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(54) Title: CULTIVATION MEDIUM AND METHOD OF CULTIVATING MICROALGAE

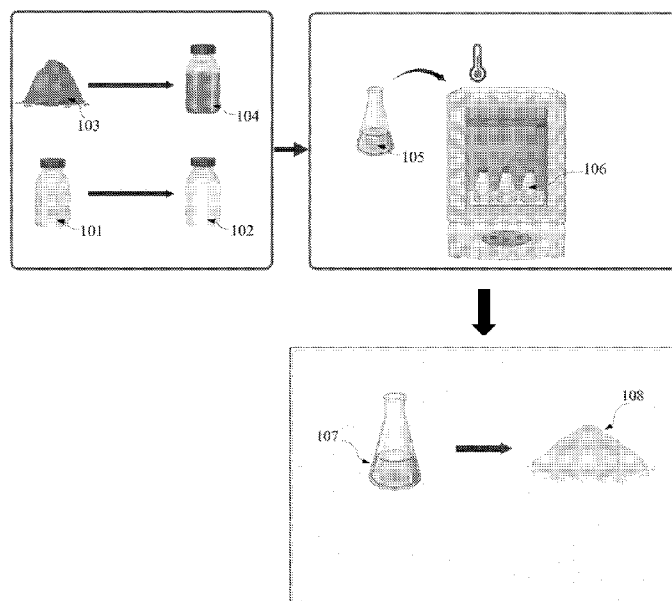


FIG. 1

(57) Abstract: The present invention relates to a cultivation medium, a method of preparing a cultivation medium and a method of cultivating heterotrophic microalgae. The cultivation medium comprises a mixed hydrolysate medium containing 15% to 25% v/v soy whey hydrolysate and 75% to 85% v/v brewer's spent grain hydrolysate, based on the total volume of the mixed hydrolysate medium, wherein the cultivation medium is rich in endogenous glucose, having an endogenous glucose density of 46.0 to 51.0 g/L, for supporting growth of heterotrophic microalgae. The method of the present invention yields relatively high amount of microalgal biomass and provides increased biomass production.

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CULTIVATION MEDIUM AND METHOD OF CULTIVATING MICROALGAE

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of priority to the Singapore application no. 10202301601W filed June 7, 2023, and the contents of which are hereby incorporated by reference in their entirety for all purposes.

TECHNICAL FIELD

[0002] The present disclosure generally relates to a cultivation medium, a method of preparing the cultivation medium and a method of cultivating heterotrophic microalgae.

BACKGROUND

[0003] To bolster resilience in food production, microalga is promising as it is one of the most land-efficient food sources compared to conventional food crops. For example, *Auxenochlorella protothecoides*, a microalgal species approved by FDA and EFSA for human consumption (European Commission, 2022; Torres-Tiji et al., 2020), achieved high dry biomass production (4.33 g/L) under heterotrophy.

[0004] Despite high biomass productivities associated with heterotrophy, the reliance on glucose hampers economic viability. Using heterotrophic *Auxenochlorella* spp. as an example, glucose accounts for 31% of total production costs. Assuming a growth yield of 0.35 kg biomass/kg sugar, the glucose and microalgal dry biomass costs 0.44 €/kg and 4.00 €/kg, respectively (Ruiz et al., 2022).

[0005] To mitigate costs and enhance resource efficiency, nutrient-dense industrial food processing side-streams have been considered and are found to be potential substitutes for starch/sugar crops.

[0006] An example of an industrial food processing side-stream is brewer's spent grains (BSG). Each year, approximately 36.4 million tonnes of BSG are generated globally from the beer brewing process, most of which is converted to animal feed or discarded (Zeko-Pivač et al., 2022). To upcycle BSG for heterotrophic microalgal cultivation, large quantities of starch (up to 30% dry mass; dm) contained within the lignocellulosic materials could be hydrolysed

to liberate glucose. Another example is soy whey (SW), which is one of the side-streams of tofu and soy protein isolate production. Per kg of soybean, 9 kg of SW is generated which is typically discharged into sewers and promotes eutrophication due to its high chemical and biological oxygen demands. To upcycle SW, moderate quantities of di- and oligosaccharides in SW (e.g., sucrose, stachyose and raffinose; approximately 8.5 g/L total) could be hydrolysed to release glucose and fructose, usable for microalgal assimilation (Chua & Liu, 2019).

[0007] Generally, there are research and development efforts directed towards microalgae cultivation in either BSG hydrolysates or SW individually, although the microalgal cell densities achieved can be further enhanced to reduce downstream harvesting costs. For SW, glucose was commonly supplied to boost cell densities, reaching up to 7.8 g/L, which unfortunately increases production costs (Mitra et al., 2012; Wang et al., 2019; Wang et al., 2018). On the other hand, efforts to cultivate microalgae in BSG hydrolysates required dilution with conventional media to enhance growth. However, this method failed to support high cell densities, with reported values ranging from 0.88 g/L and 7.25×10^5 cells in different studies (Kim et al., 2020; Yap et al., 2022).

[0008] It is therefore desirable to provide an alternative media and method for cultivating microalgae which seeks to address at least one of the problems described hereinabove, or at least to provide an alternative solution.

SUMMARY

[0009] In one aspect, the present application discloses a cultivation medium comprising a mixed hydrolysate medium containing 15% to 25% v/v soy whey hydrolysate and 75% to 85% v/v brewer's spent grain hydrolysate, based on the total volume of the mixed hydrolysate medium, wherein the cultivation medium is rich in endogenous glucose, having an endogenous glucose density of 46.0 to 51.0 g/L, for supporting growth of heterotrophic microalgae.

[0010] In a second aspect, the present application discloses a method of preparing a cultivation medium. The method comprises pre-treating soy whey by subjecting the soy whey to hydrolysis under conditions of heating the soy whey at a temperature of 95°C in a first acid for a first duration to obtain a soy whey hydrolysate; pre-treating brewer's spent grain by subjecting the brewer's spent grain to hydrolysis under conditions of heating the brewer's spent

grain at a temperature of 130°C in a second acid for a second duration to obtain a brewer's spent grain hydrolysate; mixing 15% to 25% v/v soy whey hydrolysate and 75% to 85% v/v brewer's spent grain hydrolysate to obtain a mixed hydrolysate medium; and preparing a cultivation medium comprising the mixed hydrolysate medium, wherein the cultivation medium is rich in endogenous glucose, having an endogenous glucose density of 46.0 to 51.0 g/L, for supporting growth of heterotrophic microalgae.

[0011] In a third aspect, the present application discloses a method of cultivating heterotrophic microalgae. The method comprises inoculating a culture of microalgae to a cultivation medium comprising a mixed hydrolysate medium containing 15% to 25% v/v soy whey hydrolysate and 75% to 85% v/v brewer's spent grain hydrolysate, wherein the cultivation medium is rich in endogenous glucose, having an endogenous glucose density of 46.0 to 51.0 g/L; growing the culture of microalgae aerobically to obtain a biomass of heterotrophic microalgae with a biomass density of 20 to 26 g/L; and harvesting the biomass of heterotrophic microalgae to obtain a dry biomass of heterotrophic microalgae.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Various embodiments of the present disclosure are described below with reference to the following drawings:

FIG. 1 illustrates a schematic diagram of a method of cultivating heterotrophic microalgae according to an embodiment of the present disclosure.

FIGS. 2A to 2D are graphs depicting time-course release of glucose and fructose in soy whey at different temperatures and HCl concentrations. FIG. 2A: Glucose release using 0.1 M HCl, FIG. 2B: fructose release using 0.1 M HCl, FIG. 2C: glucose release using 0.2 M HCl, and FIG. 2D: fructose release using 0.2 M HCl. Initial sucrose concentration was 9.28 ± 0.01 g/L. Values are the mean of triplicate independent experiments ($n = 3$), with error bars representing the standard deviations of the mean values.

FIGS. 3A to 3C are response surface methodology plots illustrating the effects of temperature, time, and H₂SO₄ concentration on the release of xylose + arabinose after hydrolysis of brewer's spent grains.

FIGS. 3D to 3F are response surface methodology plots illustrating the effects of temperature, time, and H₂SO₄ concentration on the release of glucose after hydrolysis of brewer's spent grains.

FIG. 4A depicts *A. protothecoides* cultivation in different ratios of soy whey (SW) and brewer's spent grain (BSG) hydrolysates. Values are the mean of duplicate independent experiments (n = 2), with error bars representing the standard deviations of the mean values.

FIG. 4B shows a simplex lattice mixture design plots of *A. protothecoides* depicting maximum biomass concentration and biomass productivity in different proportions of SW.

FIG. 4C shows a simplex lattice mixture design plot of *A. protothecoides* depicting maximum biomass concentration and biomass productivity in different proportions of BSG hydrolysates.

FIG. 5A depicts *A. protothecoides* cultivation in 15% SW-85% BSG hydrolysate medium (H) vs. BBM.

FIGS. 5B to 5H depict nutrient consumption patterns of *A. protothecoides* in hydrolysate medium (H) and Bold's Basal Medium (BBM). FIG. 5B: glucose and fructose, FIG. 5C: arabinose and xylose, FIG. 5D: total free amino acids (A.A) and NH₃ and sum of nitrogen (N) from free A.A. and NH₃, FIG. 5E: NH₃ and arginine, FIG. 5F: phosphorous, FIG. 5G: succinic acid and acetic acid, FIG. 5H: 5-hydroxymethylfurfural (5-HMF), furfural, and formic acid. Values are the mean of triplicate independent experiments (n = 3), with error bars representing the standard deviation of the mean values. Missing data for BBM indicates the absence of that nutrient.

FIGS. 6A to 6H depict *A. protothecoides* cultivation and nutrient consumption profiles on hydrolysate containing 15% soy whey and 85% brewer's spent grain mix (H), hydrolysate spiked with 20 g/L yeast extract (H+20YE), and standard cultivation media BBM spiked with 48 g/L glucose and 24.33 g/L yeast extract (BBM+G+YE). FIG. 6A: *A. protothecoides* growth, FIG. 6B: pH changes, FIG. 6C: glucose consumption, FIG. 6D: fructose consumption, FIG. 6E: xylose consumption, FIG. 6F: arabinose concentration, FIG. 6G: total nitrogen from free amino acids and NH₃, and FIG. 6H: phosphate consumption. Values are the mean of triplicate independent experiments (n = 3), with error bars representing the standard deviation of the mean values. All cultivations were adjusted to pH 6-7 daily using 2 M NaOH.

FIG. 7A depicts *A. protothecoides* growth in 15% soy whey and 85% brewer's spent grain mixed hydrolysate medium supplemented with varying amounts of KH_2PO_4 (P) and yeast extract (YE). Numbers in the legend indicate the amount (g/L) spiked.

FIGS. 7B to 7D are response surface methodology plots illustrating effects of KH_2PO_4 (A) and yeast extract (B). FIG. 7B: protein content, FIG. 7C: protein yield, and FIG. 7D: protein productivity.

FIGS. 8A to 8H depict amino acid consumption patterns of *A. protothecoides* in 15% SW-85% BSG hydrolysate medium and BBM. FIG. 8A: aspartic acid (Asp) and glutamic acid (Glu), FIG. 8B: alanine (Ala) and isoleucine (Ile), FIG. 8C: serine (Ser) and glycine (Gly), FIG. 8D: lysine (Lys) and threonine (Thr), FIG. 8E: leucine (Leu) and valine (Val), FIG. 8F: phenylalanine (Phe) and proline (Pro), FIG. 8G: tyrosine (Tyr) and tryptophan (Trp), FIG. 8H: histidine (His) and methionine (Met). Values are the mean of triplicate independent experiments ($n = 3$), with error bars representing the standard deviation of the mean values. Note: Cystine is not included due to its low concentration and negligible consumption in both the hydrolysate medium and BBM.

FIG. 9 illustrates an overall mass balance to produce *A. protothecoides* biomass using food side-streams according to an exemplary embodiment of the present invention.

FIGS. 10 A to 10H depict *A. protothecoides* cultivation and nutrient consumption profiles in hydrolysate containing 25% soy whey and 75% brewer's spent grain mix (H), hydrolysate spiked with 20 g/L yeast extract (H+20YE), and BBM spiked with 48 g/L glucose and 30 g/L yeast extract (BBM+G+YE). FIG. 10A: *A. protothecoides* growth, FIG. 10B: pH changes, FIG. 10C: glucose consumption, FIG. 10D: fructose consumption, FIG. 10E: xylose consumption, FIG. 10F: arabinose concentration, FIG. 10G: total nitrogen from free amino acids and NH_3 , and FIG. 10H: phosphate consumption. Values are the mean of triplicate independent experiments ($n = 3$), with error bars representing the standard deviation of the mean values. All cultivations were adjusted to pH 6-7 daily using 2 M NaOH.

FIGS. 11A and 11B depict *A. protothecoides* cultivation in SW-BSG hydrolysate mix spiked with different nitrogen sources. FIG. 11A: 20 g/L yeast extract, chicken carcass hydrolysate, or arginine, and FIG. 11B: $(\text{NH}_4)_2\text{CO}_3$, $\text{H}+\text{NH}_4\text{H}_2\text{PO}_4+(\text{NH}_4)_2\text{HPO}_4$, or $(\text{NH}_4)_2\text{SO}_4$. Values are the mean of duplicate independent experiments ($n = 2$), with error bars representing the standard deviation of the mean values.

FIG. 12 depicts batch cultivations of *A. protothecoides* in a 6.5-L stirred tank bioreactor containing H+20 YE.

DETAILED DESCRIPTION

[0013] The following detailed description is made with reference to the accompanying drawings, showing details and embodiments of the present disclosure for the purposes of illustration. Features that are described in the context of an embodiment may correspondingly be applicable to the same or similar features in the other embodiments, even if not explicitly described in these other embodiments. Additions and/or combinations and/or alternatives as described for a feature in the context of an embodiment may correspondingly be applicable to the same or similar feature in the other embodiments.

[0014] In the context of various embodiments, the articles “a”, “an” and “the” as used with regard to a feature or element include a reference to one or more of the features or elements.

[0015] In the context of various embodiments, the term “about” or “approximately” as applied to a numeric value encompasses the exact value and a reasonable variance as generally understood in the relevant technical field, e.g., within 10% of the specified value.

[0016] As used herein, the term “and/or” includes any, and all combinations of one or more of the associated listed items.

[0017] As used herein, “comprising” means including, but not limited to, whatever follows the word “comprising”. Thus, use of the term “comprising” indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present.

[0018] As used herein, “consisting of” means including, and limited to, whatever follows the phrase “consisting of”. Thus, use of the phrase “consisting of” indicates that the listed elements are required or mandatory, and that no other elements may be present.

[0019] As used herein, the term “heterotrophic” refers to a situation in which microalgae utilizes organic compounds of nitrogen and carbon as a source of energy and for metabolic synthesis.

[0020] As used herein, the term “soy whey” or “SW” refers to a liquid by-product of the soybean manufacture such as tofu or any soy food product.

[0021] As used herein, the term “brewer’s spent grain” or “BSG” refers to a solid by-product of the brewing industry, consisting of the insoluble solid residue after the production of beer and other malt products.

[0022] As used herein, the term “hydrolysis” refers to the release of assimilable monosaccharides (e.g., glucose, fructose) from unassimilable di-, oligo- and polysaccharides (e.g., sucrose, starch).

[0023] Heterotrophic cultivation eliminates the requirement of light, organic carbon source is provided, and the nitrogen source can be organic or inorganic depending upon the microalgal strain used.

[0024] In one aspect, the present disclosure relates to a cultivation medium comprising a mixed hydrolysate medium containing soy whey hydrolysate and brewer's spent grain hydrolysate, wherein the cultivation medium is rich in endogenous glucose, having an endogenous glucose density of 46.0 to 51.0 g/L, for supporting growth of heterotrophic microalgae.

[0025] In some embodiments, the concentration of the soy whey hydrolysate in the mixed hydrolysate medium is at least about 10% v/v, at least about 15% v/v, at least about 20% v/v, at least about 25% v/v, at least about 50% v/v, at least about 75% v/v or at least about 90% v/v based on the total volume of the mixed hydrolysate medium. In some embodiments, the concentration of the soy whey hydrolysate is 15% v/v based on the total volume of the mixed hydrolysate medium. In some embodiments, the concentration of the soy whey hydrolysate is 25% v/v based on the total volume of the mixed hydrolysate medium.

