one or more steps may be added or omitted from the exemplary processes described herein. For example, in some embodiments, an additional step of chemically treating the carbonaceous feedstock, *i.e.*, corn thin stillage (process **100**) and municipal wastewater (process **200**), before fermentation to produce the fermentate containing the fatty acids. In other embodiments, additional steps may include adjusting the pH of the carbonaceous feedstock before fermentation to produce fatty acids, or adjusting the pH of the carbonaceous feedstock after fermentation to produce fatty acids and before inoculation with PHA-producing bacteria. Further, the sterilization and/or disinfection step may be omitted (as seen in process **100**) or present (as seen in process **200**). In certain embodiments, the fatty acid-containing liquid may be directly inoculated with PHA-producing bacteria after flocculation to remove residual solids.

[0067] As used herein, the term "about" refers to an approximation of a stated value within an acceptable range. Preferably, the range is +/- 10% of the stated value.

The Carbonaceous Feedstock

[0068] While the carbonaceous feedstocks provided in the exemplary processes described above is corn thin stillage (process **100**) and municipal wastewater (process **200**), the carbonaceous feedstock is not limited to these feedstocks. As used herein, the carbonaceous feedstock can be any material that contains carbon and can serve as a source for producing PHAs. Such materials may include one or more of animal manure, pulp waste, waste from food processing plants (*e.g.*, tomato paste production waste), restaurant waste, yard waste, forest waste, other plant-based materials, biodiesel transesterification waste products (*e.g.*, glycerol), ethanol fermentation waste products (*e.g.*, thin stillage from corn or cane sugar), or a combination of these materials. In some embodiments, the carbonaceous feedstock may contain organic and inorganic solid materials suspended in a water-based liquid.

[0069] The carbonaceous feedstock used in the processes described herein typically contains organic materials, such as cellulose, hemicellulose, lignin, sugars, oils, fats, fatty acids, proteins and/or humic materials. The relative amount of such organic materials will vary from one type of feedstock to another, as well as among different batches of feedstock used in the processes described herein. Feedstock with high organic content is suitable for the process described herein because the organic materials (*e.g.*, sugars, fats, and fatty acids) serve as the feedstock for producing the fatty acids that serve as precursors for PHA production. In some embodiments, the total amount of organic material in the carbonaceous feedstock is at least above 1%, at least above 5%, at least above 10%, at least above 20%, at least above 30%, at least above 40%, or at least above 50% of the total solids content. In some embodiments, the carbonaceous feedstock may contain more fats, oils, and greases than lignocellulosic materials, which may be desirable since fats, oils, and greases are more readily convertible into fatty acids.

[0070] The total solids content in the carbonaceous feedstock may affect the efficiency of the process described herein. For example, the total solids content may affect the efficiency of the separation steps. In some embodiments, the total solids content of the carbonaceous feedstock is between 0.5% and 80%, between 0.5% and 60%, between 5% and 50%, between 10% and 40%, or between 20% and 60%.

[0071] In instances where flocculation is employed as the separation technique, when the total solids content is too high, there may not be enough free volume to flocculate. When the total solids content is too low, it may be harder to flocculate because the suspension is too dispersed. In some embodiments, the total solids content of the carbonaceous feedstock is below

15%. In other embodiments, the total solids content of the carbonaceous feedstock is below 10%. In yet other embodiments, the total solids content is between 0.5% to 10%. In yet other embodiments, the total solids content is between 0.5% to 6%.

[0072] In some embodiments, the carbonaceous feedstock is a water-based carbonaceous feedstock. In certain embodiments, at least 40%, at least 50%, at least 60%, at least 70%, or at least 80% by weight of the carbonaceous feedstock is water. The carbonaceous feedstock used in the process described herein is typically a suspension, in which the solid phase includes organic materials present as distinct particles suspended in water. As used herein, the term “suspension” refers to a heterogeneous mixture of one or more liquids and solid particles, in which the liquid forms a continuous phase and the solid particles form a discontinuous phase inside the liquid. Where the solid particles have a size of less than about one micron, the suspension may be a colloid, in which the particles are dispersed evenly throughout the liquid.

[0073] In some embodiments, the carbonaceous feedstock is a heterogeneous carbonaceous feedstock. In certain embodiments, the heterogeneous carbonaceous feedstock is a suspension of at least two, at least three, at least four, or at least five materials suspended in a water-based liquid.

[0074] It should be noted that although the process described herein is well-suited to handling heterogeneous feedstock, homogenous feedstock may also be used. For example, the carbonaceous feedstock may be a solution of glucose, fructose or carbohydrates dissolved in water, without the presence of suspended materials. As used herein, the term “solution” refers to a homogeneous mixture of two or more substances, which the solute is dissolved in a solvent such that there is no distinct boundary between solute and solvent (*i.e.*, there is one continuous phase). If a feedstock solution containing sugars is used for the process described herein, removal of solids from the fermentate upstream may not be necessary because solids are not present. As used herein, the term “fermentate” refers to the liquid effluent from a biological digestion process, such as fermentation.

[0075] In some embodiments, the carbonaceous feedstock can be treated before fermentation to produce the fermentate containing fatty acids. In some embodiments, the carbonaceous feedstock may be chemically treated using, for example, acid or base treatment to make the feedstock more susceptible to fermentation to produce fatty acids.

Fermentation of Carbonaceous Feedstock to Produce Fatty Acids

[0076] The carbonaceous feedstock is fermented to produce fatty acids and salts thereof. The fermentate generally includes a fatty-acid containing liquid and residual solids (*e.g.*, lignocellulosic materials and inorganic solids).

[0077] Fatty acids produced by the fermentation of the carbonaceous feedstock described herein may include, for example, butyrate, propionate, acetate, caproic acid, caprylic acid, capric acid, and lauric acid. It should be understood that fatty acids of any monomeric length may be produced. The fatty acid produced may depend on the fermentation bacteria employed in the process described herein. In some embodiments, the carbonaceous feedstock is fermented to produce short- and medium-chained fatty acids.

[0078] As used herein, “short-chained fatty acids” (also known as volatile fatty acids or VFAs) have a carbon chain of six carbons or fewer. “Medium-chained fatty acids” have a carbon chain between six to twelve carbons. “Long-chained fatty acids” have a carbon chain greater than twelve carbons. The salts of the fatty acids may include, for example, acetate salts (*e.g.*, sodium acetate salts of the fatty acids).

[0079] The carbonaceous feedstock may be fermented to produce fatty acids using any standard fermentation techniques known in the art. In some embodiments, as depicted in exemplary processes **100** (FIG. 1) and **200** (FIG. 2), anaerobic fermentation is employed. During the anaerobic fermentation process, fermentation bacteria partially break down the solid organic materials in the carbonaceous feedstock to generate fatty acids. As discussed above, the solid organic materials in the carbonaceous feedstock may include cellulose, hemicellulose, lignin, sugars, fats, fatty acids, proteins and/or humic materials. The sugars and fats in the carbonaceous feedstock are readily converted into fatty acids. The lignocellulosic materials in the carbonaceous feedstock may also be converted into fatty acids, although often in lower yields.

[0080] In other embodiments, aerobic fermentation may also be employed to produce fatty acids from the carbonaceous feedstock. Fermentation under aerobic conditions may in certain instances be less efficient, however, due to higher fermentation rates that may lead to greater loss of carbon through respiration and carbon dioxide emission.

[0081] Numerous factors related to the fermentation conditions, such as the operating pH and the type of fermentation bacteria may affect the type and ratio of fatty acids produced. The ratio

of fatty acids produced affects the relative amounts of co-polymers, such as polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV), polyhydroxybutyratevalerate (PHBV), and/or polyhydroxyhexanoate (PHH), present in the final PHA product.

[0082] Controlling the pH at which the fermentation to fatty acids occurs may be useful for controlling the downstream production of the final PHA product, such as the blend of PHB and PHV present in the final PHA product, and the length of the PHA polymers and/or copolymers. For example, lower operating pH may produce longer chains of fatty acids at the end of fermentation, which may lead to a higher content of PHV and other longer-chained PHAs in the final PHA product. In some embodiments, fermentation occurs at a pH between 4.0 and 8.0. In other embodiments, fermentation occurs at a pH between 5.5 and 7.0. In one embodiment, fermentation occurs at a pH between 6.0 and 7.0.

[0083] In some embodiments, the process includes determining the composition of the carbonaceous feedstock to determine the appropriate operating pH. One skilled in the art would recognize the various techniques available for analyzing the carbonaceous feedstock. For example, predictive analyses may include total solids, gas chromatography, and elemental analysis. Rough elemental analysis may be helpful to identify specific inhibitors.