[0026] In some embodiments, the concentration of the brewer's spent grain hydrolysate in the mixed hydrolysate medium is at least about 10% v/v, at least about 15% v/v, at least about 20% v/v, at least about 25% v/v, at least about 50% v/v, at least about 75% v/v, at least about 85% v/v or at least about 90% v/v based on the total volume of the mixed hydrolysate medium. In some embodiments, the concentration of the brewer's spent grain hydrolysate is 85% v/v based on the total volume of the mixed hydrolysate medium. In some embodiments, the concentration of the brewer's spent grain hydrolysate is 75% v/v based on the total volume of the mixed hydrolysate medium.

[0027] In some embodiments, the mixed hydrolysate medium comprises 15% v/v soy whey hydrolysate and 85% v/v brewer's spent grain hydrolysate, based on the total volume of the mixed hydrolysate medium. In some embodiments, the mixed hydrolysate medium comprises 25% v/v soy whey hydrolysate and 75% v/v brewer's spent grain hydrolysate, based on the total volume of the mixed hydrolysate medium.

[0028] In the present disclosure, the cultivation medium prepared from industrial food side-streams is generally rich in endogenous glucose, nitrogen, phosphorous, and minerals which promotes heterotrophic microalgae biomass production.

[0029] In some embodiments, the cultivation medium can be supplemented with other nitrogen sources when higher biomass protein contents are desired. Some examples of such nitrogen sources include, but are not limited to, arginine, ammonium, yeast extract, nitrogen rich side-streams (e.g., chicken carcass hydrolysate), peptone, urea, nitrate, nitrite, peptides, soluble proteins. In one embodiment, the cultivation medium is supplemented with a yeast extract. The addition of yeast extract enhances the protein content of microalgal biomass. In an exemplary embodiment, the addition of yeast extract in an amount of about 20 g/L can increase the protein content from 19.71% to 41.45%, which is more than double the amount of protein content of 19.71%. In some embodiments, the yeast extract is added in an amount of about 20 g/L. In various embodiments, the yeast extract is added to the cultivation medium comprising mixed hydrolysate medium containing 15% to 25% v/v soy whey hydrolysate and 75% to 85% v/v brewer's spent grain hydrolysate. In some embodiments, the yeast extract is added to the cultivation medium comprising mixed hydrolysate medium containing 15% v/v soy whey hydrolysate and 85% v/v brewer's spent grain hydrolysate. In yet other embodiments, the yeast extract is added the cultivation medium comprising mixed hydrolysate medium containing 25% v/v soy whey hydrolysate and 75% v/v brewer's spent grain hydrolysate, based on the total volume of the mixed hydrolysate medium.

[0030] In some embodiments, the cultivation medium is rich in endogenous glucose, having an endogenous glucose density ranging from 46.0 to 51.0 g/L. In some embodiments, the endogenous glucose density is at least about 30.0 g/L, at least about 40.0 g/L, at least about 46.0 g/L, at least about 46.79 g/L, at least about 50 g/L, at least about 50.75 g/L or at least about 51.0 g/L. In some embodiments, the cultivation medium has an endogenous glucose density ranging from 50.60 to 50.80 g/L. In some embodiments, the cultivation medium supplemented with yeast extract has an endogenous glucose density of about 46.79 g/L.

[0031] In some embodiments, the cultivation medium is rich in nitrogen, having a total nitrogen content ranging from 0.1 to 3.2 g/L. In some embodiments, the total nitrogen content of the cultivation medium is about 1.4 g/L. In some embodiments, the total nitrogen content of the cultivation medium supplemented with yeast extract is about 3.0 to 3.2 g/L.

[0032] In some embodiments, the cultivation medium is rich in phosphorous, having a phosphorous content ranging from 0.1 to 0.3 g/L.

[0033] In some embodiments, the cultivation medium can be supplemented with other carbon sources to enhance the lipid content of the microalgal biomass. Some examples of such carbon sources include, but are not limited to, hydrolysed molasses containing glucose and fructose, crude glycerol, fruit juices (glucose and fructose), hydrolysed maltose and starch (glucose).

[0034] The cultivation medium is suitable for cultivating heterotrophic microalgae, such as those in the genus *Auxenochlorella* spp. or previously known as *Chlorella*. The *Auxenochlorella* spp. can encompass various species capable of heterotrophic growth, including *Auxenochlorella protothecoides*.

[0035] The soy whey hydrolysate and the brewer's spent grain hydrolysate can be prepared by subjecting soy whey and brewer's spent grain to hydrolysis by fungal, enzymatic, acid/alkali, heat, or combination treatments thereof to release assimilable monosaccharides.

[0036] In a second aspect, a method of preparing the cultivation medium of the present disclosure is provided. Referring to FIG. 1, the method comprises pre-treating soy whey (101) by subjecting the soy whey to hydrolysis under conditions of heating the soy whey at a temperature of 95 °C in a first acid for a first duration to obtain a soy whey hydrolysate (102), pre-treating brewer's spent grain (103) by subjecting the brewer's spent grain to hydrolysis under conditions of heating the brewer's spent grain at a temperature of 130 °C in a second acid for a second duration to obtain a brewer's spent grain hydrolysate (104), mixing 15% to 25% v/v soy whey hydrolysate and 75% to 85% v/v brewer's spent grain hydrolysate to obtain a mixed hydrolysate medium (105), and preparing a cultivation medium comprising the mixed hydrolysate medium, wherein the cultivation medium is rich in endogenous glucose, having an endogenous glucose density of 46.0 to 51.0 g/L, for supporting growth of heterotrophic microalgae.

[0037] In some embodiments, the first acid and the second acid can be selected from the group consisting of hydrochloric acid, sulphuric acid, phosphoric acid, nitric acid, perchloric acid, maleic acid, oxalic acid. The first acid and the second acid can be the same or different.

[0038] In some embodiments, the first acid has a concentration ranging from 0.05 M to 0.2 M, or 0.1 M to 0.2 M. In some embodiments, the first acid can have a concentration of at least

about 0.05 M, at least about 0.1 M, at least about 0.15 M or at least about 0.2 M. In some embodiments, the first acid has a concentration of 0.1 M. In some embodiments, the first acid is hydrochloric acid with a concentration of 0.1 M.

[0039] In some embodiments, the second acid has a concentration ranging from 0.1 M to 0.6 M, or 0.1 M to 0.2 M. In some embodiments, the second acid can have a concentration of at least about 0.1 M, at least about 0.15 M, at least about 0.2 M, at least about 0.25 M, at least about 0.3 M, at least about 0.35 M, at least about 0.4 M, at least about 0.45 M, at least about 0.5 M, at least about 0.55 M or at least about 0.6 M. In some embodiments, the second acid has a concentration of 0.2 M. In some embodiments, the second acid is sulphuric acid with a concentration of 0.2M.

[0040] In some embodiments, the temperature suitable for the hydrolysis of soy whey includes at least about 75 °C, at least about 80 °C, at least about 85 °C, at least about 90 °C or at least about 95 °C. In some embodiments, the temperature is at least about 95°C.

[0041] In some embodiments, the temperature suitable for the hydrolysis of brewer's spent grain includes at least about 110 °C, at least about 115 °C, at least about 120 °C, at least about 125 °C or at least about 130 °C. In some embodiments, the temperature is at least about 130 °C.

[0042] In some embodiments, each of the first duration and the second duration of the hydrolysis process ranges from 30 to 45 minutes. In some embodiments, each of the first duration and the second duration is at least about 30 minutes, at least about 35 minutes, at least about 36 minutes, at least about 40 minutes or at least about 45 minutes. In some embodiments, the first duration is at least about 30 minutes. In some embodiments, the second duration is at least about 36 minutes.

[0043] In some preferred embodiments, the soy whey is hydrolysed under conditions of heating the soy whey at a temperature of about 95 °C in 0.1 M of hydrochloric acid for about 30 minutes. In some preferred embodiments, the brewer's spent grain is hydrolysed under conditions of heating the brewer's spent grain at a temperature of about 130 °C in 0.2 M sulphuric acid for about 36 minutes.

[0044] In some embodiments, the method may further include adding a yeast extract as a supplementary nitrogen source to the cultivation medium. In some embodiments, the yeast extract is added to the mixed hydrolysate medium to obtain a cultivation medium with a desired or enhanced protein content.

[0045] In some embodiments, the method may further include adding a carbon source to the cultivation medium to enhance the lipid content in the microalgal biomass. In some embodiments, carbon source such as hydrolysed molasses containing glucose and fructose are added to the mixed hydrolysate medium to obtain a cultivation medium with a desired or enhanced lipid content.

[0046] In some embodiments, the concentration of glucose released from the hydrolysed soy whey ranges from 2.4 to 6.0 g/L. In some embodiments, the concentration is at least about 2.4 g/L, at least about 4.0 g/L, at least about 4.2 g/L, at least about 4.5 g/L, at least about 5.0 g/L, at least about 5.5 g/L, at least about 5.9 g/L or at least about 6.0 g/L.

[0047] In some embodiments, the concentration of fructose released from the hydrolysed soy whey ranges from 3.4 to 8.1 g/L. In some embodiments, the concentration is at least about 3.4 g/L, at least about 4.0 g/L, at least about 4.5 g/L, at least about 5.0 g/L, at least about 5.5 g/L, at least about 6.0 g/L, at least about 6.5 g/L, at least about 7.0 g/L, or at least about 8.1 g/L.

[0048] In some embodiments, the concentration of glucose released from the hydrolysed brewer's spent grain ranges from 48.0 to 85.5 g/L. In some embodiments, the concentration is at least about 48.0 g/L, at least about 48.1 g/L, at least about 49.0 g/L, at least about 50.0 g/L, at least about 53.0 g/L, at least about 55.0 g/L, at least about 57.0 g/L, at least about 59.0 g/L, at least about 59.9 g/L, at least about 60.0 g/L, or at least about 85.5 g/L.

[0049] In a third aspect, a method of cultivating heterotrophic microalgae is provided. Referring to FIG. 1, the method comprises inoculating a culture of microalgae (106) to a cultivation medium comprising a mixed hydrolysate medium (105) containing 15% to 25% v/v soy whey hydrolysate and 75% to 85% v/v brewer's spent grain hydrolysate, wherein the cultivation medium is rich in endogenous glucose, having an endogenous glucose density of 46.0 to 51.0 g/L; growing the culture of microalgae aerobically to obtain a biomass of heterotrophic microalgae (107) with a biomass density of 20 to 26 g/L; and harvesting the biomass of heterotrophic microalgae to obtain a dry biomass of heterotrophic microalgae (108).

[0050] In some embodiments, the heterotrophic microalgae cultivated in the cultivation medium can reach a biomass density of at least about 15 g/L, at least about 17 g/L, at least about 19 g/L, at least about 20 g/L, at least about 21 g/L, at least about 22 g/L or at least about 23 g/L. In some embodiments, the heterotrophic microalgae cultivated in the cultivation medium

supplemented with yeast extract can reach a biomass density of at least about 24 g/L or at least about 26 g/L.

[0051] In some embodiments, the heterotrophic microalgae cultivation can achieve a productivity ranging from 6.7 to 7.4 g/L/day. In some embodiments, the productivity is at least about 4.3 g/L/day, at least about 4.7 g/L/day, at least about 4.9 g/L/day, at least about 5.2 g/L/day, at least about 6.7 g/L/day, at least about 6.9 g/L/day, at least about 7.06 g/L/day, at least about 7.1 g/L/day, at least about 7.3 g/L/day or at least about 7.4 g/L/day.

[0052] In some embodiments, the method may further include harvesting the biomass of heterotrophic microalgae in 96-hour growth or day 4 of growth. In some embodiments, the method may further include harvesting the biomass of heterotrophic microalgae in 120-hour growth or day 5 of growth.

[0053] In some embodiments, the heterotrophic microalgae belong to the genus *Auxenochlorella* spp. or previously known as *Chlorella*. In some embodiments, the species in the genus *Auxenochlorella* spp. is *Auxenochlorella protothecoides*.

[0054] In some embodiments, the biomass of heterotrophic microalgae is cultivated in batch mode. In some embodiments, the biomass of heterotrophic microalgae is cultivated in bioreactor.

[0055] In some embodiments, the heterotrophic microalgae cultivation is conducted in the absence of light.

[0056] In some embodiments, the method may further include adjusting the pH of the cultivation medium to about pH 6 to 7 or to about 6.5.

[0057] In some embodiments, the method of the present disclosure produces biomass of heterotrophic microalgae with a dry mass protein content of 15% to 21% w/w, based on the total weight of the dry biomass of heterotrophic microalgae. In some embodiments, the dry mass protein content is at least about 15% w/w, at least about 17% w/w, at least about 19% w/w or at least about 21% w/w, based on the total weight of the dry biomass of heterotrophic microalgae. In some embodiments, when the cultivation medium is supplemented with other nitrogen source such as yeast extract, the method of the present disclosure produces biomass of heterotrophic microalgae with a dry mass protein content ranging from 24% to 30% w/w, based on the total weight of the dry biomass of heterotrophic microalgae. In some embodiments, the dry mass protein content is about at least about 28% w/w, at least about 39% w/w, at least about

41% w/w, or at least about 43% w/w, based on the total weight of the dry biomass of heterotrophic microalgae.

[0058] In some embodiments, the method of the present disclosure produces heterotrophic microalgae with a dry mass lipid content of 26% to 29% w/w, based on the total weight of the dry biomass of heterotrophic microalgae. In some embodiments, the dry mass lipid content is at least about 26% w/w, at least about 27% w/w, at least about 28% w/w or at least about 29% w/w, based on the total weight of the dry biomass of heterotrophic microalgae. In some embodiments, when the cultivation medium is supplemented with other nitrogen source such as yeast extract, the method of the present disclosure produces biomass of heterotrophic microalgae with a dry mass lipid content of at least about 18% w/w, at least about 19% w/w, at least about 20% w/w or at least about 22% w/w, based on the total weight of the dry biomass of heterotrophic microalgae.

[0059] The cultivation medium and the method of the present disclosure provide several advantages. The method of the present disclosure utilises the cultivation medium of the present disclosure, which comprises a mixed hydrolysate medium containing soy whey hydrolysate and brewer's spent grain, and which is rich in endogenous glucose, nitrogen, phosphorous, and minerals, to effectively cultivate heterotrophic microalgae. The method achieves a relatively high biomass yield ranging from 20 to 23 g/L, and a relatively high productivity rate. The present disclosure demonstrates that industrial food side-streams can be used as the sole source of glucose, nitrogen, phosphorous and minerals to heterotrophically cultivate microalgae such as *Auxenochlorella protothecoides*, while achieving excellent biomass production. The cultivation medium and method of the present disclosure allows the production of microalgal biomass that is rich in protein and/or lipid contents.

[0060] The cultivation medium and the method of the present disclosure significantly reduce raw material costs associated with conventional media by utilizing industrial food side-streams. The method minimizes waste by upcycling discarded industrial food side-streams. It enables the selection of suitable industrial food side-streams rich in nutrients and low in inhibitory compounds for preparing the cultivation medium. Additionally, it allows the mixing of different industrial food side-streams to balance nutritional deficiencies and reduce inhibitory compounds arising from a single food side-stream. The method offers flexibility in supplementing industrial food side-streams with various nutrient sources to modify microalgal

biomass protein and lipid contents according to nutritional needs if necessary. The microalgal biomass produced by the method of the present disclosure is safe for human consumption, as the cultivation medium is prepared from food processing side-streams generated from existing food production processes. Moreover, the chemicals used for hydrolysis, which are also permitted food additives, further ensure safety.

[0061] The cultivation medium and the method of the present disclosure also offer a resource-efficient solution as high densities of microalgal biomass can be grown in bioreactors. Indoor bioreactors do not require arable land and are resilient to climate changes.

[0062] Non-limiting examples of the disclosure will be further described in greater detail by reference to specific Examples, which should not be construed as in any way limiting the scope of the disclosure.