[0084] Moreover, any fermentation bacteria that can convert the organic materials found in the carbonaceous feedstock into fatty acids may be employed in the process described herein. The bacteria causing fermentation to produce fatty acids may either intrinsically exist in the carbonaceous feedstock provided, or extrinsically be added to the carbonaceous feedstock. In some embodiments, the fermentation bacteria intrinsically exist in the carbonaceous feedstock. In other embodiments, the carbonaceous feedstock may be inoculated with one or more fermentation bacteria. In yet other embodiments, the fermentation bacteria may include bacteria that are both intrinsic to and extrinsically added to the carbonaceous feedstock.

[0085] In certain embodiments, the fermentation bacteria are acidogenic bacteria, which typically convert the organic materials in the carbonaceous feedstock described above into fatty acids, such as volatile fatty acids. The acidogenic bacteria may be intrinsic to the carbonaceous feedstock, or added to the carbonaceous feedstock. Other types of fermentation bacteria might be present intrinsically in the carbonaceous feedstock, including for example methanogenic bacteria. In certain embodiments, a greater amount of acidogenic bacteria is present for fermentation of the carbonaceous feedstock into fatty acids than other types, such as methanogenic bacteria.

[0086] Examples of suitable fermentation bacteria used in the processes described herein may include *Butyribacterium rettgeri*, *Pseudomonas aeruginosa*, *Clostridium acetobutylicum*, and *Acetobacter woodii*, as well as any genetically-engineered organisms that may produce fatty acids from a carbonaceous feedstock.

Separation of the Fermented Feedstock

[0087] The fatty acids produced by fermentation serve as feedstock for PHA-producing bacteria downstream in the process. Depending on the composition of the carbonaceous feedstock, however, not all components in the feedstock may be converted into fatty acids. As discussed above, organic materials such as sugars and fats may be more readily converted into fatty acids. The lignocellulosic materials and/or inorganic solids that are less readily converted into fatty acids may remain suspended in the fermentate. The lignocellulosic materials and/or inorganic solids typically make up the residual solids in the fermentate.

[0088] At least a portion of the residual solids are removed from the fermentate according to the processes described herein. Removal of such solids results in a fatty-acid rich feedstock for use in PHA production. Any techniques known in the art suitable for removing at least a portion of the residual solids suspended in the fermentate may be employed. Suitable techniques may involve, for example, flocculation, gravity belt separators, hydrocyclone, membranes, filtration, centrifugation, or a combination of these techniques.

[0089] In some embodiments, centrifugation (as depicted in process **100**) or filtration is employed.

[0090] In other embodiments, flocculation (as depicted in process **200**) is employed. Although flocculation removes a substantial amount of the residual solids, it should be understood that the fatty acid-containing liquid may still include some of the residual solids. In some embodiments, flocculation removes at least 90% of the residual solids. In other embodiments, flocculation removes at least 80% of the residual solids. Solid/liquid separation may offer several advantages for the processes described herein. For example, removing the residual solids upstream from the PHA-production process reduces the number of contaminant microbes that may compete with nutrition-uptake with PHA-producing bacteria, the additional reactive and/or adsorption sites that may compete with PHA-producing bacteria for the supply of oxygen in an aerobic PHA reactor, as well as the contaminants in the final PHA material. Moreover, removing solid material in the feedstock makes it easier to monitor cell growth rate

(*e.g.*, by flow cytometry or UV-spectroscopy) in a PHA bioreactor. This allows for maximizing PHA production by more accurately determining the optimal reactor harvest time needed. Thus, it is desirable after fermentation to retain the fatty acid-containing liquid fraction of the fermentate while discarding or recycling the residual solids.

[0091] In some embodiments, one or more polymers may be used as a flocculant to remove the residual solids from the fermented feedstock. It should be recognized, however, that the type of flocculation polymer used in the process described herein may vary depending on the composition of the feedstock. Moreover, a polymer that does not interfere with the growth of PHA-producing bacteria is suitable since residual polymer may be carried over into the PHA producing step.

[0092] In other embodiments, the flocculant is made up of one or more cationic polymers having a medium charge and a high molecular weight. Examples of suitable cationic polymers may include WE-509, FO-4650VHM, FO-4550SH, Praestol 610BC, Praestol 611BC, Praestol 644BC, Praestol 650BC, Praestol 655BC, Praestol 655BS, Praestol 658BS, Praestol 822BS, Praestol 835BS, Praestol 851BC, Praestol 852BC, Praestol 853BC, Praestol 855BS, Praestol 857BS, Praestol 858BS, Praestol 859BS, Zetag 7523, Zetag 7553, Zetag 7557, Zetag 7563, Zetag 7565, Zetag 7583, Zetag 7587, Zetag 7593. In some embodiments, the flocculant may include a cationic polymer selected from the group consisting of WE-509, FO-4650VHM, Praestol 835BS, Praestol 852BC, Praestol 858BS, Zetag 7557, and Zetag 7583. In other embodiments, the flocculant may include a combination of cationic polymers selected from the group consisting of Praestol 644BC, Praestol 655BS, Praestol 822BS, Zetag 7523, Zetag 7565, WE-509, Praestol 835BS, Praestol 852BC, Praestol 858BS, Zetag 7557, and Zetag 7583.

[0093] At the end of fermentation, the pH is typically acidic in nature (*e.g.*, about pH 5.0). The pH of the fermented feedstock may be increased to remove the residual solids. The pH to which the fermented feedstock should be increased may vary depending on the composition of the feedstock. In some embodiments, the pH of the fermented feedstock may be increased to a neutral pH (*e.g.*, about pH 7.0).

[0094] Furthermore, it should be noted that the flocculation techniques of adding a charged polymer or increasing the pH of the fermented feedstock may be employed alone or in combination to remove the residual solids. For example, the fermentate may first be neutralized by increasing the pH, and then flocculated by adding a charged polymer. Alternatively, the fermentate may first be flocculated, and then neutralized; however, one or more additional

flocculations may be required to remove the residual solids. The flocculated and neutralized fermentate may be stored for use as feedstock for PHA production at a later time.

[0095] After floc formation, the flocs may be removed from the supernatant containing the fatty acids by any suitable solid/liquid separation methods known in the art. For example, decanting may be used to remove the flocs. Other methods include centrifugation or screening.

[0096] The residual solids that are removed during the separation step involving the fermented feedstock can be further used as feedstock for other processes, such as the production of chemical products. Alternatively, residual solids can be transformed into liquid or gaseous materials eliminating the need for disposal in landfills.

Reduction of Fatty Acid-Competing Bacteria

[0097] In some embodiments, the content of viable bacteria in the fatty acid-containing liquid may be reduced before contacting the fatty acid-containing liquid with the PHA-producing bacteria. Such viable bacteria may compete for the fatty acids and other nutrients that are useful or necessary for producing the PHAs. Viable fatty acid-competing bacteria may be eliminated from the fatty acid-containing liquid (*i.e.*, sterilization), or viable fatty acid-competing bacteria may be reduced to a lower content (*i.e.*, disinfection). Moreover, some techniques used to reduce the viable bacteria in the fatty acid-containing liquid may include the use of ozonation, UV light, peroxides (*e.g.*, hydrogen peroxide, peracetic acid), which may reduce the odor of the fatty acid-containing liquid and has desirable downstream effects of reducing the unpleasant odor in the final PHA product.

[0098] Any methods known in the art may be used to reduce the fatty acid-competing bacteria in the fatty acid-containing liquid. Suitable methods may include, for example, the addition of ozone, bleaching, pasteurization, irradiation, heat, filtration, antibiotics, chlorination, sulfur dioxide, electroporation, pH, or peroxides (*e.g.*, hydrogen peroxide, peracetic acid), or a combination of these methods. One skilled in the art would recognize how to employ these various techniques to the process described herein. For example, if bleach is used, it may be necessary to expose the liquid to a neutralization agent, such as sunlight or UV light, to neutralize residual bleach before inoculating with PHA-producing bacteria.

[0099] It should be recognized, however, that the reduction of fatty acid-competing bacteria in the fatty acid-containing liquid is an optional step in the process described herein. In some

embodiments, as depicted in process **100**, the fatty acid-containing liquid may be directly inoculated with PHA-producing bacteria for PHA production.

Fermentation of Fatty Acid-Rich Feedstock to Produce PHAs

[0100] The fatty acid-rich feedstock is used for PHA production. One skilled in the art would recognize that various techniques that may be employed to inoculate the fatty acid-rich feedstock with PHA-producing bacteria. Any PHA-producing bacteria that can convert fatty acids into PHAs may be employed in the process described herein. Examples of bacteria suitable for PHA production may include *Cupriavitus necator*, *Alcaligenes latus*, *Azotobacter*, *Comamonas*, *Pseudomonads*, *Burkholderia*, and *Delftia acidovorans*. Genetically-engineered organisms that produce PHA, such as *Cupriavitus*, *Escherichia coli*, *Klebsiella*, and *Delftia*, may also be used.