EXAMPLES

[0063] **Example 1 - Methods**

[0064] **1.1 Hydrolysis of soy whey**

[0065] Fresh soy whey (SW), which was collected from local food manufacturer and stored at -20 °C, was defrosted overnight at 4 °C prior to use. Next, 10 mL aliquots of SW were placed into 15-mL propylene centrifuge tubes and adjusted to 0.05, 0.1, or 0.2 M HCl using a 5 M HCl stock solution (Sigma-Aldrich, Missouri, USA). For the control (0 M HCl), deionised water was used instead of HCl. The tubes were then placed in a shaking water bath (Julabo SW-22, Seelbach, Germany), set at 100 rpm, at either 75, 85, or 95 °C. Time-course sampling was conducted at 0, 5, 10, 20, 30, 50, 60, and 120 min, with a tube removed at each time point and immediately placed in an ice bath. Once cooled, the SW was neutralised to pH 6.5 with 5 M NaOH (Goodrich Chemical Enterprise, Singapore), followed by centrifugation (10,000 × g, at 4 °C for 5 min) and filtration through a 0.22-µm hydrophilic polytetrafluoroethylene (PTFE) filter (Sartorius, Göttingen, Germany). The hydrolysates obtained were analysed for sugars. All hydrolysis conditions were performed in triplicate.

[0066] Prior to *A. protothecoides* cultivation, SW was subjected to the same hydrolysis procedure using the chosen hydrolysis condition (0.1 M HCl, 95 °C for 30 min). However, instead of 10 mL, 100-mL aliquots of SW were placed into 250-mL glass capped bottles and hydrolysed in an autoclave (Hirayama HG-80, Tokyo, Japan).

[0067] 1.2 Hydrolysis of brewer's spent grain

[0068] Fresh brewer's spent grain (BSG) consisting of 100% malted barley was collected from RedDot Brewhouse (Singapore) and immediately stored at -20 °C, before defrosting overnight at 4 °C prior to use. Thawed BSG was suspended in deionised water (15 g dry BSG in 100 mL of water) and pulverised with a household blender to obtain a slurry. Hydrolysis was conducted in 250-mL glass capped bottles, containing 100 mL of the slurry, with variable amounts of added 5 M H₂SO₄ (Sigma-Aldrich) to achieve 0.2 to 0.6 M acid concentrations. The bottles were then placed in an autoclave under different time (15 to 45 min) and temperature (110 to 130 °C) conditions. Once hydrolysed, the bottles were cooled in an ice bath, and the slurry neutralised to pH 6.5 with 5 M NaOH, followed by centrifugation (10, 000 ×g, at 4 °C for 5 min) and filtration through 0.22-µm hydrophilic PTFE filters. The hydrolysates obtained were analysed for sugars. All hydrolysis conditions were carried out in triplicate.

[0069] Unlike SW hydrolysis in a water bath, BSG hydrolysis in an autoclave impedes time-course sampling. Therefore, optimal BSG hydrolysis parameters were chosen with the aid of response surface methodology (RSM) based on a Box-Behnken design comprising of 17 runs and 5 centre points, created using the Design-Expert 13 statistical software (Stat-Ease Inc., Minneapolis, USA). Temperature (A: 110, 120, 130 °C), time (B: 15, 30, 45 min), and acid concentration (C: 0.2, 0.4, 0.6 M H₂SO₄) were assigned as factors, while glucose and xylose, and arabinose concentrations were assigned as responses. Actual values of factors, together with experimental and predicted values of responses are given in Table 1. Analysis of variance (ANOVA) for the quadratic models of sugars released and coded coefficients are given in Table 2 and 3 respectively.

[0070] Table 1. Box-Behnken design to evaluate the effects of acid hydrolysis parameters (temperature (A), time (B), and H₂SO₄ concentration (C)) on pentoses (xylose + arabinose) and glucose yields from brewer's spent grain.

Run ^a	Experimental conditions			Responses ^b					
	A: Temperature (°C)	B: Time (min)	C: H ₂ SO ₄ concentration (M)	Xylose (g/L)	Arabinose (g/L)	Xylose + Arabinose (g/L)	Predicted xylose+ arabinose (g/L)	Glucose (g/L)	Predicted glucose (g/L)
1	130	30	0.2	20.34 ± 4.10	8.79 ± 0.77	29.14 ± 4.72	28.56	66.37 ± 5.59	66.09
2	110	15	0.4	29.06 ± 1.62	8.67 ± 1.21	37.73 ± 2.82	36.91	42.05 ± 0.75	41.28
3	110	30	0.2	12.00 ± 0.97	8.80 ± 0.19	20.81 ± 0.79	20.86	35.56 ± 2.55	35.39
4	110	45	0.4	30.41 ± 4.50	8.90 ± 1.19	39.31 ± 5.68	39.50	60.94 ± 7.98	61.60
5	120	30	0.4	32.60 ± 1.20	9.59 ± 1.12	42.20 ± 1.79	42.62	68.24 ± 2.97	67.73
6	120	15	0.2	13.62 ± 0.65	8.89 ± 0.17	22.50 ± 0.61	23.27	40.23 ± 1.59	41.17
7	120	45	0.2	18.51 ± 1.87	9.34 ± 0.43	27.85 ± 2.21	27.60	62.16 ± 3.12	61.67
8	130	45	0.4	31.56 ± 1.35	8.95 ± 0.35	40.51 ± 1.49	41.33	68.68 ± 3.08	69.45
9	120	30	0.4	33.60 ± 0.48	9.19 ± 0.42	42.80 ± 0.29	42.62	67.97 ± 1.47	67.73
10	130	30	0.6	33.78 ± 0.06	9.42 ± 0.53	43.20 ± 0.58	43.15	71.09 ± 1.83	71.26
11	120	15	0.6	34.88 ± 0.28	10.34 ± 0.35	45.22 ± 0.51	45.47	68.87 ± 3.16	69.36
12	110	30	0.6	34.54 ± 1.46	10.02 ± 1.99	44.56 ± 3.45	45.14	65.98 ± 2.32	66.26
13	120	30	0.4	33.75 ± 0.47	9.62 ± 0.82	43.37 ± 0.75	42.62	68.72 ± 1.48	67.73
14	130	15	0.4	31.69 ± 0.68	9.30 ± 0.15	40.99 ± 0.62	40.80	69.77 ± 1.71	69.11
15	120	30	0.4	33.31 ± 1.56	8.83 ± 1.35	42.14 ± 2.25	42.62	65.12 ± 7.71	67.73
16	120	30	0.4	32.70 ± 1.22	9.88 ± 0.76	42.57 ± 0.89	42.62	68.58 ± 1.14	67.73
17	120	45	0.6	34.98 ± 3.74	10.05 ± 3.25	45.03 ± 4.39	44.26	70.46 ± 5.11	69.52

^a Run order was randomised and analysed in triplicate independent batches.

^b Values are mean ± SD of three values.

[0071] Table 2. Analysis of variance (ANOVA) for the adjusted quadratic model of sugars released from hydrolysis of brewer's spent grain.

Source	Sum of Squares	Degree of freedom	Mean Square	F-value	p-value
<i>Xylose + Arabinose</i>					
Model	1008.16	9	112.02	178.29	< 0.0001
A-Temperature	16.33	1	16.33	25.99	0.0014
B-Time	4.90	1	4.90	7.80	0.0268
C-Acid concentration	754.86	1	754.86	1201.43	< 0.0001
AB	1.06	1	1.06	1.69	0.2350
AC	23.47	1	23.47	37.36	0.0005
BC	7.67	1	7.67	12.21	0.0101
A ²	14.44	1	14.44	22.98	0.0020
B ²	5.37	1	5.37	8.55	0.0222
C ²	169.07	1	169.07	269.09	< 0.0001
Residual	4.40	7	0.6283		
Lack of Fit	3.39	3	1.13	4.51	0.0899
Pure Error	1.00	4	0.2510		
Cor Total	1012.55	16			
<i>Glucose</i>					
Model	2070.19	9	230.02	120.58	< 0.0001
A-Temperature	636.89	1	636.89	333.86	< 0.0001
B-Time	213.42	1	213.42	111.87	< 0.0001
C-Acid concentration	649.44	1	649.44	340.44	< 0.0001
AB	99.80	1	99.80	52.32	0.0002
AC	165.12	1	165.12	86.56	< 0.0001
BC	103.43	1	103.43	54.22	0.0002
A ²	68.15	1	68.15	35.72	0.0006
B ²	47.06	1	47.06	24.67	0.0016
C ²	65.79	1	65.79	34.49	0.0006
Residual	13.35	7	1.91		
Lack of Fit	4.52	3	1.51	0.6825	0.6075
Pure Error	8.83	4	2.21		
Cor Total	2083.54	16			

[0072] Table 3. Coded coefficients for the second order 8 quadratic polynomial equation using xylose + arabinose and glucose as the responses during hydrolysis of brewer's spent grain.

	Intercept	A	B	C	AB	AC	BC	A ²	B ²	C ²
Xylose + Arabinose	42.616	1.42875	0.7825	9.71375	-0.515	-2.4225	-1.385	-1.85175	-1.12925	-6.33675
<i>p</i> -values		0.0014	0.0268	<0.0001	0.2350	0.0005	0.0101	0.0020	0.0222	<0.0001
Glucose	67.726	8.9225	5.165	9.01	-4.995	-6.425	-5.085	-4.023	-3.343	-3.953
<i>p</i> -values		<0.0001	<0.0001	<0.0001	<0.0002	<0.0001	<0.0002	0.0006	0.0016	0.0006

A-Temperature; B-Time; C-Acid concentration

[0073] Using the optimal condition (0.2 M H₂SO₄, 130 °C, 36 min) to maximise glucose recovery while minimising xylose + arabinose release and acid usage, the hydrolysis process was repeated thrice in 250-mL glass capped bottles for model validation. Once validated, the same hydrolysis procedure was applied to produce BSG hydrolysates for microalgal cultivations except for the following modification: Instead of NaOH, Ca(OH)₂ (Sigma-Aldrich) was used as the neutralising agent to remove excess SO₄²⁻ ions in the form of CaSO₄ precipitate, thereby alleviating salt stress (e.g., Na⁺, Cl⁻) during cultivation. The precipitate was removed via centrifugation, and the supernatant subsequently sterile-filtered (0.22-μm) prior to *A. protothecoides* cultivation and analyses. To characterise the composition of BSG, the solids were washed thrice with deionised water via centrifugation, freeze-dried, and characterised for starch, structural carbohydrates, lignin, extractives, and ash according to the protocols described by the National Renewable Energy Laboratory (Michel et al., 2021; Sluiter et al., 2008).

[0074] 1.3 Preparation of microalgal strain

[0075] *A. protothecoides* SAG 211-7a was obtained from the culture collection of algae at the University of Goettingen, Germany. Microalgal stock cultures were maintained at room temperature and under natural light, as agar plates containing sterile BBM with 6.7 g/L yeast extract (Oxoid Ltd., Hampshire, United Kingdom), and were sub-cultured on to new agar plates monthly. Prior to each experiment, two subculture cycles from the stock culture were conducted: i) A single microalgal colony from the agar plate was propagated in 125-mL Erlenmeyer flasks, each containing 20 mL of BBM (20 g/L glucose; Thomas Coopers Breweries, South Australia, Australia and 6.7 g/L yeast extract); ii) 10% (v/v) of *A. protothecoides* cells from the first subculture were inoculated into 250-mL Erlenmeyer 6 flasks

containing 45 mL of BBM, to make up to a total volume of 50 mL. All subcultures were conducted in the dark, in an OrbiCult™ IBS shaking incubator (Esco Lifesciences, Singapore) with a 25 mm orbit size. Temperature and shaking speed were maintained at 25 °C and 210 rpm, respectively, for 3 days corresponding to the late exponential phase.

[0076] Prior to final experimental cultivation, twice sub-cultured *A. protothecoides* was centrifuged at 1,500 ×g for 5 min at 25 °C to remove the medium, washed twice with 0.25 g/L saline, followed by resuspension using a fresh medium (either hydrolysates or BBM). All experiments started with an inoculum of 1 g/L *A. protothecoides* cells into a 100-mL working volume cultivation medium (either hydrolysates or BBM) contained in a 500-mL Erlenmeyer flask. Cultivations were carried out in the absence of light at 25°C and 210 rpm, with the initial pH standardised to 6.50 ± 0.25 . Additionally, blank samples (without an inoculum) were subjected to identical cultivation conditions, which demonstrated that any metabolite changes were a result of *A. protothecoides* growth and metabolism, in addition to ensuring sterility (data not shown).

[0077] 1.4 Optimisation of soy whey-brewer's spent grain mixed hydrolysate medium

[0078] Five mixtures of SW and BSG hydrolysates (in % v/v: 0% SW-100% BSG, 25% SW-75% BSG, 50% SW-50% BSG, 75% SW-25% BSG, 100% SW-0% BSG) were prepared, following a simplex lattice mixture design by Design-Expert 13 software. The design space comprised of 8 runs consisting of replicated vertices and centre points, which were conducted in duplicate, in randomised order. Microalgal maximum biomass concentration (g/L) and biomass productivity (g/L/day) were selected as responses, and their actual and predicted response values, along with ANOVA for the quartic and quadratic equations, are presented in Table 4 and 5 respectively.

[0079] Table 4. Simplex lattice mixture design to evaluate the effects of mixtures of brewer's spent grain (A) and soy whey (B) hydrolysates on *A. protothecoides* maximum biomass concentration and productivity.

Run ^a	Component		Responses ^b		
	A: Brewer's spent grain hydrolysate (%)	B: Soy whey hydrolysate (%)	Maximum biomass (g/L)	Predicted maximum biomass (g/L)	Predicted Productivity (g/L/day)

1	25	75	10.46 ± 0.10	10.45	4.73 ± 0.05	5.12
2	0	100	4.61 ± 0.50	4.51	3.61 ± 0.51	3.44
3	100	0	21.79 ± 0.01	21.92	5.20 ± 0.00	5.26
4	0	100	4.42 ± 0.19	4.51	3.42 ± 0.19	3.44
5	100	0	22.04 ± 0.47	21.92	5.26 ± 0.12	5.26
6	50	50	13.16 ± 0.99	13.26	6.08 ± 0.49	5.99
7	75	25	19.22 ± 0.30	19.22	6.07 ± 0.10	6.04
8	50	50	13.36 ± 1.37	13.26	6.18 ± 0.68	5.99

^a Run order was randomised and analysed in duplicate independent batches.

^b Values are mean ± SD of two values.

[0080] Table 5. Analysis of variance (ANOVA) for the quartic and quadratic models of *A. protothecoides* maximum biomass and productivity cultivated in mixtures of soy whey and brewer's spent grain hydrolysates.

	Sum of Squares	Degree of freedom	Mean square	F-value	p-value
<i>Maximum biomass quartic model</i>	690.61	4	172.65	528.08	< 0.0001
Linear Mixture	682.86	1	682.86	2088.62	< 0.0001
AB	0.0057	1	0.0057	0.0174	0.8973
AB(A-B)	0.0033	1	0.0033	0.0102	0.9214
AB(A-B) ²	6.32	1	6.32	19.32	0.0011
Pure Error	3.60	11	0.3269		
Cor Total	694.21	15			
<i>Biomass productivity quadratic model</i>	16.11	2	8.06	70.04	< 0.0001
Linear Mixture	7.50	1	7.50	65.19	< 0.0001
AB	8.61	1	8.61	74.89	< 0.0001
Residual	1.50	13	0.1150		
Lack of Fit	0.4254	2	0.2127	2.19	0.1587
Pure Error	1.07	11	0.0973		
Cor Total	17.61	15			

A-Brewer's spent grain hydrolysate %; B- Soy whey hydrolysate %

[0081] After the optimum SW-BSG hydrolysate mixture (15% SW-85% BSG) was

determined, the predicted maximum biomass concentration and productivity were validated thrice. The validated results were concomitantly compared to the growth performance of *A. protothecoides* in BBM supplemented with 6.7 g/L yeast extract and 50 g/L glucose, the latter concentration matching that found in the 15% SW-85% BSG mixture (approximately 50 g/L endogenous glucose). The pH of all cultivations was adjusted daily with 2 M NaOH to maintain the level within an optimal range of 6 to 7. Additionally, changes in chemical compositions of the optimum SW-BSG hydrolysate medium and conventional BBM were analysed.

[0082] 1.5 Analytical methods

[0083] 1.5.1 Growth monitoring

[0084] The dry cell weight (DCW) of *A. protothecoides* was determined gravimetrically in duplicates, whereby 2 mL of a microalgal suspension was aliquoted into a pre-weighed Eppendorf tube, and centrifuged at 8,000 ×g for 15 min. The microalgal pellets were then washed with distilled water twice and dried at 105°C until constant weight.