[0101] The operating pH of the fermentation to produce PHAs may vary depending on the PHA-producing bacteria used. In some embodiments, fermentation involving the PHA-producing bacteria occurs within a pH range of 5.0 and 11.0. In other embodiments, fermentation involving the PHA-producing bacteria occurs within a pH range of 7.0 and 10.0.

[0102] In some embodiments, batch fermentation may be employed to convert the fatty acids into PHAs. In one embodiment, a fed-batch reactor may be employed for fermentation of fatty acids to produce a fatty acid-rich feedstock.

[0103] In other embodiments, continuous fermentation may be employed to convert the fatty acids into PHAs. In certain embodiments, one or more continuous reactors, such as continuous stirred tank reactors, may be employed for fermentation of fatty acids to produce a fatty acid-rich feedstock. One of skill in the art would recognize that continuous reactors typically have constant in-feed and constant out-feed. In one embodiment, one continuous stirred tank reactor is employed. In another embodiment, two continuous stirred tank reactors are employed. When two or more continuous stirred tank reactors are arranged in series, plug-flow characteristics may be created. This can enable handling of a higher percentage of PHAs in biomass and higher volumetric productivity of PHAs. Further, the use of a multi-stage fermentation scheme of the fatty acids allows for the promotion of microbial growth in conditions that are different from that of PHA accumulation in the PHA-producing bacteria.

[0104] With reference to FIG. 3, fatty acid-rich feedstock **302** may be produced according to any of the processes described above, including, for example, in processes **100** (FIG. 1) and **200**

(FIG. 2). In step 320, at least a portion of fatty acid-rich feedstock 302 is provided into the first reactor (growth reactor 304). Fatty acid-rich feedstock 302 is inoculated with PHA-producing bacteria at a constant flow rate. The conditions of growth reactor 304 may be optimized to grow the PHA-producing bacteria. In some embodiments, the temperature in the growth reactor is between 22°C and 35°C. In other embodiments, the dissolved oxygen content in the growth reactor is between 10% and 50%. In yet other embodiments, the pH in the growth reactor is between 5 and 9, or between 6.5 and 9. In yet other embodiments, the dilution rate for the growth reactor is between 0.1 h⁻¹ and 0.3 h⁻¹.

[0105] The dilution rate, expressed as an inverse of the residence time, equals to the bacterial specific growth rate at a steady state. In some embodiments, the dilution rate is set to a value slightly smaller than the maximal specific growth rate to allow rapid growth, as well as to avoid wash-out of the bacterial cells.

[0106] The contacting of fatty acid-rich feedstock 302 with the PHA-producing bacteria in cell broth forms a suspension in growth reactor 304. In step 322, at least a portion of the suspension is transferred into a second reactor (production reactor 306). Multiple production reactors may also be employed.

[0107] With reference again to FIG. 3, one or more optional steps may be performed to improve PHA production. For example, in step 328, at least a portion of fatty acid-rich feedstock 302 may optionally be transferred into production reactor 306 (or multiple production reactors), where PHAs accumulate in the PHA-producing cells. Additionally, the suspension in growth reactor 306 may optionally be transferred in step 324 to a third reactor (harvest reactor 308), where the PHAs can be harvested. Further, in step 330, at least a portion of the suspension in harvest reactor 308 can be optionally recycled to dilute the fatty acid-rich feedstock 302.

[0108] In step 326, PHAs 310 and residual cells 312 can be harvested from harvester reactor 308 (if used), or directly from production reactor 306. Residual cells 312 are a bioproduct that is generally a repository for nitrogen (which may be in protein form) and phosphate.

[0109] The conditions of the second reactor may be optimized for PHA accumulation in the PHA-producing cells. It should be understood, however, that growth may still occur in production reactor 306. In some embodiments, the temperature in the production reactor is between 20°C and 32°C. In other embodiments, the dissolved oxygen content in the production reactor is between 0.2% and 30%. In yet other embodiments, the pH in the production reactor is

between 6.5 and 9. In yet other embodiments, the dilution rate for the growth reactor is between 0.04 h^{-1} and 0.2 h^{-1} . If multiple production reactors are used, it should be understood that the specific conditions inside each production reactor may vary.

Separation to Obtain PHA-Containing Cell Paste

[0110] A suspension made up of PHA-producing cells suspended in a cell broth results from contacting the fatty acid-rich feedstock with PHA-producing bacteria. A separation step upstream of PHA-production allows the processes described herein to handle heterogeneous suspensions in very large flow rates and allow for high volume processing. The process described herein can reduce large volumes of carbonaceous feedstock to smaller volumes of PHA-containing solids, allowing the use of smaller reactor vessels and other equipment to extract and isolate the PHA product.

[0111] Any techniques suitable for isolating a PHA-containing cell paste from the cell broth may be employed. Suitable techniques may involve, for example, flocculation, gravity belt separators, hydrocyclone, membranes, filtration, centrifugation, or combinations of these techniques.

[0112] In some embodiments, as depicted in process **100**, removing at least a portion of the cell broth by centrifugation produces a cell paste. In other embodiments, filtration is employed.

[0113] In yet other embodiments, as depicted in process **200**, flocculation is employed to isolate the PHA-containing solids. The flocculation techniques described above may be employed after completion of cell growth and PHA production within the cells, to isolate the PHA-containing cell paste from the cell broth.

[0114] With reference again to process **200**, a pH increase induces partial flocculation of the PHA-containing cells, and causes the outer cell wall or membrane of the PHA-producing cells to become more permeable. The pH increase may also lyse the PHA-producing cells by breaking open the cell wall or membrane. In some embodiments, the pH of the cell broth containing PHA-containing cells is increased to a pH between 10.0 and 13.5. In other embodiments, the pH of the cell broth containing PHA-containing cells is increased to a pH between 11.0 and 12.5. The cell broth may be agitated for up to 30 minutes at this increased pH. In some embodiments, this pH increase flocculates over 90% of the solid materials in the cell broth. In other embodiments, this pH increase flocculates over 95% of the solid materials in the cell broth. In

yet other embodiments, this pH increase flocculates over 99% of the solid materials in the cell broth.

[0115] After this pH increase, an optional pH adjustment step may be performed to further increase the flocculation yield. In some embodiments, the pH of the cell broth may then be lowered to a pH between 6.0 and 11.0. In other embodiments, the pH of the cell broth may then be lowered to a pH between 10.0 and 11.5.

[0116] With reference again to process **200**, after pH adjustment for flocculation, the PHA-containing solids are isolated from the supernatant. Any suitable solid/liquid separation methods known in the art may be employed to isolate the cell paste. The resulting cell-free supernatant typically has an elevated pH, and may be reused upstream as a neutralizing agent for an acidic stream. Alternatively, the supernatant may be re-inoculated to grow additional PHA-producing cells, if nutrients remain.

[0117] In some embodiments, the isolated cell paste has a PHA content per dry cell weight of at least 30%, at least 40% at least 50%, at least 60%, at least 70%, at least 80% or at least 90%. In other embodiments, the cell paste has a PHA content per dry cell weight of between 30% and 90%, between 40% and 60%, between 50% and 80%, or between 60% and 90%. Any suitable methods known in the art to determine PHA content per dry cell weight may be employed.

Formation of Dried PHA-Containing Cells

[0118] The PHA-containing cell paste isolated from the supernatant typically contains both PHA granules and cellular debris. In some embodiments, as depicted in process **200**, the cell paste may be dried to form dried PHA-containing cells. These dried cells have a reduced weight and volume compared to the cell paste. As such, the dried cells can more easily be transported or stored. The ability to store and transport dried PHA-containing cells also allows for the possibility of batch processing and/or centralized processing for the extraction of the PHA product. Additionally, drying the PHA-containing solids before extraction to isolate the PHA product may help to reduce or avoid emulsification in the extraction.

[0119] To form the dried PHA-containing cells, the PHA-containing solids are formed into a cell paste. For example, with reference to process **200**, the PHA-containing flocs isolated from the cell broth in step **214** are made into a cell paste. The PHA-containing solids may be re-

suspended in water to form the cell paste. After forming the cell paste, at least a portion of the liquid from the cell paste may be removed to obtain dried PHA-containing cells.

[0120] It should be recognized that upon isolation from the supernatant, the PHA-containing solids may entrap a large amount of water. For example, up to 90% of the PHA-containing flocs may be made up of water. In such instances, the cell paste may be formed without adding water to the PHA-containing solids.

[0121] To aid in removing the liquid from the cell paste, the cell paste may be ground using a high shear instrument or other grinding instruments known in the art. The grinding reduces the size of the solids, which may hinder drying in some instances. Moreover, since some of the PHA granules may be able to withstand the grinding, the grinding may help release more PHA granules from the cell debris and increase the overall PHA production yield.

[0122] The resulting cell paste may be dried by any methods or instruments known in the art. For example, the cell paste may be dried using an air convection oven, a fluidized bed, or a hot plate operated at high temperatures (*e.g.*, between 100°C and 170°C).