[0085] The maximum biomass concentration (X_{max} ; g/L) was determined based on the DCW at the end of the exponential phase. The biomass productivity (PX_{max} ; g/L/day) and biomass yield coefficient ($Y_{X/S}$; g biomass/g glucose + fructose) were calculated using equations 1 and 2:

[0086] $PX_{ax} = (DCW_n - DCW_0) / t_n$ ----- (1)

[0087] $Y_{X/S} = (DCW_n - DCW_0) / (C_0 - C_n)$ ----- (2)

[0088] where DCW_0 and DCW_n are the biomass concentrations (g/L) on day 0 and at the end of the exponential phase, respectively, and t_n is the total duration leading up to the end of the exponential phase expressed in days. C_0 and C_n are the concentrations of assimilable sugar (glucose + fructose) on day 0 and at the end of the exponential phase, respectively.

[0089] 1.5.2 Quantification of sugars, organic acids, and furans in hydrolysates and BBM

[0090] Sugars, organic acids (succinic, acetic, lactic, citric, and formic acids) and furans (furfural and 5-hydroxy-5-methylfurfural; 5-HMF) were quantified following the protocol of Zhou et al. (2022). However, sugar quantification had the following modifications: glucose, fructose, and sucrose were quantified using the Agilent 1260 Infinity II prime liquid chromatography system, connected to an evaporative light scattering detector (gain: 5; 40 °C, Agilent Technologies, California, USA). A XBridge Amide column (150 x 4.6 mm, Waters, Massachusetts, USA) was maintained at 35 °C, with an isocratic flow rate of 0.6 mL/min. For

better separation, xylose and arabinose were quantified separately using a refractive index detector (Agilent Technologies), using a 7.8 x 300 mm Aminex® HPX-87P column maintained at 85°C, attached to a 4.6 x 30 mm Microguard Carbo-P refill cartridge (Bio-Rad Laboratories, California, USA). Ultrapure water served as the eluent, with an isocratic flow rate of 0.6 mL/min. All chemical standards were obtained from Sigma-Aldrich.

[0091] 1.5.3 Quantification of amino acids and NH₃ in hydrolysates and BBM

[0092] Filtered supernatants were diluted with 5% (w/v) trichloroacetic acid (Sigma-Aldrich), centrifuged 10,000 ×g for 5 min at 4 °C, and filtered through a 0.22-µm syringe filter. Free amino acids and NH₃ were detected according to the pre-set hydrolysate separation program on the Hitachi LA8080 amino acid analyser (Hitachi, Tokyo, Japan) equipped with a 4.6 mm x 60 mm custom ion exchange column and photometers at 570 and 440 nm. Eluents PF1 to PF4, and PF-RG served as the mobile phase as provided by the manufacturer.

[0093] 1.5.4 Elemental composition analysis

[0094] Elemental composition was determined using Shimadzu ICPE-9820 Inductively coupled plasma optical emission spectroscopy (ICP-OES; Shimadzu, Kyoto, Japan). The operating parameters were as follows: 1.20 kW radio frequency power, 10 L/min plasma flow rate, 0.6 L/min auxiliary gas flow rate, 0.7 L/min nebulizer flow rate, 30 s sample flush time. Samples were diluted in 2% (v/v) aqueous nitric acid, centrifuged 10,000 ×g for 5 min at 4 °C, and filtered through a 0.22-µm hydrophilic PTFE syringe filter. All measurements were conducted in duplicate.

[0095] 1.5.5 Protein and lipid analyses of microalgal biomass

[0096] At the end of exponential growth, microalgal biomass was harvested by centrifugation at 8,000 ×g for 15 min and washed with distilled water twice before being lyophilised. The biomass was then ground with a mortar and pestle and stored in a desiccator before subsequent protein and lipid analyses, which were performed in duplicate. The total protein content of *A. protothecoides* was determined according to AOAC 2001.11, using the Kjeltac™ 8400 and Tecator™ Digestor 2520 (FOSS, Hillerød, Denmark) and a conversion factor of 4.78 (Lourenço et al., 2004). Lipid measurements were conducted according to the Folch method (Folch et al., 1957), whereby homogenised *A. protothecoides* had their cell walls disrupted by bead-beating using the TissueLyser II (QIAGEN, Venlo, Netherlands) at 30 Hz for 15 min.

[0097] 1.5.6 Statistical analyses

[0098] All cultivations were conducted in duplicate or triplicate ($n = 2$ or 3) where stated and data are presented as mean values $\pm$ standard deviations. Further statistical tests were not performed as the assumptions for parametric tests (i.e., normality and equal variance) could not be tested robustly with a small sample size, which also limited the application of non-parametric tests.

[0099] **Example 2 – Conditions for hydrolysis of soy whey and brewer's spent grains**

[00100] Blending SW with BSG hydrolysates may yield better growth parameters compared to cultivation in a single side-stream: the former as a diluent to reduce the concentration of inhibitory/non-preferred compounds from BSG, and the latter as a glucose-rich source as well as providing complementary nutrients. To ensure maximal recoveries of assimilable monosaccharides (glucose and fructose) from SW and BSG that could support high cell densities of *A. protothecoides*, mild acid hydrolysis parameters were first optimised.

[00101] For SW, this was achieved by assessing the effects of different HCl concentrations (0, 0.05, 0.1, and 0.2 M) and temperatures (75 °C, 85 °C, 95 °C) on glucose and fructose release over the course of 120 min as shown in FIGS. 2A to 2D. The releases of glucose and fructose were not detected at 0 and 0.05 M HCl (data not shown). At 0.1 M HCl (FIGS. 2A and 2B), hexose release was the fastest at 95 °C (within 30 min; glucose: 5.15 ± 0.75 g/L; fructose: 7.02 ± 1.10 g/L), compared to that at 75 and 85 °C (within 30-45 min). Notably, increasing the HCl concentration to 0.2 M (FIGS. 2C and 2D) accelerated glucose and fructose release, peaking at the 10-20 min mark regardless of temperature (e.g., at 75 °C for 20 min, glucose: 5.48 ± 1.13 g/L; fructose: 6.85 ± 0.95 g/L). However, this acceleration would be at the expense of increased acid usage which raises raw material cost, in addition to NaCl accumulation upon NaOH neutralisation which imposes salt stress and diminishes biomass yield.

[00102] The effects of subjecting SW to harsher hydrolysis conditions (130 °C and 30-45 min) are presented in Table 6. Although higher temperatures and longer durations further enhanced sugar recovery (from stachyose and raffinose degradation), inhibitory compounds such as furfural, formic acid, phenolic compounds were also produced.

[00103] Table 6. Effects of increasing time and temperature conditions on sugar and inhibitory compound release in soy whey containing 0.1 M HCl.

	Time and temperature conditions		
	95 °C, 30 min	130 °C, 30 min	130 °C, 45 min
<i>Sugar (g/L)</i>			
Glucose	4.71 ± 0.51a	7.39 ± 1.86b	6.82 ± 0.40ab
Fructose	6.65 ± 0.03a	6.62 ± 1.25a	7.04 ± 0.47a
Galactose	n.d.	3.28 ± 0.72a	3.55 ± 0.90a
<i>Inhibitory compounds (g/L)</i>			
Furfural	n.d.	0.17 ± 0.02a	0.27 ± 0.02b
Formic acid	0.13 ± 0.00a	0.58 ± 0.02b	0.75 ± 0.05c
TPC (gallic acid equivalent g/L)	0.45 ± 0.02 a	0.52 0.02b	

n.d.: Not detected.

Values are expressed as the mean of triplicate independent experiments ± SD.

Different lowercase letters in a row indicate statistical differences ($P < 0.05$) between different hydrolysis treatments.

[00104] Therefore, 0.1 M HCl, 95 °C, 30 min were adopted as the SW hydrolysis treatment in subsequent research.

[00105] For BSG, the effects of different combinations of temperature (110-130 °C), time (15-45 min), and H₂SO₄ (0.2-0.6 M) on xylose + arabinose and glucose releases are depicted as RSM plots in FIGS. 3A to 3C. The data from each response was fitted into second order polynomial equations, and it was evident that all three factors positively enhanced sugar release in the following order: H₂SO₄ > temperature > time (Table 3). Due to good model fit and predictability, the polynomial equations were further utilised to predict the optimal hydrolysis conditions which would maximise glucose release, while minimising acid usage and the amount of xylose + arabinose generated. Using an optimisation method developed by Derringer and Suich (1980), the optimal conditions recommended by Design-Expert were 130 °C, 36 min, 0.2 M H₂SO₄, with predicted values of 68.0 g/L glucose and 29.0 g/L xylose + arabinose. Upon validation with triplicate, actual values obtained were 67.20 ± 1.39 g/L glucose and 31.33 ± 3.84 g/L xylose + arabinose, which were within 95% prediction intervals. Therefore, 130 °C, 36 min, 0.2 M H₂SO₄ were applied as the BSG hydrolysis treatment in subsequent experiments.

[00106] **Example 3 - Composition of soy whey hydrolysate and brewer's spent grain hydrolysate**

[00107] Prior to *A. protothecoides* cultivation, the corresponding optimal hydrolysis conditions were applied to produce SW hydrolysate and BSG hydrolysate, with modifications described in Examples 1.1 and 1.2 (i.e., SW: 10-mL aliquot was raised to 100-mL aliquot; BSG:

NaOH was changed to Ca(OH)₂. The compositions of the BSG solids and hydrolysates are detailed in Tables 7 and 8, respectively.

[00108] Table 7. Chemical compositions of unhydrolysed and hydrolysed brewer's spent grain (BSG) solids, under optimal hydrolysis condition of 130 °C, 36 min, and 0.2 M H₂SO₄.

	Unhydrolysed BSG	Hydrolysed BSG
<i>Composition of the solid fraction (% dm)</i>		
Starch	34.93 ± 0.54	0.50 ± 0.03
Glucan	7.57 ± 0.25	39.53 ± 0.04
Xylan	10.14 ± 0.01	3.72 ± 0.05
Arabinan	5.86 ± 0.02	0.16 ± 0.00
Acid-insoluble lignin	12.88 ± 0.24	15.90 ± 0.32
Acid-soluble lignin	1.21 ± 0.07	1.74 ± 0.10
Protein [#]	17.13 ± 0.20	10.62 ± 0.05
Ash	2.88 ± 0.07	4.48 ± 0.02
Extractives	38.03 ± 0.32	24.58 ± 0.69
Solid recovery %	N.A.	19.46 ± 1.44

[#]Protein content was determined using the Kjeldahl method with a nitrogen to protein conversion factor of 5.83 for barley (FAO, 2003).

N.A.: Not applicable

[00109] Table 8. Chemical compositions of unhydrolysed soy whey (SW), SW hydrolysate, brewer's spent grain (BSG) hydrolysate, the optimal mixed hydrolysate medium, and Bold's basal media (BBM)

	SW unhydrolysed	SW hydrolysate	BSG hydrolysate	Hydrolysate medium (15% SW-85% BSG)	BBM (50 g/L glucose; 6.7 g/L yeast extract)
Sugars (g/L)					
Sucrose	9.28 ± 0.01	n.d.	n.d.	n.d.	n.d.
Glucose	n.d.	4.71 ± 0.51	54.02 ± 5.88	50.75 ± 2.49	50.44 ± 2.30
Fructose	n.d.	6.65 ± 0.03	n.d.	1.71 ± 0.13	n.d.
Xylose	n.d.	n.d.	15.94 ± 2.02	13.90 ± 0.21	n.d.
Arabinose	n.d.	n.d.	8.59 ± 0.86	8.36 ± 0.13	n.d.
Free amino acids & NH₃ (mg/L)					
Aspartic acid	22.89 ± 2.29	30.54 ± 0.77	638.18 ± 59.10	661.36 ± 0.00	118.18 ± 1.70
Threonine	13.48 ± 0.21	13.81 ± 0.55	30.47 ± 4.22	31.03 ± 0.47	110.76 ± 1.94
Serine	17.42 ± 1.01	17.03 ± 0.51	40.19 ± 5.99	39.78 ± 0.87	137.35 ± 2.03
Glutamic acid	146.20 ± 8.39	138.89 ± 4.36	90.22 ± 15.04	114.88 ± 1.63	501.93 ± 7.91
Glycine	68.35 ± 4.52	54.62 ± 1.87	44.83 ± 7.34	48.33 ± 0.92	71.02 ± 1.27
Alanine	90.16 ± 3.23	87.94 ± 3.00	64.28 ± 10.00	69.95 ± 1.50	259.43 ± 4.29
Cystine	1.73 ± 0.05	n.d.	15.75 ± 2.16	17.74 ± 0.28	5.49 ± 0.98
Valine	1.16 ± 0.03	3.76 ± 0.34	11.28 ± 2.58	52.74 ± 0.85	161.69 ± 2.96
Methionine	10.06 ± 0.34	8.59 ± 0.44	17.03 ± 2.44	19.42 ± 0.72	50.38 ± 0.99
Isoleucine	9.38 ± 0.75	10.00 ± 0.38	39.30 ± 5.17	58.52 ± 11.19	139.06 ± 2.33
Leucine	14.49 ± 0.19	14.89 ± 0.48	77.92 ± 10.94	78.64 ± 1.57	249.73 ± 4.17
Tyrosine	22.22 ± 0.83	25.20 ± 0.93	79.05 ± 9.35	81.38 ± 1.88	37.89 ± 1.00
Phenylalanine	32.43 ± 0.77	35.85 ± 1.42	173.89 ± 2.43	202.74 ± 4.64	179.40 ± 2.45
Tryptophan	23.04 ± 0.87	24.12 ± 1.88	n.d.	45.00 ± 3.34	45.71 ± 0.87
Lysine	44.97 ± 1.41	45.24 ± 1.74	45.54 ± 6.16	48.56 ± 0.99	151.40 ± 2.30
NH ₃	19.94 ± 1.68	22.54 ± 0.53	346.64 ± 27.50	304.07 ± 4.97	16.47 ± 0.36
Histidine	19.83 ± 0.72	19.83 ± 0.59	26.55 ± 3.02	26.79 ± 0.58	47.40 ± 0.54
Arginine	246.50 ± 10.30	244.06 ± 7.44	52.46 ± 6.80	80.36 ± 1.85	98.57 ± 2.78
Proline	57.78 ± 2.10	54.72 ± 2.67	81.55 ± 11.54	83.93 ± 2.10	57.33 ± 1.08
Σ Free amino acids & NH ₃	862.05 ± 36.22	851.64 ± 27.77	1696.87 ± 186.93	2065.24 ± 58.10	2439.2 ± 39.66
Σ N from free amino acids & NH ₃	168.21 ± 7.94	167.55 ± 5.41	458.96 ± 42.96	466.70 ± 10.09	330.30 ± 5.60

Organic acids & furans (g/L)					
Succinic acid	1.09 ± 0.20	1.01 ± 0.03	1.72 ± 0.27	1.79 ± 0.03	0.62 ± 0.08
Formic acid	n.d.	0.13 ± 0.00	0.46 ± 0.03	0.13 ± 0.02	0.11 ± 0.03
Citric acid	5.84 ± 0.05	4.75 ± 0.06	n.d.	0.66 ± 0.05	n.d.
Acetic acid	n.d.	n.d.	0.70 ± 0.16	0.82 ± 0.01	n.d.
Furfural	n.d.	n.d.	0.59 ± 0.06	0.38 ± 0.00	n.d.
5-Hydroxymethylfurfural	n.d.	n.d.	0.35 ± 0.05	0.29 ± 0.00	n.d.
Elements (mg/L)					
Boron	3.21 ± 0.40	3.21 ± 0.39	n.d.	n.d.	19.83 ± 2.07
Calcium	552.67 ± 47.51	571.00 ± 7.00	1053.00 ± 188.41	1323.33 ± 23.09	13.77 ± 1.66
Iron	5.38 ± 1.15	6.51 ± 2.38	4.80 ± 0.36	6.68 ± 0.06	n.d.
Potassium	2960.00 ± 30.00	3023.33 ± 80.21	110.80 ± 10.57	598.67 ± 10.02	337.67 ± 53.67
Magnesium	301.67 ± 3.79	267.33 ± 19.50	385.67 ± 40.15	376.00 ± 4.92	6.57 ± 1.29
Sodium	13.31 ± 0.43	1480.00 ± 26.46	5.86 ± 1.34	212.17 ± 2.89	n.d.
Phosphorous	252.67 ± 2.08	238.00 ± 3.61	351.00 ± 37.99	296.67 ± 24.54	291.33 ± 61.04
Sulphur	697.00 ± 39.89	684.00 ± 18.52	1060.00 ± 45.83	1055.67 ± 101.62	30.10 ± 6.93
Selenium	3.20 ± 0.42	4.11 ± 1.50	5.83 ± 1.89	n.d.	n.d.
Silicon	2.73 ± 0.45	6.54 ± 0.94	23.11 ± 3.89	2.73 ± 0.45	6.54 ± 0.94

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- 1

Optimal SW hydrolysis parameters: 95 °C, 30 min, and 0.1 M HCl.
- 2

Optimal BSG hydrolysis parameters: 130 °C, 36 min, and 0.2 M H₂SO₄.
- 3

n.d. Not detected.