[0123] The dried PHA-containing cells may contain at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% by weight of the dried cells is one or more PHAs. For example, between 15% and 20%, between 15% and 99%, between 20% and 80%, between 30% and 70%, between 50% and 99%, or between 60% to 90% by weight of the dried cells is the one or more PHAs.

[0124] Furthermore, it should be understood that the dried PHA-containing cells may still contain some residual liquid, and that the residual liquid content in the cell paste may vary. In some embodiments, the dried cells may contain between about 0-50% of liquid by weight (*e.g.*, residual water and/or cell broth). In other embodiments, the dried cells may contain between about 0-20% of liquid by weight. In yet other embodiments, the dried cells may contain between about 0-5% of liquid by weight. In yet other embodiments, the dried cells may contain between about 0-1% of liquid by weight.

Extraction of PHAs

[0125] The PHAs may be extracted from the dried cells using any extraction methods known in the art. Any polar organic solvent or mixture of polar organic solvents may be used to solubilize the PHAs. For example, suitable solvents may include chloroform, dichloromethane,

ethyl acetate, isobutanol, ethanol, propanol, propylene carbonate, ethylene carbonate, isopropanol, amyl alcohol, or mixtures of these solvents. In some embodiments, the solvent includes chloroform, dichloromethane, or a combination of these solvents. In other embodiments, a water-based system may be used for the extraction.

[0126] The isolated PHA product may be stored dry after solvent removal (*i.e.*, solvent evaporation). It should be noted, however, that while the PHA product may be stored dry after extraction, the PHA product may also be stored after extraction into the solvent (*i.e.*, without solvent evaporation).

[0127] In some embodiments, the processes described herein allow for production and isolation of PHA product in relatively higher yields. In certain embodiments, the yield of the isolated PHA product is at least about 80%, at least about 85%, at least about 90%, or at least about 95%.

[0128] In some embodiments, the processes described herein allow for production and isolation of PHA products in relatively higher purity. In certain embodiments, the purity of the isolated PHA product is at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99%. In yet other embodiments, the isolated PHA product has a purity of at least 70-100%, 80-100%, 90-100%, 70-95%, 80-95%, or 90-95%. In yet another embodiment, the isolated PHA has a purity of about 100%. One skilled in the art would recognize the different techniques that may be used to determine purity, such as GPC, HPLC, GCMS, or GC-FID. In one embodiment, the use of a crotonic acid assay by HPLC may also be used to determine purity.

The PHA Product

[0129] The PHA products produced by the process described herein is typically one type of PHAs or a blend of PHA polymers and/or co-polymers including, for example, polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV), polyhydroxyhexanoate (PHH), polyhydroxyoctanoate (PHO), polyhydroxydecanoate, and polyhydroxybutyratevalerate (PHBV). It should be understood, however, that the blend of polymers and/or co-polymers may include straight-chained or branched PHAs that may be substituted with different functional groups.

[0130] In some embodiments, the PHA product includes a blend of PHB and PHV. In one embodiment, the PHA product includes a 90:10 ratio of PHB to PHV. In another embodiment,

the PHA product includes an 85:15 ratio of PHB to PHV. In yet another embodiment, the PHA product includes a 80:20 ratio of PHB to PHV. In yet another embodiment, the PHA product includes a 70:30 ratio of PHB to PHV.

[0131] In some embodiments, the PHAs produced by the process described herein may be further processed and converted into one or more plastics. The biocompatible nature of PHAs enables PHA-based plastics to be used in variety of biological applications, including medical sutures, tissue repair devices, or other biomedical uses.

EXAMPLES

[0132] The following Examples are merely illustrative and are not meant to limit any aspects of the present disclosure in any way.

Example 1: Preparation of Feedstock for PHA Production Using One Flocculation Step

[0133] Municipal wastewater from Davis (CA) was obtained and fed into a 2000-L fermentation reactor. No additional fermentation bacteria were added to the waste stream. The stream was fermented under anaerobic conditions in the reactor. The pH of the fermentate after fermentation was 6.0, and the fermentate volume was 1750 L.

[0134] The fermentate was then flocculated by adding a cationic polymer solution to remove the residual solids suspended in the fermentate. Specifically, 170.8 kg of a WE-509 polymer solution was added and stirred in the reactor for 10 minutes. 1500 L of flocculated fermentate was produced.

[0135] The flocculated fermentate was neutralized before use as feedstock for PHA production. The pH of the flocculated fermentate was 6.0. To this flocculated fermentate was added 0.625 L of a NaOH solution (50% w/v), and stirred for 5 minutes. After addition of the base, the final pH of the fermentate was 6.8. After neutralization, the fermentate was clear, suggesting that a second flocculation was not required.

[0136] The neutralized fermentate was stored in ambient conditions (about 20°C, inside the tank) prior to use as feedstock for PHA production at a later date.

Example 2: Preparation of Feedstock for PHA Production Using Multiple Flocculation Steps

[0137] Municipal wastewater from Davis (CA) was obtained and fermented under anaerobic conditions according to the procedure described in Example 1 above. 1705 L of the fermentate was used. The pH of the fermentate was 5.3.

[0138] The fermentate was flocculated by adding a WE-509 polymer solution (0.20% concentration, *i.e.*, 2 g/L). 176.9 kg of polymer solution was consumed, and 1535 L of flocculated fermentate was recovered. To this fermentate was added more WE-509 polymer solution. 35.4 kg of polymer was consumed. After this second flocculation step, about 1500 L of flocculated fermentate was recovered (88% recovery).

[0139] The flocculated fermentate was neutralized before use as feedstock for PHA production. The pH of the flocculated fermentate was 5.3. To this flocculated fermentate was added 2.635 L of NaOH solution (50% w/v). The pH of the flocculated fermentate after neutralization was 7.2. The neutralized fermentate was stored in ambient conditions (about 20°C, inside the tank) prior to use as feedstock for PHA production at a later date.

Example 3: Cell Flocculation and Extraction

[0140] Municipal wastewater from Davis (CA) was obtained and fermented under anaerobic conditions according to the procedure described in Example 1 above. The resulting fermentate was flocculated according to the procedure described in Example 1 to remove residual solids, such as lignocellulosic material that was unaffected by fermentation.

[0141] In a separate 1L reactor, DEA01 (*Delftia acidovorans*; PHA-producing bacteria) was grown to provide the cell broth used in this example. The flocculated fermentate was combined with 1 L of cell inoculum to produce a suspension of PHA-producing cells suspended in the cell broth. The pH of this suspension was 8.3.

[0142] 15 mL of NaOH solution (50% w/v) was added to the suspension, and stirred for 25 minutes. After addition of the base, the pH of the suspension was 13.0. It was observed that most of the PHA-producing cells were lysed, and spontaneously flocculated.

[0143] 15 mL of H₂SO₄ solution (93% w/v) was then added. After addition of the acid, the pH was 11.2. More flocs were observed in the cell broth. The supernatant was decanted, and flocs containing the PHA granules and lysed cell debris were separated at the bottom of the beaker. A small amount of water was added to have enough material for the high shear mixer to

form a cell paste containing PHA granules. The wet cells were dispersed at high shear to form a wet cell paste, which was left to dry overnight.

[0144] The dry weight of the ground cell paste was 0.84 g. To extract the PHAs from the dried cell paste, 16.8 mL of chloroform was added to the cell paste. The chloroform layer was extracted, and evaporated to obtain 0.4 g of PHA solids. The PHA solids were analyzed by GC-MS. The PHA solids had a purity of 98%, containing 91% PHB and 7% PHV.

Example 4: Cell Flocculation and Extraction

[0145] In a 300-L reactor, DEA01 (*Delftia acidovorans*; PHA-producing bacteria) was grown to provide the cell broth used in this example. Fermentate obtained from a synthetic media was combined with 1L of cell inoculum to produce a suspension of PHA-producing cells suspended in the cell broth. The pH of this suspension was 8.24.

[0146] 25 mL of NaOH solution (50% w/v) was added to the suspension, and stirred for 10 minutes. After addition of the base, the pH of the suspension was 13.3. It was observed that most of the PHA-producing cells were lysed, and spontaneously flocculated.

[0147] 13 mL of H₂SO₄ solution (93% w/v) was then added. After addition of the acid, the pH was 11.2. More flocs were observed in the cell broth. The supernatant was decanted, and flocs containing the PHA granules and lysed cell debris were separated at the bottom of the beaker. A small amount of water was added to have enough material for the high-shear mixer to form a cell paste containing PHA granules. The wet cells were dispersed at high shear to form a wet cell paste, which was left to dry overnight.