[00110] Table 8 shows that mild acid hydrolysis was successful in releasing large amounts of assimilable hexoses from unassimilable di-, oligo- and polysaccharides in both SW and BSG. For SW, increasing the aliquot size did not cause differences in maximum glucose and fructose recoveries (10-mL: 5.15 ± 0.75 g/L glucose, 7.02 ± 1.10 g/L fructose; 100-mL: 4.71 ± 0.51 g/L glucose; 6.65 ± 0.03 g/L fructose). Also notable for SW was complete sucrose hydrolysis, which would yield equimolar amounts of glucose and fructose. Yet, fructose was in excess of glucose, indicating that the α -Glc-(1 $\rightarrow$ 2)- β -Fru glycosidic linkage was also cleaved within stachyose (α -Gal-(1 $\rightarrow$ 6)- α -Gal-(1 $\rightarrow$ 6)- α -Glc-(1 $\rightarrow$ 2)- β -Fru) and raffinose (α -Gal-(1 $\rightarrow$ 6)- α -Glc-(1 $\rightarrow$ 2)- β -Fru) (Rehms & Barz, 1995). Although stachyose and raffinose were not quantified here, their concentrations typical in SW (5.48-6.40 g/L and 0.30-1.80 g/L respectively) are a sizeable source of fructose (Chua & Liu, 2019).

[00111] For BSG, a remarkably high glucose concentration (54.02 ± 5.88 g/L) was achieved in the hydrolysate due to an abundance of starch ($34.93 \pm 0.54\%$ dm) which was completely hydrolysed (Table 7). Such a high concentration is remarkable despite glucose losses (from 67.20 ± 1.39 to 54.02 ± 5.88 g/L) after substituting NaOH with $\text{Ca}(\text{OH})_2$ as the neutralising agent, possibly due to the binding of Ca^{2+} with the ionic intermediates of sugars at increasing pH values. With regards to the solubilisation of other polysaccharides, glucose recovery from cellulose was negligible due to harsher hydrolysis conditions required (Table 7). Conversely, hemicellulose solubilisation occurred more readily (95.3 % solubilisation; Table 7), which led to the release of xylose and arabinose (without glucose release). Also noteworthy is that the hydrolysis treatment liberated appreciable amounts of free amino acids and NH_3 from proteins, as well as minerals and phosphorus (Tables 7 and 8), which could replace the exogenous mineral salts and yeast extract in BBM.

[00112] Potentially inhibitory compounds (e.g., furfural, 5-HMF, acetic and formic acids) were more extensively liberated in BSG hydrolysate compared to SW (Table 8). Furans and formic acid are derived from sugar degradation owing to the harsher hydrolysis treatment applied to BSG, while acetic acid is derived from the acetyl groups located on hemicellulose chains. In excess amounts, these inhibitory compounds may generate ROS that impact cell membrane stability and impair biomass yields. Therefore, it was hypothesised that by blending BSG hydrolysate with SW hydrolysate, better growth parameters could be attained due to the

former's role as a glucose-rich source to support biomass production, and the latter's role as a diluent to reduce the concentration of inhibitory/non-preferred compounds from BSG, besides complementing other nutrients.

[00113] **Example 4 - Heterotrophic cultivation of *A. protothecoides* in mixtures of soy whey and brewer's spent grain hydrolysates**

[00114] To determine the optimal SW-BSG mix that could best support *A. protothecoides* growth, SW hydrolysate and BSG hydrolysate were blended in different ratios and their effects on maximum biomass concentration ($X_{\max}$) and productivity ($P_{X_{\max}}$) were obtained and shown in FIGS. 4A to 4C. Although growth was observed in all SW-BSG blends, $X_{\max}$ and $P_{X_{\max}}$ differed across ratios. By increasing the proportion of BSG hydrolysate, $X_{\max}$ values increased correspondingly, reaching 21.92 ± 0.31 g/L in 0% SW-100% BSG (FIGS. 4A and 4B). In contrast, increasing BSG proportion led to enhancement in $P_{X_{\max}}$ values only up to 25% SW-75% BSG (6.07 ± 0.10 g/L/day), before decreasing to 5.23 ± 0.06 g/L/day in 0% SW-100% BSG (FIGS. 4A and 4C). Such observations indicate that by increasing the BSG hydrolysate proportion, elevations in glucose levels promoted biomass production, while increments in inhibitory compounds or glucose inhibition possibly inhibited biomass productivity (Table 8). Therefore, to maximise both $X_{\max}$ and $P_{X_{\max}}$, blending BSG hydrolysate and SW hydrolysate together in an optimal proportion would be necessary.

[00115] With the goal of maximising both $X_{\max}$ and $P_{X_{\max}}$, quartic and quadratic models with good model fit and predictability were generated from the simplex lattice design in FIGS. 4B and 4C, and the equations were used to predict the optimal hydrolysate mix ratio that would achieve this goal. A ratio of 15% SW-85% BSG was determined, with predicted $X_{\max}$ and $P_{X_{\max}}$ values of 21.41 g/L and 5.84 g/L/day, respectively. Following validation with triplicates, the actual $X_{\max}$ was 22.17 ± 0.98 g/L, which fell within the 95% prediction interval. Productivities suffered with increasing BSG hydrolysate ratios beyond 60% BSG, possibly due to the amount of inhibitory compounds present. However, the actual $P_{X_{\max}}$ exceeded the 95% prediction interval and reached a higher-than-expected value of 7.06 ± 0.33 g/L/day, possibly due to inconsistencies in pH control owing to manual adjustment daily with 2 M NaOH.

[00116] **Example 5 - Growth of *A. protothecoides* in optimal mixed hydrolysate medium vs. BBM**

[00117] To assess whether the optimal SW-BSG hydrolysate medium could wholly replace the conventional BBM, *A. protothecoides* was cultivated in BBM (50.44 g/L glucose and 6.7

g/L yeast extract added) and the growth performance was compared to that in the 15% SW-85% BSG hydrolysate medium (50.75 g/L and 1.71 g/L endogenous glucose and fructose respectively). The growth performance observed in each medium is illustrated in FIG. 5A and Table 9.

[00118] Despite containing comparable amounts of assimilable sugars (glucose + fructose), cultivations in BBM led to slightly weaker $X_{\max}$, $P_{X_{\max}}$, and $Y_{X/S}$ values compared to the SW-BSG hydrolysate medium (Table 9). This suggests that the mixed hydrolysate medium is capable of wholly replacing conventional BBM, attributed to the abundance of endogenous assimilable sugars, free amino acids and NH_3 , phosphorous, and other minerals within the mixed hydrolysate medium (Table 8). Such results are remarkable, considering that no researchers have attained similar $X_{\max}$, $P_{X_{\max}}$, and $Y_{X/S}$ values in batch mode flask cultivations of heterotrophic *A. protothecoides* in other single food side-streams. For example, in either expired fruit and vegetable juices, hydrolysed carrot pomace, whey permeate, or waste molasses containing about 30 g/L endogenous reducing sugars each, $X_{\max}$, $P_{X_{\max}}$, and $Y_{X/S}$ values obtained were 9.10-14.50 g/L, 1.01-2.90 g/L/day and 0.29-0.48 g biomass/g carbon substrate respectively, compared to the mixed hydrolysate medium in our study ($X_{\max}$: 22.17 g/L, $P_{X_{\max}}$: 7.06 g/L/day, $Y_{X/S}$: 0.41 g biomass/g utilised sugar). Although a lower reducing sugar content of 30 g/L might have contributed to weaker $X_{\max}$ in the abovementioned studies, superior $P_{X_{\max}}$ and $Y_{X/S}$ values achieved here suggest the mixed hydrolysate medium might offer a more complete nutritional profile than single food side-streams.

[00119] Table 9. Growth parameters and biomass compositions of *A. protothecoides* cultivated in 15% SW-85% BSG hydrolysate medium and Bold's basal medium (BBM).

	Mixed hydrolysate medium	BBM
$X_{\max}$ (g/L)	22.17 ± 0.98	20.02 ± 0.39
$P_{X_{\max}}$ (g/L/day)	7.06 ± 0.33	6.34 ± 0.13
$Y_{X/S}$ (g biomass/g utilised sugar)	0.41 ± 0.02	0.39 ± 0.01
Protein (% dry mass)	15.17 ± 0.93	9.86 ± 0.54
Lipid (% dry mass)	26.56 ± 1.91	36.43 ± 2.61

$X_{\max}$: maximum biomass concentration

$P_{X_{\max}}$: biomass productivity

$Y_{X/S}$: biomass yield coefficient

[00120] **Example 6 - Nutrient consumption patterns of *A. protothecoides* in optimal mixed hydrolysate medium vs. BBM**

[00121] To understand how endogenous nutrients in the mixed hydrolysate medium supported *A. protothecoides* growth as compared to BBM, and to guide future nutrient manipulations to produce targeted biomolecules (e.g., proteins, lipids), nutrient consumption patterns were further examined which are presented in FIGS. 5B to 5H.

[00122] **6.1 Sugars**

[00123] For glucose that was present in equivalent amounts in both BBM and the mixed hydrolysate medium, its consumption by *A. protothecoides* followed a similar trend, with depletions observed by day 3 (FIG. 5B). For fructose, which was only present in the mixed hydrolysate medium, its consumption was initiated only on day 2 when glucose was largely consumed, implying a preference for glucose over fructose. Fructose utilisation by *A. protothecoides* for growth has been documented, and its absence in BBM may explain why slightly higher X_{max} values were observed in the mixed hydrolysate medium (Table 9). For xylose and arabinose that were present only in the mixed hydrolysate medium (FIG. 5C), their consumption by *A. protothecoides* was only evident on day 4 when glucose and fructose were depleted. This suggests that the pentoses are potential albeit less preferred carbon sources for *A. protothecoides*, consistent with others who demonstrated less preferential xylose and arabinose uptake by the microalgal species via: xylose $\rightarrow$ xylitol $\rightarrow$ xylulose $\rightarrow$ xylulose-5-phosphate $\rightarrow$ pentose phosphate pathway (PPP); and arabinose $\rightarrow$ xylulose-5-phosphate $\rightarrow$ PPP (Chen et al., 2019; Mu et al., 2015). Noteworthy is that despite xylose assimilation on day 4, growth was not observed, possibly because xylose requires more energy (e.g., ATP) and reducing power (e.g., NADPH) for entry into the PPP (Chen et al., 2019). Overall, these sugar consumption patterns indicate the diauxic sugar uptake by *A. protothecoides* in order of preference: glucose>fructose>xylose and arabinose.

[00124] **6.2 Total amino acids and NH_3**

[00125] Total amino acids and NH_3 were depleted in BBM and the mixed hydrolysate medium by day 1 and 3, respectively, with their respective total nitrogen (N) following a similar trend (FIG. 5D). This indicates that amino acids and NH_3 — intrinsically present in the mixed hydrolysate medium and extrinsically supplied to BBM by yeast extract – were effectively assimilated by *A. protothecoides*.

[00126] While total amino acids were present in higher amounts in BBM than in the mixed

hydrolysate medium, the latter medium contained a higher quantity of total N arising from an abundance of NH_3 (FIG. 5E). Evidently, NH_3 was a preferred N source for *A. protothecoides* as it was rapidly depleted by day 2 in the mixed hydrolysate medium compared to amino acids (FIGS 8A-8H). In the genome sequence of *A. protothecoides* (NCBI accession SRA115225), NH_3 is converted into glutamate via α -ketoglutarate and glutamate dehydrogenase (NADP+; activity is usually enhanced in heterotrophic conditions) or glutamine synthetase, before being further metabolised into other amino acids (e.g., aspartic acid) via transamination for subsequent protein synthesis (Gao et al., 2014; Hellebust & Ahmad, 1989; Perez-Garcia et al., 2011). Unlike NH_3 which is directly absorbed by an ammonium transporter, amino acids usually require an additional extracellular deamination step via L-amino acid oxidase, to liberate NH_3 for absorption by microalgae (Murphree et al., 2017). This makes NH_3 an energetically efficient N source for protein biosynthesis, which may explain why the mixed hydrolysate medium enabled greater biomass protein accumulation (15.17% dm) compared to BBM (9.86% dm; Table 9).

[00127] Arginine was another preferred N source for *A. protothecoides*, being depleted by day 1 in both the mixed hydrolysate medium and BBM (FIG. 5E), compared to other amino acids which were only fully consumed on day 2 or 3 in the mixed hydrolysate medium (FIGS. 8A-8H). This preference could be due to the presence of a high affinity arginine-specific carrier which has been demonstrated in several other microalgal species such as *Chlamydomonas reinhardtii* and *Chlorella pyrenoidosa* (Kirk & Kirk, 1978). Once absorbed, arginine is deaminated to form ammonium by arginine deiminase, or converted to nitric oxide and citrulline for further incorporation into the tricarboxylic acid (TCA) cycle.

[00128] 6.3 Phosphorous

[00129] As phosphorous is crucial for building cellular components such as nucleotides and phospholipids, its consumption patterns were monitored in FIG. 5F. Phosphorous was present in similar amounts in both the mixed hydrolysate medium and BBM, and it was rapidly consumed by the first day of cultivation. The rapid consumption could result from two different simultaneous processes – adsorption onto extracellular polymeric substances and incorporation into intracellular components, as observed in heterotrophic *Synechocystis* sp. (Zhou et al., 2019). The rapid uptake also suggests that phosphorous is limiting in the mixed hydrolysate medium compared to carbon and nitrogen sources, although further work is required to validate this as microalgae are capable of storing excess phosphorous in the form of polyphosphate

vacuoles NH_3 . Also noteworthy, phosphorous in both media was not depleted, possibly due to the presence of unassimilable reduced chemical forms of phosphorous such as phosphite and hydrophosphite.

[00130] 6.4 Organic acids and furans

[00131] Acetic acid that was present only in the mixed hydrolysate medium was rapidly depleted by day 1 (FIG. 5G), due to conversion by *A. protothecoides* into acetyl-CoA, before entering the glyoxylate or TCA cycles to generate ATP and NADH. Being present only in the mixed hydrolysate medium and not BBM, acetic acid could provide additional ATP and NADH for growth, thereby contributing to higher X_{max} and $P_{X_{\text{max}}}$ values in the mixed hydrolysate medium (Table 9). Separately, levels of succinic acid increased in both media throughout cultivation (FIG. 5G), potentially as products of the glyoxylate and TCA cycles.

[00132] Despite being potential inhibitory compounds, furfural and 5-HMF present in the mixed hydrolysate medium were depleted by day 1 of cultivation (FIG. 5H). This phenomenon could be a result of furfural degradation into formic acid and 5-HMF degradation into formic and levulinic acids, supported by the concurrent spike in formic acid concentration in the mixed hydrolysate medium on day 1. After the initial spike, formic acid concentrations in the mixed hydrolysate medium and BBM decreased during cultivation, possibly due to decomposition into molecular hydrogen and carbon dioxide by formate hydrogen lyase, as demonstrated in bacteria and fungi (Ciani et al., 2008; Ward, 2015). Therefore, while potential inhibitory compounds were present in the mixed hydrolysate medium, they may be inconsequential due to rapid degradation, thereby enabling excellent *A. protothecoides* growth in the mixed hydrolysate medium.

[00133] **Example 7 - Enhancing biomass protein content cultivated in BSG-SW mixed hydrolysate medium via yeast extract supplementation**

[00134] The 15% SW-85% BSG mixed hydrolysate medium was used as the basal media, and varying amounts of monopotassium phosphate (KH_2PO_4) (0.17-1 g/L; Sigma Aldrich, Missouri, USA) and yeast extract (4-20 g/L) were added according to a central composite design (CCD, generated by Design-Expert) comprising of 13 runs (4 axial, 4 factorial, and 5 centre points). KH_2PO_4 was initially used here due to anticipated phosphate depletion (FIG. 5F), while yeast extract was added as it is a source of organic nitrogen for enhancing biomass protein content. Prior to microalgal cultivation, the hydrolysates were adjusted to pH 6.5 with 2 M NaOH. Thereafter, *A. protothecoides* was cultivated, with approximately 1 g/L initial

inoculum, at 25 °C, 210 rpm, and shake throw 26 mm. pH was adjusted daily to 6 to 7 using 2 M NaOH, and biomass measurements were taken daily. For each of the 13 runs, a single replicate was conducted.