[0148] The dry weight of the ground cell paste was 0.87 g. To extract the PHAs from the dried cell paste, 17.4 mL of chloroform was added to the cell paste. The chloroform layer was extracted, and evaporated to obtain 0.31 g of PHA solids. The PHA solids were analyzed by GC-MS. The PHA solids had a purity of 91%, containing 84% PHB and 7% PHV.

Example 5: Scale-Up of Cell Flocculation and Extraction

[0149] In a 300-L reactor, DEA01 (*Delftia acidovorans*; PHA-producing bacteria) was grown to provide the cell broth used in this example. Fermentate obtained from a synthetic media was combined with 286.4 L of the cell inoculum to produce a suspension of PHA-producing cells suspended in the cell broth. The pH of this suspension was 8.4.

[0150] 9.975 L of NaOH solution (50% w/v) was added to the suspension, and stirred for 10 minutes. After addition of the base, the pH of the suspension was 13.2. It was observed that most of the PHA-producing cells were lysed, and spontaneously flocculated.

[0151] 4.11 L of H₂SO₄ solution (93% w/v) was then added. After addition of the acid, the pH was 11.2. More flocs were observed in the cell broth. The supernatant was decanted and neutralized to a pH of 7.0 by adding 610 mL of NaOH (50% w/v). Flocs containing the PHA granules and lysed cell debris were separated at the bottom of the beaker. A small amount of water added to have enough material for the high-shear mixer to form a cell paste containing PHA granules. The wet cells were dispersed at high shear to form a wet cell paste, which was left to dry overnight.

[0152] The dry weight of the ground cell paste was 178 g. To extract the PHAs from the dried cell paste, 3.56 L of chloroform was added to the cell paste. The chloroform layer was extracted, and evaporated to obtain 66 g of PHA solids. The PHA solids were analyzed by GC-MS. The PHA solids had a purity of 87%, containing 80% PHB and 7% PHV.

Example 6: Effect of Solid Removal from VFA-Rich Fermentate of Corn Ethanol Process Waste on PHA Production

[0153] A syrup (a concentrated thin stillage from corn ethanol process, 35% w/w solid) was obtained from a corn ethanol producer and 2 L of the syrup was fed into a 5-L fermentation reactor with 2 L of water. After raising the pH to 6.0 with 40 % sodium hydroxide solution, 1 L of a previously-fermented syrup was combined with the half-diluted syrup to inoculate additional fermentation bacteria. The stream was fermented for a week under anaerobic conditions in the reactor to generate volatile fatty acids (VFA). The pH was maintained at 6.0 with 40 % sodium hydroxide solution using a PI controller. The final VFA concentration (measured using Hach TNT 872 kit) was 29.7 g/L.

[0154] The fermentate containing suspended solid was sterilized by autoclaving in 1-L bottles at 121°C for 30 min. The autoclaved fermentate was then neutralized to pH at 7.0-7.2 with 20% sodium hydroxide solution (autoclaved). 800 mL of the fermentate was combined with 3200 mL of autoclaved deionized water to make a 5-fold diluted fermentate. Half of the diluted fermentate was transferred to autoclaved 450-mL centrifuge tubes and centrifuged at 8300 x g for 10 min to remove suspended solid. The fermentate in which solid was removed (NSF) and the fermentate containing suspended solid (SF) were transferred (1425 mL each) into 2-L autoclaved baffled flasks.

[0155] A culture of PHA-synthesizing bacterial strain DEA01 (*Delftia acidovorans*), grown on nutrient broth overnight, was inoculated to NSF and SF (75 mL each). The solid contents including bacterial cells of NSF and SF, immediately after inoculation, were 0.76 and 10.36 g/L (measured by collecting solid with biomass by centrifugation at $4000 \times g$ for 10 min and dried at 120°C for 24 h), respectively. The initial VFA concentrations were 5.63 g/L (NSF) and 6.18 g/L (SF). The cultures in flasks were incubated on a shaker (190 rpm) at 30°C for 48 h. Optical densities at 600 nm were measured periodically along with solid contents, to monitor bacterial growth. Changes in PHA concentrations were monitored by converting PHB (the expected major component of PHA copolymer polyhydroxybutyrate-co-valerate (PHBV) synthesized by DEA01) to crotonic acid by heating ethanol-washed and dried cell pellet/solid mixtures in concentrated sulfuric acid at 100°C for 10-15 min and analyzing crotonic acid concentrations by HPLC (column: Sepax Carbomix H-NP5:8%, 7.8 x 50 mm; mobile phase: 18 mM sulfuric acid in water; flow rate: 1 mL/min; temperature: 60 °C; sample injection: 7 µL; detection: UV at 210 nm; elution time: 2.32 min). The final solid/cell weights (at 48 h) of NSF and SF cultures were 1.63 and 12.6 g/L, with 27.9 % and 6.2 % PHB contents per solid (PHB concentrations were 455 mg/L (NSF) and 750 mg/L (SF), assuming 100 % conversion of PHB to crotonic acid), respectively.

[0156] To extract PHA from bacterial cells, 500 mL of dichloromethane was added to the culture broth (1.39 L each) in the 2-L flask, and the mixture was stirred by an impeller rotating at 400 rpm for 36 h at room temperature ($22 \pm 3^\circ\text{C}$). The organic layer and emulsion, which included cells and some water, were crudely separated from the bulk aqueous layer using a separatory funnel. This organic mixture was centrifuged ($1500 \times g$, 10 min) to give a homogeneous organic solution. The solvent was removed under reduced pressure to give crude PHA. The crude PHA was re-suspended in dichloromethane (200mL). Methanol (200mL) was added, and then resulting mixture was precipitated by removing the dichloromethane under reduced pressure. The solution was chilled (0°C) and the precipitate was filtered and dried overnight under reduced pressure. A white opaque solid was recovered (precipitate weight: 0.536g) from the NSF culture, and a yellow opaque solid (precipitate weight: 2.02g) was recovered from the SF culture.

[0157] The PHA purities of the recovered precipitates, as well as PHV % in the PHBV copolymer, were analyzed by acid-catalyzed hydrolysis of the polymeric chain and trans-esterification with methanol followed by GC analysis of the methyl esters. Approximately 30 mg of the samples was placed in a 10 mL screw-top vial with 2 mL of methanol containing 3%

b.v. sulfuric acid and 2 mL of chloroform. The mixture was then heated to 100°C for 60 min. After cooling the samples, 5 mL of toluene and 1 mL of water were added. The vials were shaken for five minutes, and the layers were allowed to separate. A portion of the organic layer was placed in vials for analysis by GC (Agilent 6890 with FID detection; column: Agilent Innowax, 180µm ID x 20 m long x 0.18 µm film thickness; injection: 2 µL, 50:1 split ratio; carrier gas: helium; flow rate: 1.1 mL/min; temperature program: ramped from an initial 35°C to 240°C at 60°C/min with a final hold for two minutes. PHBV formed methyl-3-hydroxybutyrate and methyl-3-hydroxyvalerate, and the elution times were 2.54 and 2.75 min, respectively. Quantitation was performed by comparing the peak areas of methyl-3-hydroxybutyrate and methyl-3-hydroxyvalerate from the digested polymer to peak areas from standards which contained approximately 30 mg of each of the two methyl esters and which were taken through the same process. To convert the observed quantities of methyl ester back into PHA there was a gravimetric factor required. The observed quantity of methyl-3-hydroxybutyrate times 86/118 provides the amount of PHB and the observed quantity of methyl-3-hydroxyvalerate times 100/132 provides the amount of PHV from the sample. The PHA mass contents of the precipitates, recovered from extraction of NSF and SF cultures, were 0.509g and 0.707g with PHV contents 1.85% and 2.55%, respectively. Therefore, the purity percentages in the recovered precipitates were 95% (NSF) and 35% (SF) with and without solid removal in the fermentate.

[0158] Thus, this example demonstrates that removing solids from VFA-rich feedstock used for PHA production results in a purer PHA product.

Example 7: PHA fermentation from corn ethanol process waste

[0159] Syrup (concentrated thin stillage) was obtained from a corn ethanol producer and 1L was fed into a 2-L fermentation reactor. 1L of municipal wastewater was combined with the syrup to inoculate additional fermentation bacteria and to dilute the syrup which contains total solids 30-35% (w/w). The stream was fermented for a week under anaerobic conditions in the reactor. The pH of the fermentate was adjusted to 5.5 at the onset and was 4.5 after fermentation. The final volatile acids concentration was 23 g/L with an increase in acetic acid concentration 6.0 g/L.

[0160] The fermentate was then centrifuged at 6000 rpm for 10 min to remove the solids suspended in the fermentate. Approximately 900 mL of the fermentate was produced. The fermentate was sterilized by autoclaving at 121°C for 20 min. The fermentate was neutralized

and the pH was 7.2 before use of as feedstock or PHA production. The fermentate was filtrated down to a 0.2- μ m membrane to remove the residual solids which can interfere with optical density measurements.

[0161] Prior to PHA production, the growth curves of DEA01 on the fermentate were obtained using a 96-well microplate by measuring optical density at 600 nm. The fermentate was diluted (1x, 2x, 5x, and 10x) to alleviate the inhibitory effects by a high salinity or high concentrations of any compounds. DEA01 (*Delftia acidovorans*) was well grown in the five-fold dilution of the fermentate.

[0162] In a 250-mL flask, 142.5 mL of the five-fold diluted fermentate was combined with 7.5 mL of cell inoculum. The cell broth was aerobically fermented to produce PHA-containing Dea01 cells for 41 hours. The final PHB content per dry cell weight was observed to 62%. Moreover, 79% of volatile fatty acids were observed to be consumed during the PHA fermentation.

Example 8: Continuous PHA fermentation using two-stage continuous stirred tank reactors (CSTRs)

[0163] Municipal wastewater from Fairfield (CA) was obtained and anaerobically fermented according to the procedure described in Example 1 above. The resulting fermentate was flocculated according to the procedure described in Example 1 to remove residual solids and autoclaved to eliminate viable contaminant microbes. The pH of the fermentate was adjusted to between 7 and 8.

[0164] In two 1L reactors (working volume 900 mL) connected in series, the fermentate was filled, and DEA01 (*Delftia acidovorans*; PHA-producing bacteria) was inoculated and grown under aerobic conditions. The dissolved oxygen (DO) in the culture maintained at 30% of air saturation with varying agitation speed and aeration rate. The pH was maintained at 8.0 by adjusting it with 3N sulfuric acid. The temperature was set at 27°C. Once the growth of the culture reached a late log-phase, the fermentate was introduced into the first reactor continuously at a flow rate of 1.8 mL/min (dilution rate 0.12 h⁻¹). The culture broth in the first reactor was harvested and introduced into the second reactor at a flow rate of 0.9 mL/min (dilution rate 0.06 h⁻¹). The volumes of the both reactors were maintained at 900 mL by detecting the liquid levels by level sensors and pumping out the liquid. The DO content in the second reactor was maintained at 20% of air saturation.

[0165] DEA01 was observed to produce a copolymer PHA, polyhydroxybutyrate-co-valerate (PHBV) with valerate monomer composition 20 - 40% from the municipal wastewater fermentate. The PHA content per dry cell weight was observed to be 40 - 60%.

Example 9: Reduction of contaminant microbes by flocculation and disinfection

[0166] Municipal wastewater from Fairfield (CA) was obtained and fermented under anaerobic conditions according to the procedure described in Example 1 above. After one week of anaerobic fermentation, viable microbes approximately 10^{10} cells/mL, including 10^8 cells/mL aerobes which will compete with PHA-producing bacteria in the later stage, were present. The resulting fermentate was flocculated according to the procedure described in Example 1 to remove residual solids. The process reduced the number of viable microbes to approximately 10^7 cells/mL.

[0167] Peracetic acid or hydrogen peroxide was used for disinfection of the flocculated fermentate. In 1L of the fermentate, 0.25 mL of 40 % (w/v) of peracetic acid (final concentration 100 mg/L) or 2.5 mL of 40 % (w/v) hydrogen peroxide (final concentration 1000 mg/L) was added and mixed constantly. After 1 hour of reaction, any residual peroxides were quenched with thiosulfate solution (for peracetic acid) or catalase (for hydrogen peroxide). The numbers of culturable aerobic microbes in the fermentate reduced from 10^7 cells/mL to less than 10^4 cells/mL.

CLAIMS

What is claimed is:

1. A method for producing one or more polyhydroxyalkanoates (PHAs), the method comprising:
 - (a) providing a carbonaceous feedstock;
 - (b) contacting the carbonaceous feedstock with fermentation bacteria under conditions suitable to produce a fermentate, wherein the fermentate comprises a fatty acid-containing liquid and residual solids;
 - (c) separating at least a portion of the residual solids from the fermentate to produce a fatty acid-rich feedstock;
 - (d) contacting the fatty acid-rich feedstock with PHA-producing bacteria in a cell broth under conditions suitable to produce one or more PHAs, wherein the contacting of the fatty acid-rich feedstock with the PHA-producing bacteria in the cell broth forms a suspension; and
 - (e) separating at least a portion of the cell broth from the suspension to produce a cell paste, wherein the cell paste comprises the one or more PHAs.
2. The method of claim 1, wherein the cell paste has a PHA content per dry cell weight of at least 30%.
3. The method of claim 1 or 2, further comprising extracting the one or more PHAs from the cell paste.
4. The method of claim 3, wherein the one or more extracted PHAs have a purity of at least 40%.
5. The method of claim 3 or 4, wherein the one or more PHAs are extracted from the cell paste using a polar organic solvent.
6. The method of claim 5, wherein the polar organic solvent is selected from the group consisting of chloroform, dichloromethane, ethyl acetate, isobutanol, ethanol, propanol, propylene carbonate, ethylene carbonate, isopropanol, and amyl alcohol, or a mixture thereof.
7. The method of any one of claims 1 to 6, wherein the fermentation bacteria are acidogenic bacteria.

8. The method of any one of claims 1 to 7, wherein the fatty acid-containing liquid further comprises viable bacteria, and the method further comprises reducing the viable bacteria in the fatty acid-containing liquid before contact with the PHA-producing bacteria.
9. The method of claim 8, wherein the viable bacteria is reduced by ozone, bleach, pasteurization, irradiation, heat, filtration, antibiotics, chlorination, sulfur dioxide, electroporation, pH, or peroxides, or a combination thereof.
10. The method of any one of claims 1 to 9, further comprising sterilizing and/or disinfecting the fermentate or the fatty acid-rich feedstock.
11. The method of claim 10, wherein the sterilization and/or disinfection reduces the odor of the fatty acid-containing liquid in the fermentate or the fatty acid-rich feedstock.
12. The method of claim 10 or 11, wherein the sterilization and/or disinfection employs ozone, bleach, pasteurization, irradiation, heat, filtration, antibiotics, chlorination, sulfur dioxide, electroporation, pH, or peroxides, or a combination thereof.
13. The method of any one of claims 1 to 12, wherein the carbonaceous feedstock is chemically treated before fermentation to produce the fermentate.
14. The method of any one of claims 1 to 13, wherein the carbonaceous feedstock is fermented under anaerobic conditions.
15. The method of any one of claims 1 to 14, wherein the carbonaceous feedstock is fermented at a pH between 4.0 and 8.0.
16. The method of claim 15, wherein the carbonaceous feedstock is fermented at a pH between 6.0 and 7.0.
17. The method of any one of claims 1 to 16, wherein the fatty acid-containing liquid comprises short-chain fatty acids, medium-chain fatty acids, or a combination thereof.
18. The method of any one of claims 1 to 17, wherein the one or more PHAs are selected from the group consisting of polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV), polyhydroxybutyratevalerate (PHBV), and polyhydroxyhexanoate (PHH).
19. The method of any one of claims 1 to 18, wherein the carbonaceous feedstock comprises organic solid materials, inorganic solid materials, or a combination thereof.

20. The method of claim 19, wherein the organic solid materials comprise cellulose, hemicellulose, lignin, fats, fatty acids, saccharides, protein, or humic materials, or a combination thereof.
21. The method of any one of claims 1 to 20, wherein the carbonaceous feedstock is dispersed in a water-based liquid.
22. The method of any one of claims 1 to 21, wherein at least 40% by weight of the carbonaceous feedstock is water.
23. The method of any one of claims 1 to 22, wherein the carbonaceous feedstock is wastewater, animal manure, pulp waste, food processing plant waste, restaurant waste, yard waste, forest waste, biodiesel transesterification waste products, ethanol process waste, or a combination thereof.
24. The method of any one of claims 1 to 23, wherein the carbonaceous feedstock is a heterogeneous carbonaceous feedstock.
25. The method of any one of claims 1 to 24, wherein the fatty acid-rich feedstock is contacted with the PHA-producing bacteria in one or more continuous reactors.
26. The method of claim 25, wherein the fatty acid-rich feedstock is contacted with the PHA-producing bacteria in two or more continuous reactors,
- wherein one of the two or more continuous reactors is a growth reactor and one or more of the two or more continuous reactors are production reactors,
- wherein the fatty acid-rich feedstock is contacted with the PHA-producing bacteria in the growth reactor to produce an inoculated suspension under conditions suitable for growing the PHA-producing bacteria, and
- wherein at least a portion of the inoculated suspension is transferred into the one or more production reactors, wherein the conditions in the one or more production reactors are suitable for PHA accumulation in the PHA-producing bacteria.
27. The method of claim 26, wherein one or more of the following conditions are present in the growth reactor:

- (i) the temperature in the growth reactor is between 22°C and 35°C;

- (ii) the dissolved oxygen content in the growth reactor is between 10% and 50%; and
- (iii) the pH in the growth reactor is between 5 and 9.