[00135] Using the CCD, 20 g/L yeast extract without KH₂PO₄ supplementation was chosen to obtain maximal protein yield and productivity. The final formulation, 15% SW-85% BSG mixed hydrolysate medium spiked with 20 g/L yeast extract, was then validated in triplicate independent batches of cultivations. As comparisons, triplicate cultivations in hydrolysates without any supplementation, as well as BBM supplemented with 48 g/L glucose and 24.33 g/L yeast extract (estimated to contain equivalent assimilable hexose and total nitrogen to 15% SW-85% BSG mixed hydrolysate medium) were also conducted in parallel. Biomass measurements and pH adjustments to 6 to 7 were conducted daily. Biomasses were harvested on day 4 of cultivation, and lipid content was measured using the Bligh-Dyer method while protein content was measured using the Kjeldahl method, with 6.25 as the nitrogen to protein conversion factor.

[00136] To demonstrate the flexibility of the 15% SW-85% BSG mixed hydrolysate medium for use as a basal media, experiments were carried out to enhance *A. protothecoides* biomass protein yields and productivities using yeast extract as a supplement.

[00137] The effect of varying amounts of KH₂PO₄ and yeast extract on *A. protothecoides* growth, protein contents, yields, and productivities are displayed in FIGS. 7A to 7D. Linear and quadratic models obtained for each response are as follows:

[00138] Protein content (% dry mass) = 19.833 – 2.73182A + 1.79596B – 0.0262959AB + 2.13675A² – 0.0286714B² (R² = 0.9799; R² adjusted = 0.9656; R² predicted = 0.9306, lack of fit = 0.6407) ----- (3)

[00139] Protein yield (g/L) = 4.87759 – 0.840551A + 0.216814B (R² = 0.9797; R² adjusted = 0.9757; R² predicted = 0.9710, lack of fit = 0.8534) ----- (4)

[00140] Protein productivity (g/L/day) = 1.16544 – 0.208826B + 0.0515852A (R² = 0.9786; R² adjusted = 0.9743; R² predicted = 0.9704, lack of fit = 0.9078) ----- (5)

[00141] From FIG. 7A, biomass yields and productivities remained unaffected regardless of KH₂PO₄ and yeast extract additions. Conversely, protein content, protein yield, and protein productivity were influenced by KH₂PO₄ and yeast extract additions as shown in FIGS. 7B, 7C, 7D and Equations 3 to 5. KH₂PO₄ had a slightly negative effect, while yeast extract (up to 20 g/L) had a strongly positive effect on biomass protein content, protein yield and productivity.

Therefore, subsequent protein-centric cultivations omitted KH_2PO_4 , while using 20 g/L yeast extract as a supplement.

[00142] Notably, excessive yeast extract dosages should be avoided as dosages beyond 20 g/L led to minimal increases in protein content (quadratic Equation 3 and FIG. 7B). Indeed, preliminary data indicated that protein yield and protein productivity were similar at a dosage level of 30 g/L (8.56 g/L and 2.14 g/L/day respectively) compared to 20 g/L yeast extract (8.52 g/L and 2.13 g/L/day respectively).

[00143] FIGS. 6A to 6H and Table 10 show the growth, protein contents, yields, and productivities of *A. protothecoides* when cultivated in 15% SW-85% BSG mixed hydrolysate medium (H), 15%-85% BSG mixed hydrolysate medium supplemented with 20 g/L yeast extract (H+20YE), and BBM spiked with 48 g/L glucose and 24.33 g/L yeast extract (BBM+G+YE). The results show that supplementation with 20 g/L yeast extract enhanced biomass protein content from $19.71 \pm 0.58\%$ (H) to $41.45 \pm 1.71\%$ (H+20YE), without compromising biomass yield and productivity. Therefore, we prove that the 15% SW-85% BSG mixed hydrolysate medium can be used as a basal medium for supplementation with nitrogen sources if higher biomass protein contents are desired.

[00144] Table 10. Yields, productivities, and protein content of *A. protothecoides* cultivated on hydrolysate (H: 15% SW, 85% BSG), hydrolysate spiked with 20 g/L yeast extract (H+20YE) and BBM spiked with 48 g/L glucose and 24.33 g/L yeast extract (BBM+G+YE).

	H	H+20YE	BBM+G+YE
$X_{\max}$ (g/L)	$20.42 \pm 0.63\text{b}$	$20.56 \pm 1.15\text{b}$	$17.86 \pm 0.69\text{a}$
$P_{X\max}$ (g/L/day)	$4.87 \pm 0.16\text{b}$	$4.91 \pm 0.28\text{b}$	$4.23 \pm 0.17\text{a}$
$Y_{x/s}$ (g biomass/g glucose)	0.40 ± 0.01	0.61 ± 0.06	0.42 ± 0.09
Protein (% dry mass)	$19.71 \pm 0.58\text{a}$	$41.45 \pm 1.71\text{b}$	$42.17 \pm 0.79\text{b}$
Protein yield (g/L)	$4.02 \pm 0.06\text{a}$	$8.52 \pm 0.13\text{c}$	$7.53 \pm 0.21\text{b}$
Protein productivity (g/L/day)	$1.01 \pm 0.01\text{a}$	$2.13 \pm 0.03\text{c}$	$1.88 \pm 0.05\text{b}$
Lipid (% dry mass)	$27.97 \pm 0.78\text{c}$	$19.54 \pm 0.63\text{b}$	$16.86 \pm 3.17\text{a}$

Biomass was harvested on day 4 for lipid and protein measurements. Values are expressed as the mean of triplicate independent experiments $\pm$ SD.

Different lowercase letters in a row indicate statistical differences ($P < 0.05$) between different treatments. H contains 48 g/L glucose and 1451.67 mg/L total nitrogen. H+20YE contains 48 g/L glucose and 3040.12 mg/L total nitrogen. BBM+G+YE contains 48 g/L glucose and 2703.18 mg/L total nitrogen.

[00145] It is noteworthy that the protein yield and protein productivity obtained when *A. protothecoides* was cultivated in H+20YE (8.52 ± 0.13 g/L and 2.13 ± 0.03 g/L/day

respectively) were significantly better than that in BBM+G+YE (7.53 ± 0.21 g/L and 1.88 ± 0.05 g/L/day respectively). This is despite similar glucose and total nitrogen contents between the two different cultivation media (Table 10). Hence, hydrolysate (H: 15% SW, 85% BSG) is better than BBM for use as basal media, as the former only requires about 82% of yeast extract to achieve significantly higher protein yields and productivities. Raw material costs arising from nitrogen supplementation are thus lowered, thereby improving economic viability.

[00146] **Example 8 - Overall mass balance for *A. protothecoides* cultivation**

[00147] To summarise the input, output, and losses in the entire *A. protothecoides* biomass production process using SW and BSG mixed hydrolysate medium, a mass balance is computed and shown in FIG. 9 in accordance with an exemplary embodiment of the present disclosure. The dilute acid treatments for SW (95 °C, 30 min, 0.1 M HCl) and BSG (130 °C, 36 min, 0.2 M H₂SO₄) followed by their subsequent mixing (15% SW-85% BSG) yielded a sugar-rich hydrolysate medium (94.08% overall glucose recovery). Aided by design of experiments, the dilute acid treatment also minimised raw material costs (HCl, H₂SO₄) and the production of inhibitory compounds and non-preferred substrates (e.g., furans, 95.3% overall pentoses recovery). When *A. protothecoides* was cultivated in the mixed hydrolysate medium, glucose and fructose were completely assimilated, while 96.08% nitrogen (from amino acids and NH₃) and 85.75% of phosphorous were consumed, yielding an excellent maximum biomass concentration of 22.17 g/L comprising of 15.17 and 26.56% dm protein and lipid contents respectively. Therefore, the present disclosure has successfully demonstrated the use of industrial food side-streams as the sole source of glucose, nitrogen, phosphorous and minerals to heterotrophically cultivate microalgae such as *A. protothecoides*, while achieving excellent biomass production.

[00148] **Example 9 - Cost estimation for heterotrophic microalgae cultivation**

[00149] To assess if wholly replacing glucose and BBM with the mixed hydrolysate medium was economically viable, cost estimates to produce dry *A. protothecoides* biomass in mixed hydrolysate medium vs. BBM is shown in Table 11. Considering raw material costs alone, *A. protothecoides* cultivated in mixed hydrolysate medium amounted to 0.15 USD/kg biomass, in contrast to 3.42 USD/kg biomass for BBM, representing a 96% cost reduction (Table 11). Such a remarkable cost reduction was possible as exogenous glucose (1.17 USD/kg dry biomass in BBM) and yeast extract (2.22 USD/kg dry biomass in BBM) were not required in the hydrolysate medium.

[00150] Table 11. Raw material cost to produce 100 kg of dry *A. protothecoides* biomass

using 15% SW-85% BSG hydrolysate medium vs. Bold's Basal Medium (BBM).

Parameter	Unit price (USD/kg dried raw material) [#]	Mass to produce 100 L of medium (g)		Raw material costs to produce 100 kg of <i>A. protothecoides</i> (USD/100 kg)	
		Mixed hydrolysate medium	BBM	Mixed hydrolysate medium	BBM
Ca(OH) ₂	0.14 ¹	1944.00	-	12.28	-
NaOH	0.11 ²	56.00	-	0.28	-
HCl	0.02 ²	55.00	-	0.04	-
H ₂ SO ₄	0.03 ²	1667.00	1.84	2.57	0.00
Glucose	0.49 ¹	-	4800.00	-	117.48
Yeast extract	6.63 ¹	-	670.00	-	221.88
CaCl ₂ ·2H ₂ O	0.15 ¹	-	2.50	-	0.02
MgSO ₄ · 7H ₂ O	0.08 ¹	-	7.50	-	0.03
K ₂ HPO ₄	1.00 ¹	-	7.50	-	0.37
KH ₂ PO ₄	1.00 ¹	-	17.50	-	0.87
NaCl	0.03 ¹	-	2.50	-	0.00
H ₃ BO ₃	1.07 ¹	-	11.40	-	0.61
EDTA	2.00 ¹	-	5.00	-	0.50
KOH	0.82 ¹	-	3.01	-	0.12
FeSO ₄ ·7H ₂ O	0.80 ¹	-	0.50	-	0.02
ZnSO ₄ ·7H ₂ O	0.54 ¹	-	0.88	-	0.02
MnCl ₂ ·4H ₂ O	0.50 ¹	-	0.14	-	0.00
MoO ₃	22.00 ¹	-	0.07	-	0.08
CuSO ₄ ·5H ₂ O	2.00 ¹	-	0.16	-	0.02
Co(NO ₃) ₂ ·6H ₂ O	8.00 ¹	-	0.05	-	0.02
Σ Imported raw material		3722.00	5530.55	15.16	342.06

Costs of SW and BSG were assumed to be zero.

[#] Unit price was determined based on the purchase/transport of 1 tonne of raw material.

¹: Alibaba.com (Retrieved on 28th May 2024).

²: Sunsirs.com (Retrieved on 28th May 2024).

EDTA: Ethylenediaminetetraacetic acid.

[00151] The mixed hydrolysate medium developed in the present disclosure is the first step to produce resource-efficient, protein-rich or lipid-rich microalgal biomass capable of bolstering food security. Diauxic effects observed in Example 6 reveal the preferred carbon and nitrogen substrates to boost biomass lipid and protein contents, respectively. For example, hydrolysed molasses containing preferred carbon sources such as glucose and fructose could be added into the mixed hydrolysate medium to improve lipid content (FIG. 5B) (Yan et al., 2011).

To enhance protein content, preferred nitrogen sources such as arginine or ammonium (FIG. 4E) could be added.

[00152] **Example 10 – Validating *A. protothecoides* growth and biomass composition when cultivated in new batches of BSG and SW hydrolysates**

[00153] Batch-to-batch variations in industrial food side-streams may result in variable nutrient compositions in hydrolysates and consequently, inconsistencies in biomass yields and protein contents.

[00154] Soy whey was collected from another local food manufacturer. Two batches of BSG (batch 1 and batch 2) were obtained from the same local manufacturer at different times, batch 2 was obtained two months later after batch 1. BSG from both batches were hydrolysed in a similar manner according to Example 1.2 and mixed in a 1:1 proportion. SW was similarly hydrolysed according to Example 1.1 and mixed with the BSG hydrolysate in a 25:75 ratio to maintain about 50 g/L glucose concentration. The 25%-75% SW-BSG hydrolysate mix was then spiked with 20 g/L yeast extract according to Example 7 to maintain about 1,333 mg/L N (H+20YE; from free amino acids and NH₃). As comparisons, cultivations were also conducted, in parallel, in hydrolysates without any supplementation (H), as well as BBM supplemented with 48 g/L glucose and 30 g/L yeast extract (BBM+G+YE; estimated to contain equivalent assimilable hexose and assimilable nitrogen).

[00155] *A. protothecoides* cultivations were conducted in triplicate, with about 1 g/L initial inoculum, at 25 °C, 210 rpm, and shake throw 26 mm. pH was adjusted daily to 6-7 using 2 M NaOH, and biomass measurements were taken daily. Biomasses were harvested on days 4 and 5 of cultivation, with lipid and protein contents analyses and time-course nutrient consumption patterns determined. The amino acid composition of the biomass was quantified via 6 N HCl hydrolysis, except for tryptophan which was quantified using 4 N methanesulfonic acid hydrolysis.

[00156] Table 12 shows the composition of new batches of SW and BSG hydrolysates, while growth parameters of *A. protothecoides* in the hydrolysate mix, together with nutrient consumption patterns, are shown in FIGS. 10A-10H and Table 13. Notably, glucose concentrations were higher in the new batches of BSG, hence the proportion of SW was increased to 25% SW-75% BSG to maintain about 50 g/L glucose concentration (46.79 g/L; FIG. 10C), consistent with the earlier hydrolysate batch 1 (50.13 g/L; FIG. 6C). Besides glucose, there was a 30% difference in other macronutrients between the previous and new

batches of hydrolysates, such as the sum N from free amino acids (518.95 mg/L in FIG. 10G vs 404.61 mg/L in FIG. 6G) and phosphate levels (0.16 g/L in FIG. 10H vs 0.22 g/L in FIG. 6H). Nevertheless, growth kinetics and biomass composition for *A. protothecoides* were still comparable when grown in previous and new batches of hydrolysates (H; Tables 10 and 13).

[00157] Consistent with the old batch of 15% SW-85% BSG mix (batch 1), protein yield and protein productivity obtained in the new batch of 25% SW-75% BSG mix (batch 2) spiked with 20 g/L yeast extract (H+20YE) were greater than that in BBM+G+YE. This is despite similar glucose and total free amino nitrogen contents between the two media (FIGS. 10A – 10H). As the new hydrolysate mix requires only about 67% of yeast extract to achieve a superior protein yield and productivity, there is potential to replace BBM with the SW and BSG hydrolysate mix as a basal medium for nitrogen supplementation.

[00158] Table 12. Sugar, organic acid, furan, amino acid, ammonium, phosphate, and mineral compositions of new batches of soy whey and brewer's spent grain (BSG) hydrolysates.