28. The method of claim 26 or 27, wherein one or more of the following conditions are independently present in each of the one or more production reactors:

- (i) the temperature in each of the one or more production reactors is independently between 20°C and 32°C;
- (ii) the dissolved oxygen content in each of the one or more production reactors is independently between 0.2% and 30%; and
- (iii) the pH in each of the one or more production reactors is independently between 6.5 and 9.

29. The method of any one of claims 1 to 28, further comprising producing one or more plastics from the cell paste.

30. The method of any one of claims 3 to 28, further comprising converting the one or more extracted PHAs into one or more plastics.

31. The method of any one of claims 1 to 28, further comprising removing any remaining cell broth from the cell paste to form dried PHA-containing cells.

32. The method of any one of claims 1 to 30, wherein the cell paste further comprises residual cells.

33. Dried cells produced by the method of claim 31, wherein at least 15% by weight of the dried cells is one or more PHAs.

34. Residual cells produced by the method of claim 32, wherein the residual cells comprise nitrogen, phosphate, or a combination thereof.

35. A method for producing polyhydroxyalkanoates (PHAs), the method comprising:

- (a) providing a carbonaceous feedstock;
- (b) contacting the carbonaceous feedstock with fermentation bacteria under conditions suitable to produce a fermentate, wherein the fermentate comprises a fatty acid-containing liquid and residual solids;

- (c) separating at least a portion of the residual solids from the fermentate to produce a fatty acid-rich feedstock;
- (d) transferring at least a portion of the fatty acid-rich feedstock into a growth reactor;
- (e) contacting the fatty acid-rich feedstock in the growth reactor with PHA-producing bacteria in a cell broth under conditions suitable for growing the PHA-producing bacteria, wherein the contacting of the fatty acid-rich feedstock with the PHA-producing bacteria in the cell broth forms a suspension in the growth reactor;
- (f) transferring at least a portion of the suspension from the growth reactor into one or more production reactors, wherein the conditions in the one or more production reactors are suitable for PHA accumulation in the PHA-producing bacteria; and
- (g) separating at least a portion of the cell broth from the suspension in the one or more production reactors to produce a cell paste, wherein the cell paste comprises the one or more PHAs.

36. The method of claim 35, wherein one or more of the following conditions are present in the growth reactor:

- (i) the temperature in the growth reactor is between 22°C and 35°C;
- (ii) the dissolved oxygen content in the growth reactor is between 10% and 50%; and
- (iii) the pH in the growth reactor is between 5 and 9.

37. The method of claim 35 or 36, wherein one or more of the following conditions are independently present in each of the one or more production reactors:

- (i) the temperature in each of the one or more production reactors is independently between 20°C and 32°C;
- (ii) the dissolved oxygen content in each of the one or more production reactors is independently between 0.2% and 30%; and
- (iii) the pH in each of the one or more production reactors is independently between 6.5 and 9.

38. The method of any one of claims 35 to 37, wherein the growth reactor is a continuous reactor.

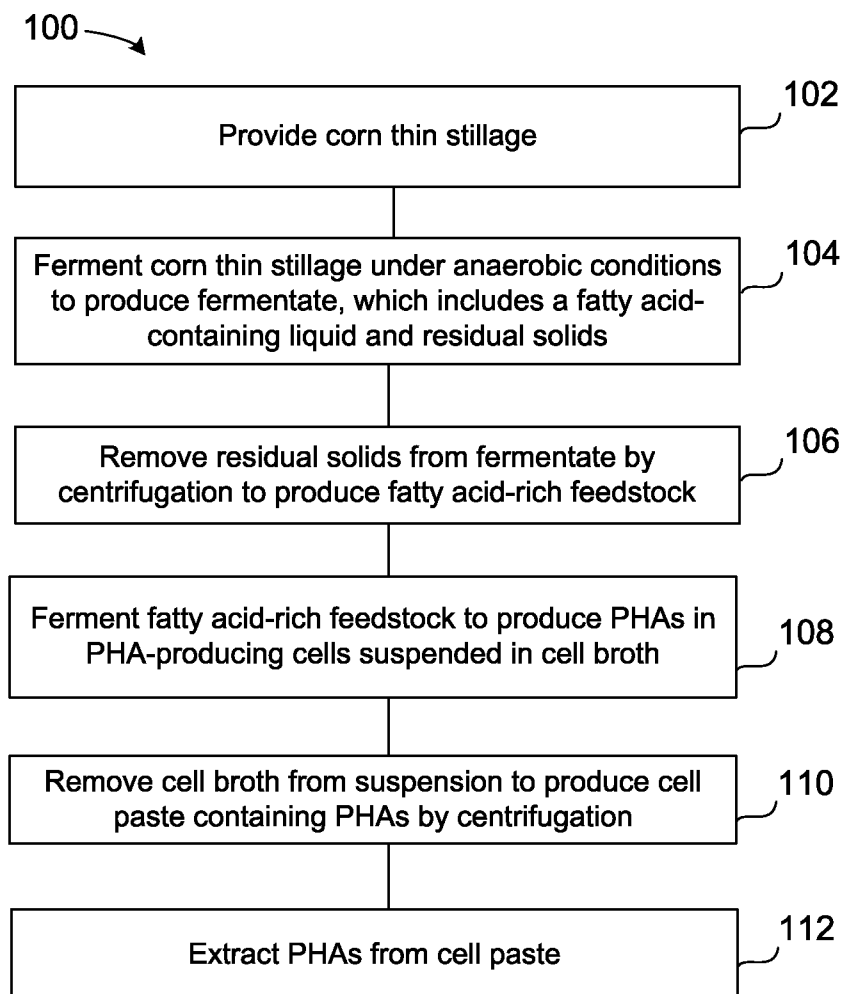
39. The method of any one of claims 35 to 38, wherein the one or more production reactors are each a continuous reactor.

40. The method of any one of claims 35 to 39, further comprising:
transferring at least a portion of the fatty acid-rich feedstock into the one or more production reactors; and
producing one or more PHAs from the fatty acid-rich feedstock in the one or more production reactors.
41. The method of any one of claims 35 to 40, wherein the cell paste has a PHA content per dry cell weight of at least 30%.
42. The method of any one of claims 35 to 41, further comprising extracting the one or more PHAs from the cell paste.
43. The method of claim 42, wherein the one or more extracted PHAs have a purity of at least 40%.
44. The method of claim 42 or 43, wherein the one or more PHAs are extracted from the cell paste using a polar organic solvent.
45. The method of claim 44, wherein the polar organic solvent is selected from the group consisting of chloroform, dichloromethane, ethyl acetate, isobutanol, ethanol, propanol, propylene carbonate, ethylene carbonate, isopropanol, and amyl alcohol, or a mixture thereof.
46. The method of any one of claims 35 to 45, wherein the carbonaceous feedstock is fermented by acidogenic fermentation bacteria.
47. The method of any one of claims 35 to 46, wherein the fatty acid-containing liquid further comprises viable bacteria, and the method further comprises reducing the viable bacteria in the fatty acid-containing liquid before contacting the fatty acid-containing liquid with the PHA-producing bacteria.
48. The method of claim 47, wherein the viable bacteria is reduced by ozone, bleach, pasteurization, irradiation, heat, filtration, antibiotics, chlorination, sulfur dioxide, electroporation, pH, or peroxides, or a combination thereof.
49. The method of any one of claims 35 to 48, further comprising sterilizing and/or disinfecting the fermentate or the fatty acid-rich feedstock.