	Soy whey hydrolysate	BSG hydrolysate (Batch 1)	BSG hydrolysate (Batch 2)
<i>Sugars (g/L)</i>			
Glucose	2.44 ± 0.04	58.33 ± 0.44	73.53 ± 11.99
Fructose	3.40 ± 0.02	0.74 ± 0.06	0.81 ± 0.15
Xylose		16.06 ± 0.32	13.22 ± 0.12
Arabinose		8.42 ± 0.60	7.22 ± 0.11
Galactose		1.43 ± 0.02	0.74 ± 0.03
<i>Organic acids & furans (g/L)</i>			
Succinic acid	0.16 ± 0.01	1.40 ± 0.02	2.58 ± 0.42
Formic acid	n.d.	0.26 ± 0.02	0.31 ± 0.05
Citric acid	1.57 ± 0.03	n.d.	n.d.
Acetic acid	n.d.	0.65 ± 0.04	0.62 ± 0.03
Furfural	n.d.	0.44 ± 0.00	1.00 ± 0.27
HMF	n.d.	0.26 ± 0.01	0.26 ± 0.01
<i>Amino acids (mg/L)</i>			
Aspartic acid	9.76 ± 0.24	603.05 ± 25.51	619.79 ± 33.46
Threonine	5.94 ± 0.08	27.93 ± 0.84	53.60 ± 6.91
Serine	4.97 ± 0.01	41.17 ± 1.52	68.39 ± 7.54
Glutamic acid	58.95 ± 0.65	105.71 ± 5.62	175.08 ± 21.43
Glycine	13.50 ± 0.49	43.97 ± 3.00	59.19 ± 5.73
Alanine	21.44 ± 0.44	57.37 ± 2.20	107.18 ± 12.28
Cystine	n.d.	11.59 ± 0.59	22.53 ± 1.10
Valine	5.32 ± 0.17	43.46 ± 1.41	99.08 ± 12.63
Methionine	2.16 ± 0.02	12.50 ± 0.47	36.93 ± 3.59
Isoleucine	3.31 ± 0.11	39.81 ± 2.60	97.82 ± 9.04
Leucine	5.27 ± 0.63	74.50 ± 2.60	147.30 ± 18.18

Tyrosine	5.69 ± 1.43	100.61 ± 2.88	98.52 ± 15.05
Phenylalanine	13.81 ± 2.52	193.85 ± 6.42	255.38 ± 18.81
Tryptophan	12.60 ± 0.14	12.41 ± 4.84	25.38 ± 0.79
Lysine	14.66 ± 0.10	43.20 ± 0.62	91.88 ± 10.85
NH ₃	5.93 ± 0.01	394.06 ± 12.9	364.14 ± 19.38
Histidine	5.48 ± 0.20	24.20 ± 0.00	45.73 ± 4.88
Arginine	36.46 ± 0.62	45.86 ± 0.68	94.24 ± 13.30
Proline	14.86 ± 0.11	87.93 ± 0.94	210.66 ± 38.63
Sum amino acids & NH ₃	240.12 ± 2.53	1963.20 ± 73.77	2672.83 ± 253.60
Sum free N from amino acids & NH ₃	41.69 ± 0.03	509.74 ± 17.53	587.49 ± 46.45
Elements (mg/L)			
Boron	1.38 ± 0.04	2.30 ± 0.00	2.28 ± 0.02
Calcium	659.50 ± 89.80	945.50 ± 4.95	1050.00 ± 28.28
Copper	1.03 ± 0.10	0.30 ± 0.01	0.29 ± 0.01
Iron	0.68 ± 0.01	1.38 ± 0.04	1.23 ± 0.13
Potassium	866.50 ± 91.22	76.35 ± 0.78	202.50 ± 41.72
Magnesium	119.50 ± 0.71	248.00 ± 22.63	264.00 ± 14.14
Manganese	0.23 ± 0.00	2.59 ± 0.21	2.67 ± 0.08
Sodium	1595.00 ± 205.06	8.77 ± 0.28	7.25 ± 0.40
Phosphorous	29.45 ± 0.49	65.45 ± 4.45	56.85 ± 1.20
Sulfur	82.40 ± 1.13	313.00 ± 18.38	327.50 ± 4.95
Silicon	2.00 ± 0.07	21.60 ± 4.67	24.90 ± 1.27
Zinc	0.72 ± 0.01	0.69 ± 0.00	0.76 ± 0.06
Others			
Phosphate (g/L)	0.08 ± 0.01	0.18 ± 0.00	0.22 ± 0.02

Values are expressed as the mean of triplicate independent experiments ± SD.

[00159] Table 13. Growth parameters and biomass composition of *A. protothecoides* cultivated in hydrolysate (H; 25% soy whey, 75% brewer's spent grain), hydrolysate spiked with 20 g/L yeast extract (H+20YE) and BBM spiked with 48 g/L glucose and 30 g/L yeast extract (BBM+G+YE).

	H	H+20YE	BBM+G+YE
X _{max} (g/L)	18.99 ± 0.91	25.06 ± 1.07	19.38 ± 3.80
PX _{max} (g/L/day)	4.53 ± 0.23	4.84 ± 0.21	3.70 ± 0.76
Y _{X/S} (g biomass/g glucose)	0.39 ± 0.01	0.57 ± 0.00	0.58 ± 0.02
Lipid content (% dry mass)	27.84 ± 1.98	21.44 ± 1.10	16.57 ± 0.74
Protein content (% dry mass)	18.07 ± 1.78	28.55 ± 0.40	32.89 ± 0.38
Protein yield (g/L)	3.59 ± 0.44	7.55 ± 0.01	6.67 ± 0.59
Protein productivity (g/L/day)	0.90 ± 0.11	1.51 ± 0.00	1.33 ± 0.12

Biomass measurements were conducted on day 4 for H, and day 5 for H+20YE and BBM+G+YE. Values are expressed as the mean of triplicate independent experiments ± SD.

[00160] To understand the nutritional quality of *A. protothecoides* biomass as a protein source, the amino acid composition of biomass protein yielded in H+20YE was determined and is shown in Table 14. While tryptophan, methionine, and histidine were present in relatively low quantities, their levels met or exceeded the FAO/WHO requirements (0.60, 1.60 and 1.50 g/100g protein respectively). Together with other essential amino acids (EAA) that were abundant, particularly lysine and leucine, an EAA of 39.46% of total protein on day 4 of harvest and 39.87% on day 5 of harvest were achieved. These figures exceed other plant proteins such as oat and soy, while also exceeding the 27.7% FAO/WHO requirement (Gorissen et al., 2018; WHO/FAO, 2007). Nevertheless, the bioavailability of *A. protothecoides* as a protein source warrants further investigation, due to the recalcitrant nature of the cell wall.

[00161] Table 14. Amino acid composition of *A. protothecoides* cultivated in 25% SW-75% BSG hydrolysate spiked with 20 g/L yeast extract.

	Biomass harvested on day 4	Biomass harvested on day 5
<i>Essential amino acids (g/100g protein)</i>		
Lysine	8.57 ± 0.72	7.18 ± 0.20
Leucine	8.94 ± 0.59	8.25 ± 0.21
Isoleucine	4.28 ± 0.33	3.51 ± 0.09
Valine	7.17 ± 0.80	5.80 ± 0.17
Threonine	6.94 ± 0.58	5.78 ± 0.23
Histidine	3.09 ± 0.25	2.64 ± 0.02
Phenylalanine	4.83 ± 0.33	4.07 ± 0.09
Methionine	1.87 ± 0.12	1.57 ± 0.07
Tryptophan	1.24 ± 0.14	1.08 ± 0.07
<i>Non-essential anhydrous amino acids (g/100g protein)</i>		
Alanine	9.48 ± 0.67	8.86 ± 0.25
Arginine	13.07 ± 1.54	10.31 ± 0.14
Aspartic acid	9.39 ± 0.64	8.72 ± 0.33
Cystine	0.65 ± 0.11	0.56 ± 0.08
Glutamic acid	14.03 ± 1.14	13.17 ± 0.63

Glycine	5.68 ± 0.39	5.35 ± 0.19
Proline	9.58 ± 0.39	6.79 ± 1.06
Serine	5.13 ± 0.27	4.86 ± 0.17
Tyrosine	4.00 ± 0.37	3.38 ± 0.09
Protein (% ; Kjeldahl K=4.78)	34.27 ± 0.87	28.81 ± 1.00
EAA (% of total protein)	39.46 ± 3.05	39.87 ± 0.96

Values are expressed as the mean of triplicate independent experiments ± SD.

[00162] Example 11 - Protein yields obtained with other organic and inorganic nitrogen sources vs yeast extract

[00163] As yeast extract is an expensive nitrogen source, other types of nitrogen sources are assessed to be an alternative supplement in the hydrolysate medium to generate high *A. protothecoides* protein yields and productivities.

[00164] The 25%-75% SW-BSG hydrolysate mix was prepared according to Example 10, prior to spiking with various nitrogen sources (about 929.02 mg/L total N from free amino acids and NH_3) which included: i) 20 g/L yeast extract, ii) 2.96 g/L arginine, iii) 3.19 g/L $(\text{NH}_4)_2\text{CO}_3$, iv) 2.39 g/L $\text{H}^+\text{NH}_4\text{H}_2\text{PO}_4$ + 3.01 g/L $(\text{NH}_4)_2\text{HPO}_4$ buffer mixture of about pH 6.5, and v) 4.38 g/L $(\text{NH}_4)_2\text{SO}_4$. The final nitrogen source was 9.16 mL of chicken carcass hydrolysate (CCH) in 90.83 mL of 15% SW-85% BSG mix (instead of 25% SW-75% BSG), to maintain ~50 g/L of glucose levels arising from dilution by CCH. CCH was prepared according to Zhang et al. (2023).

[00165] *A. protothecoides* cultivation methods were identical to Example 1.3, with about 1 g/L initial inoculum, at 25 °C, 210 rpm, and shake throw 26 mm. pH was adjusted daily to 6-7 using 2 M NaOH, and biomass measurements were taken daily. All cultivations were conducted in duplicate. Biomasses were harvested on days 4 and 5 of cultivation. Protein contents analysis was conducted with a total elemental analyser or Kjeldahl method.

[00166] FIGS. 11A to 11B and Table 15 show the growth parameters of *A. protothecoides* when cultivated in the SW-BSG hydrolysate mix spiked with various inorganic and organic nitrogen sources. It is evident that yeast extract was still the ideal nitrogen source to obtain the greatest protein yields and productivities, owing to superior X_{max} and $P_{X_{\text{max}}}$ values.

Nevertheless, inexpensive inorganic nitrogen sources or high nitrogen food side-streams (c.g., chicken carcasses) are still an option to obtain a high protein biomass, although the lowered substrate cost must be weighed against possible lowered protein yields and revenues.

[00167] Table 15. $X_{\max}$, $P_{X\max}$, and protein content of *A. protothecoides* cultivated in hydrolysate mix spiked with an equivalent of 929.02 mg/L total N of yeast extract, chicken carcass hydrolysate, arginine, $(\text{NH}_4)_2\text{CO}_3$, $\text{H}+\text{NH}_4\text{H}_2\text{PO}_4+(\text{NH}_4)_2\text{HPO}_4$, or $(\text{NH}_4)_2\text{SO}_4$.

	Yeast extract	Chicken carcass hydrolysate	Arginine*	Ammonium carbonate	Ammonium phosphate	Ammonium sulfate
$X_{\max}$	26.06 ± 0.30	20.92 ± 0.16	17.73 ± 0.96	16.40 ± 0.09	17.87 ± 0.82	15.68 ± 0.43
$P_{X\max}$ (g/L/day)	5.02 ± 0.06	3.99 ± 0.04	4.19 ± 0.25	3.09 ± 0.02	3.41 ± 0.15	2.97 ± 0.10
Protein content (% dry mass; elemental analyser)	24.58 ± 0.16	21.96 ± 0.60	26.28 ± 5.18	24.08 ± 0.04	Not determined	Not determined
Protein yield (g/L; elemental analyser)	6.41 ± 0.03	4.59 ± 0.16	4.66 ± 0.11	3.95 ± 0.30	Not determined	Not determined
Protein productivity (g/L/day; elemental analyser)	1.23 ± 0.00	0.90 ± 0.85	1.09 ± 0.15	0.75 ± 0.01	Not determined	Not determined
Protein content (% dry mass; Kjeldahl)	28.81 ± 1.00	Not determined	Not determined	Not determined	29.11 ± 0.55	32.27 ± 1.40
Protein yield (g/L; Kjeldahl)	7.55 ± 0.01	Not determined	Not determined	Not determined	5.20 ± 0.14	5.06 ± 0.22
Protein productivity (g/L/day; Kjeldahl)	1.44 ± 0.00	Not determined	Not determined	Not determined	1.04 ± 0.02	1.01 ± 0.07

*All data were obtained from day 5 corresponding to the end of exponential phase, except for arginine (day 4). Values are expressed as the mean of duplicate independent experiments ± SD.

[00168] **Example 12 - *A. protothecoides* batch cultivations in a 6.5-L stirred tank bioreactor**

[00169] To assess the growth of *A. protothecoides* under controlled conditions which are more representative of pilot and industrial scale upstream bioprocesses, batch cultivation conditions from Example 10 were scaled up from flask to a 6.5-L bioreactor (containing 3.4 L of hydrolysate).

[00170] The 25%-75% SW-BSG hydrolysate mix spiked with 20 g/L yeast extract (H+20YE) was prepared according to Example 10. Duplicate batch cultivations were conducted in a Jupiter

6.5 stirred tank bioreactor (Solaris Biotech, Mantova, Italy) containing 3.4 L of H+20YE, and equipped with 3 Rushton impellers (set at 500 rpm) and a microsparger. The temperature was set at 25 °C, while dissolved oxygen was regulated above 50% via automatic compressed air (0-0.5 NL/min) and O₂ (0-200 NmL/min) flow rates. Foaming and a pH of 6.5 were controlled via automatic additions of a 10% v/v silica-based antifoam (DOW Silicones Corporation, Michigan, USA) and 2 M NaOH respectively. During cultivations, the entire vessel was wrapped in aluminum to facilitate heterotrophy, and biomass was harvested when the in-line Excell 231 near infrared biomass sensor measuring at 850 nm with an optimal pathlength of 5 mm (Exner Process Equipment GmbH, Ettingen, Germany) started to taper off, concomitant with a rise in pH>6.50 which are indicators of late exponential/early stationary phase based on prior trials. *A. protothecoides* inoculum size was standardized to about 1 g/L, with biomass measurements at set time intervals.

[00171] FIG. 12 and Table 16 show the growth kinetics and parameters of *A. protothecoides* during batch cultivation in the 6.5-L stirred tank bioreactor containing H+20 YE. From an initial inoculum of ~ 1 g/L, productivity and specific growth rates were 10.72 g/L/day and 1.73 day⁻¹ respectively, which are 2.21 and 3.76-fold greater than in flask cultivations (4.84 g/L/day, 0.46 day⁻¹). Significantly greater growth rates achieved in the bioreactor are attributed to the maintenance of growth parameters (e.g., pH, dissolved oxygen) which was only possible in a bioreactor setup compared to that in flasks. For example, in flasks, dissolved oxygen could not be controlled while pH was adjusted manually daily which led to wide pH fluctuations (FIG. 10B)

[00172] Table 16. Growth parameters of *A. protothecoides* when cultivated in hydrolysate (H; 25% soy whey, 75% brewer's spent grain) spiked with 20 g/L yeast extract (H+20YE) in batch cultivations in flask and in a 6.5-L bioreactor.

	Cultivation mode	X _{max} (g/L)	Productivity (g/L/day)	Specific growth rate (day ⁻¹)	Doubling time (day)
Flask (500 mL) [^]	Batch	25.06 ±1.07	4.84 ± 0.21	0.46 ± 0.02	1.51 ± 0.07
Bioreactor (6.5 L) [*]	Batch	22.86 ±0.47	10.72 ± 0.88	1.73 ± 0.04	0.40 ± 0.01

[^] Data was obtained from triplicate independent cultivations derived from Example 10.

^{*} Values are expressed as the mean of duplicate independent experiments ± SD.

[00173] All examples described herein, whether of apparatus, methods, materials, or

products, are presented for the purpose of illustration and to aid understanding and are not intended to be limiting or exhaustive. Modifications may be made by one of ordinary skill in the art without departing from the scope of the invention as claimed.

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The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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CLAIMS

1. A cultivation medium comprising:
a mixed hydrolysate medium comprising 15% to 25% v/v soy whey hydrolysate and 75% to 85% v/v brewer's spent grain hydrolysate, based on the total volume of the mixed hydrolysate medium,
wherein the cultivation medium is rich in endogenous glucose, having an endogenous glucose density of 46.0 to 51.0 g/L, for supporting growth of heterotrophic microalgae.
2. The cultivation medium of claim 1, wherein the cultivation medium is rich in nitrogen, having a total nitrogen content ranging from 0.1 to 3.2 g/L.
3. The cultivation medium of claim 1, wherein the cultivation medium is rich in phosphorous, having a phosphorous content ranging from 0.1 to 0.3 g/L.
4. The cultivation medium of claim 1, wherein the mixed hydrolysate medium comprises 15% v/v soy whey hydrolysate and 85% v/v brewer's spent grain hydrolysate, based on the total volume of the mixed hydrolysate medium.
5. The cultivation medium of claim 1, wherein the mixed hydrolysate medium comprises 25% v/v soy whey hydrolysate and 75% v/v brewer's spent grain hydrolysate, based on the total volume of the mixed hydrolysate medium.
6. The cultivation medium of claim 5, wherein the cultivation medium is rich in endogenous glucose, having an endogenous glucose density of 46.79 g/L, for supporting growth of heterotrophic microalgae.
7. The cultivation medium of claim 4 or 5, wherein the cultivation medium further comprises a yeast extract as a supplementary nitrogen source for the cultivation medium.