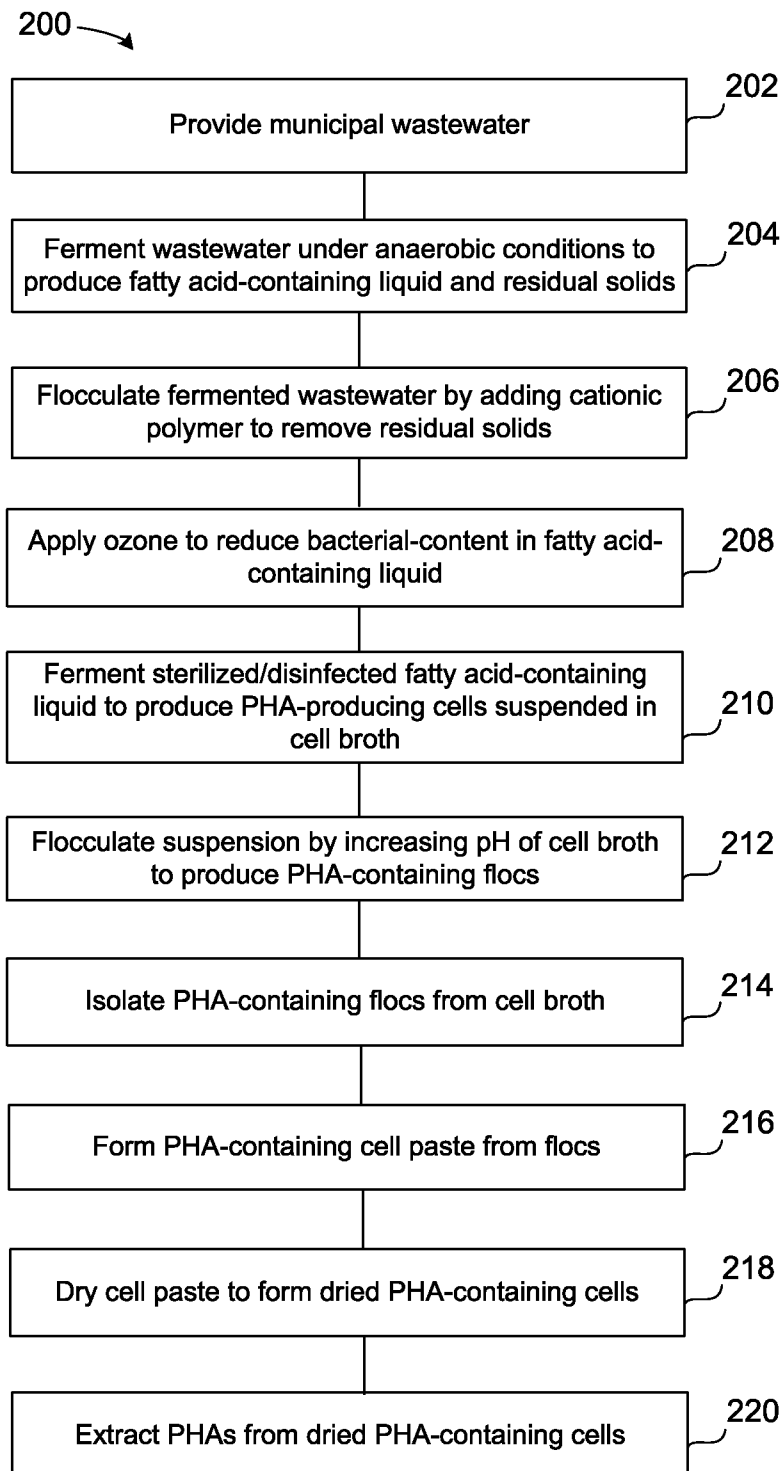
50. The method of claim 49, wherein the sterilization and/or disinfection reduces the odor of the fatty acid-containing liquid in the fermentate or the fatty acid-rich feedstock.
51. The method of claim 49 or 50, wherein the sterilization and/or disinfection employs ozone, bleach, pasteurization, irradiation, heat, filtration, antibiotics, chlorination, sulfur dioxide, electroporation, pH, or peroxides, or a combination thereof.
52. The method of any one of claims 35 to 51, wherein the carbonaceous feedstock is chemically treated before fermentation to produce the fermentate.
53. The method of any one of claims 35 to 52, wherein the carbonaceous feedstock is fermented under anaerobic conditions.
54. The method of any one of claims 35 to 53, wherein the fatty acid-containing liquid comprises short-chain fatty acids, medium-chain fatty acids, or a combination thereof.
55. The method of any one of claims 35 to 54, wherein the one or more PHAs are selected from the group consisting of polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV), polyhydroxybutyratevalerate (PHBV), or polyhydroxyhexanoate (PHH).
56. The method of any one of claims 35 to 55, wherein the carbonaceous feedstock comprises organic solid materials, inorganic solid materials, or a combination thereof.
57. The method of claim 56, wherein the organic solid materials comprise cellulose, hemicellulose, lignin, fats, fatty acids, saccharides, protein, or humic materials, or a combination thereof.
58. The method of any one of claims 35 to 57, wherein the carbonaceous feedstock is dispersed in a water-based liquid.
59. The method of any one of claims 35 to 58, wherein at least 40% by weight of the carbonaceous feedstock is water.
60. The method of any one of claims 35 to 59, wherein the carbonaceous feedstock is wastewater, animal manure, pulp waste, food processing plant waste, restaurant waste, yard waste, forest waste, biodiesel transesterification waste products, ethanol process waste, or a combination thereof.

61. The method of any one of claims 35 to 60, wherein the carbonaceous feedstock is a heterogeneous carbonaceous feedstock.
62. The method of any one of claims 35 to 61, further comprising producing one or more plastics from the cell paste.
63. The method of any one of claims 42 to 61, further comprising converting the one or more extracted PHAs into one or more plastics.
64. The method of any one of claims 35 to 63, further comprising removing any remaining cell broth from the cell paste to form dried PHA-containing cells.
65. The method of any one of claims 35 to 64, wherein the cell paste further comprises residual cells.
66. Dried cells produced by the method of claim 64, wherein at least 15% by weight of the dried cells is one or more PHAs.
67. Residual cells produced by the method of claim 65, wherein the residual cells comprise nitrogen, phosphate, or a combination thereof.

1/3

**FIG. 1**

2/3

**FIG. 2**

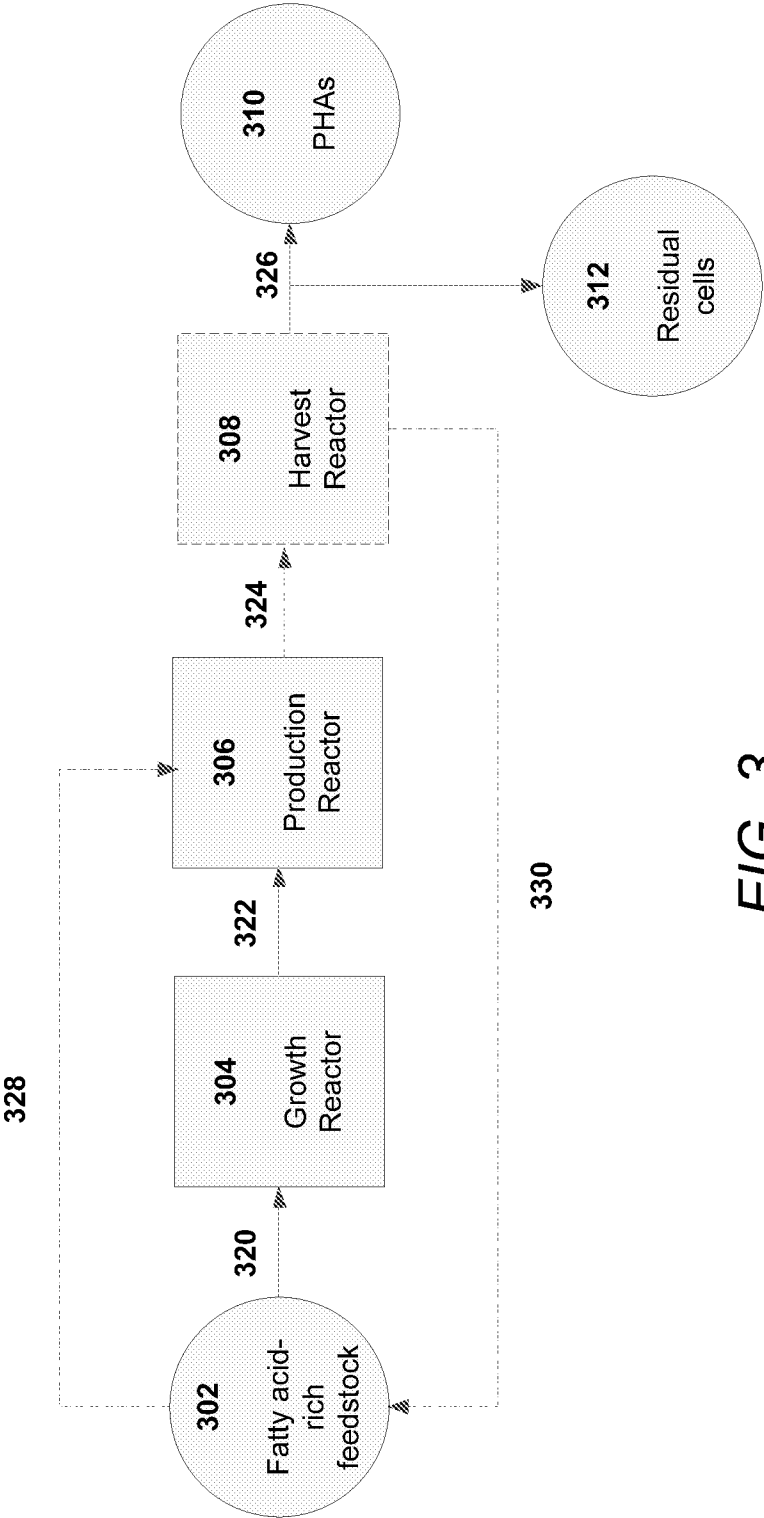


FIG. 3