8. The cultivation medium of claim 7, wherein the supplementary nitrogen source includes chicken carcass hydrolysate, arginine, ammonium carbonate, ammonium phosphate and ammonium sulfate.
9. The cultivation medium of claim 1, wherein the heterotrophic microalgae belong to the genus *Auxenochlorella* spp.
10. The cultivation medium of claim 9, wherein the species in the genus *Auxenochlorella* spp. is *Auxenochlorella protothecoides*.
11. A method of preparing a cultivation medium, the method comprising:
pre-treating soy whey by subjecting the soy whey to hydrolysis under conditions of heating the soy whey at a temperature of 95°C in a first acid for a first duration to obtain a soy whey hydrolysate;
pre-treating brewer's spent grain by subjecting the brewer's spent grain to hydrolysis under conditions of heating the brewer's spent grain at a temperature of 130°C in a second acid for a second duration to obtain a brewer's spent grain hydrolysate;
mixing 15% to 25% v/v soy whey hydrolysate and 75% to 85% v/v brewer's spent grain hydrolysate to obtain a mixed hydrolysate medium; and
preparing a cultivation medium comprising the mixed hydrolysate medium, wherein the cultivation medium is rich in endogenous glucose, having an endogenous glucose density of 46.0 to 51.0 g/L, for supporting growth of heterotrophic microalgae.
12. The method of claim 11, wherein the step of mixing comprises mixing 15% v/v soy whey hydrolysate and 85% v/v brewer's spent grain hydrolysate to obtain the mixed hydrolysate medium.
13. The method of claim 11, wherein the step of mixing comprises mixing 25% v/v soy whey hydrolysate and 75% v/v brewer's spent grain hydrolysate to obtain the mixed hydrolysate medium.

14. The method of claim 13, wherein the endogenous glucose density of the cultivation medium is 46.79 g/L, for supporting growth of heterotrophic microalgae.
15. The method of claim 11, wherein each of the first acid and the second acid has a concentration ranging from 0.1M to 0.2M.
16. The method of claim 11, wherein each of the first duration and the second duration ranges from 30 to 45 mins.
17. The method of claim 11, further comprising:
adding a yeast extract as a supplementary nitrogen source to the cultivation medium.
18. The method of claim 11, wherein the soy whey hydrolysate contains 2.4 to 6.0 g/L glucose and 3.4 to 8.1 g/L fructose.
19. The method of claim 11, wherein the brewer's spent grain hydrolysate contains 48.0 to 85.5 g/L glucose.
20. A method of cultivating heterotrophic microalgae comprising:
inoculating a culture of microalgae to a cultivation medium comprising a mixed hydrolysate medium containing 15% to 25% v/v soy whey hydrolysate and 75% to 85% v/v brewer's spent grain hydrolysate, wherein the cultivation medium is rich in endogenous glucose, having an endogenous glucose density of 46.0 to 51.0 g/L;
growing the culture of microalgae aerobically to obtain a biomass of heterotrophic microalgae with a biomass density of 20 to 26 g/L; and
harvesting the biomass of heterotrophic microalgae to obtain a dry biomass of heterotrophic microalgae.
21. The method of claim 20, wherein the mixed hydrolysate medium comprises 15% v/v soy whey hydrolysate and 85% v/v brewer's spent grain hydrolysate.

22. The method of claim 20, wherein the mixed hydrolysate medium comprises 25% v/v soy whey hydrolysate and 75% v/v brewer's spent grain hydrolysate.
23. The method of claim 22, wherein the mixed hydrolysate medium is supplemented with 20 g/L yeast extract, wherein the cultivation medium has an endogenous glucose density of 46.79 g/L.
24. The method of claim 20, further comprising:
harvesting the biomass of microalgae in 96-hour or 120-hour growth.
25. The method of any one of claims 20 to 22, wherein the heterotrophic microalgae belong to the genus *Auxenochlorella* spp.
26. The method of claim 25, wherein the species in the genus *Auxenochlorella* spp. is *Auxenochlorella protothecoides*.
27. The method of claim 21, wherein the biomass of heterotrophic microalgae has a dry mass protein content of 15% to 21% w/w, based on the total weight of dry biomass of heterotrophic microalgae.
28. The method of claim 22, wherein the biomass of heterotrophic microalgae has a dry mass protein content of 24% to 30% w/w, based on the total weight of dry biomass of heterotrophic microalgae.
29. The method of claim 20, wherein the heterotrophic microalgae is cultivated in batch mode.
30. The method of claim 20, further comprising adjusting the pH of the cultivation medium to pH 6 to 7.

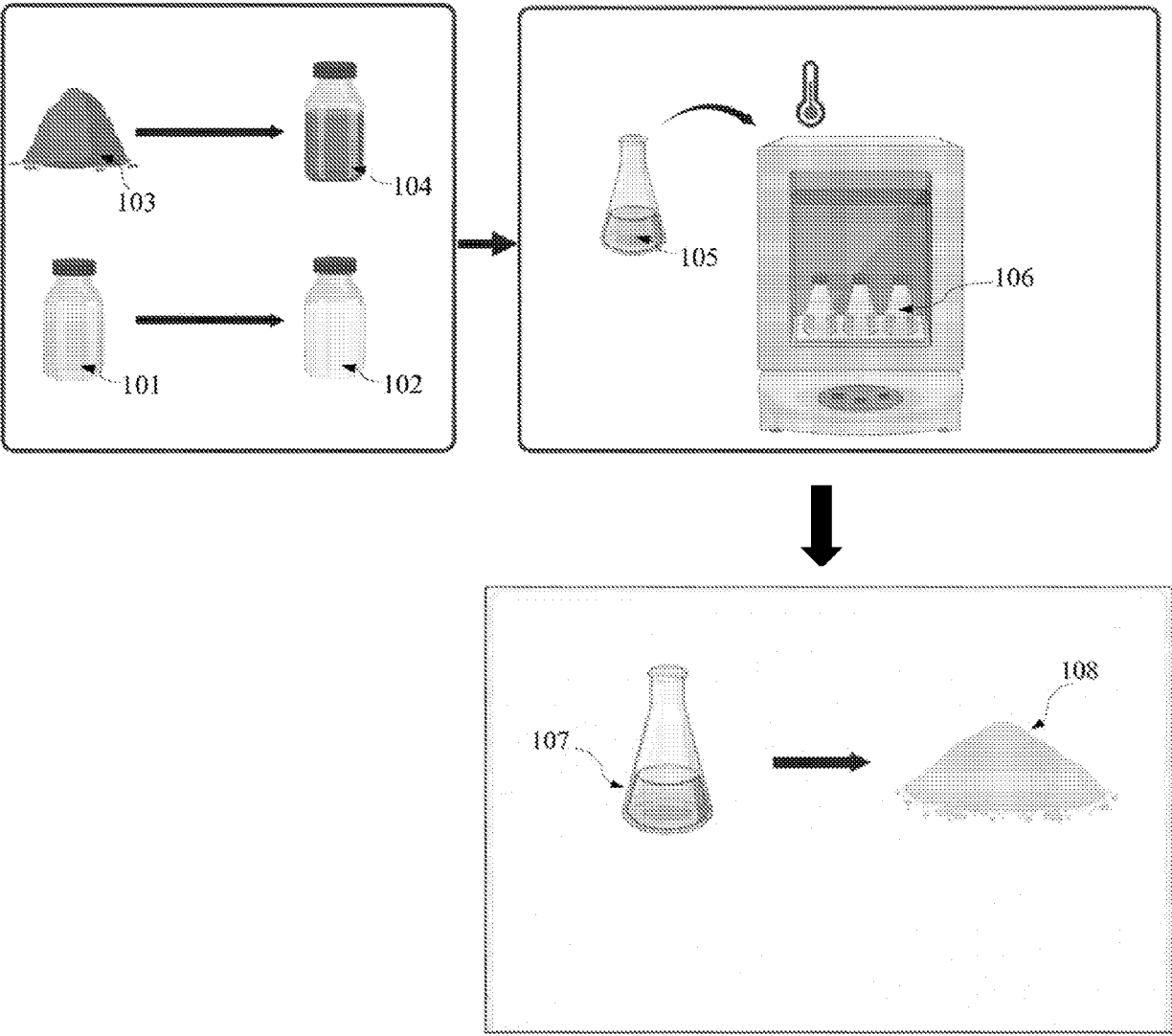


FIG. 1

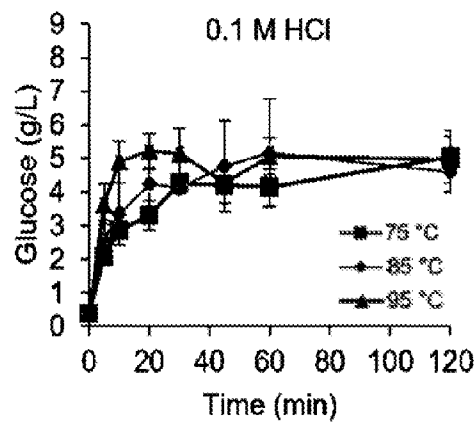


FIG. 2A

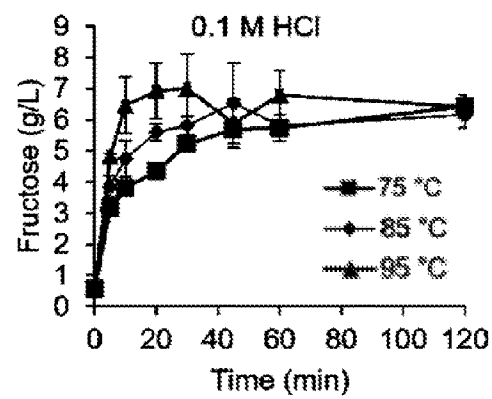


FIG. 2B

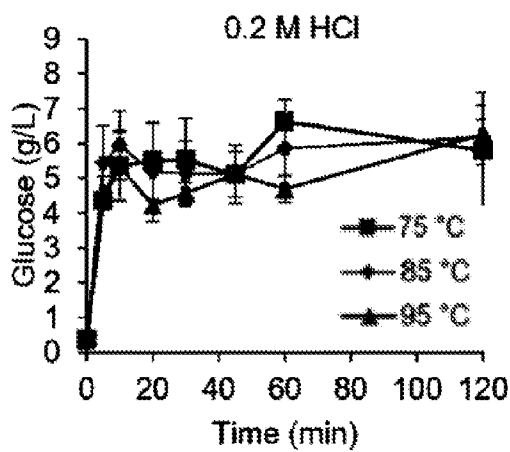


FIG. 2C

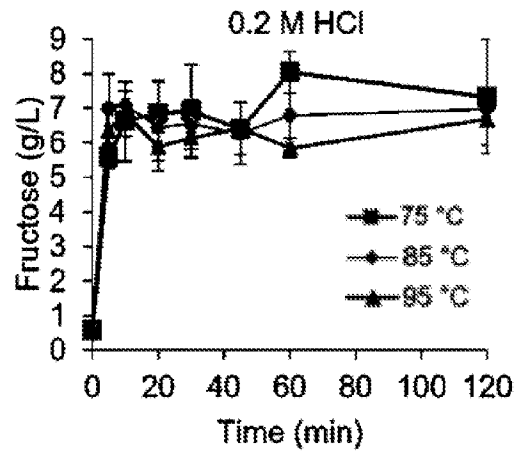


FIG. 2D

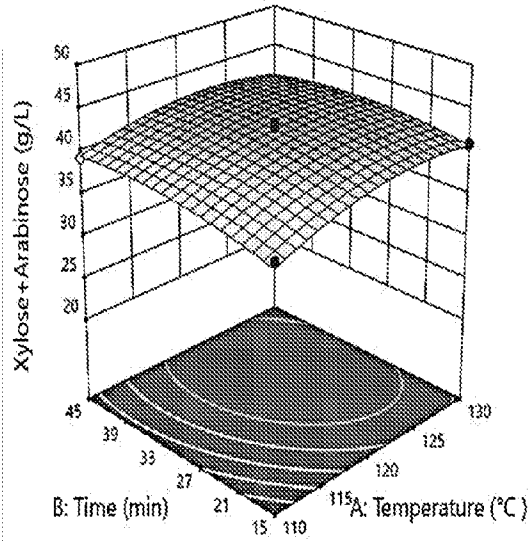


FIG. 3A

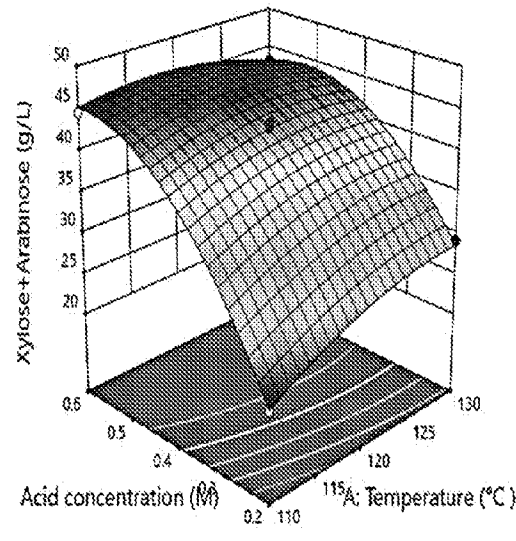


FIG. 3B

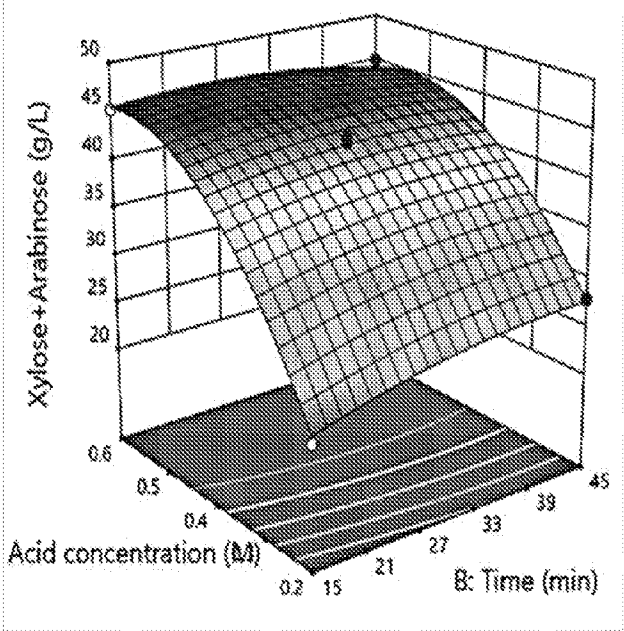


FIG. 3C

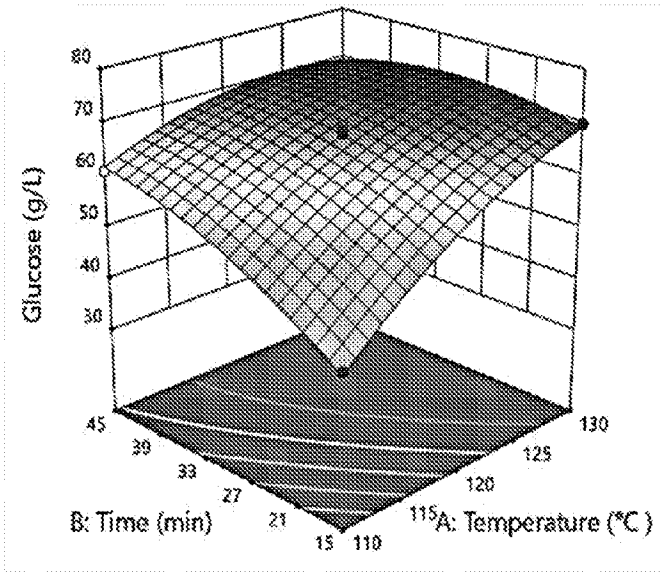


FIG. 3D

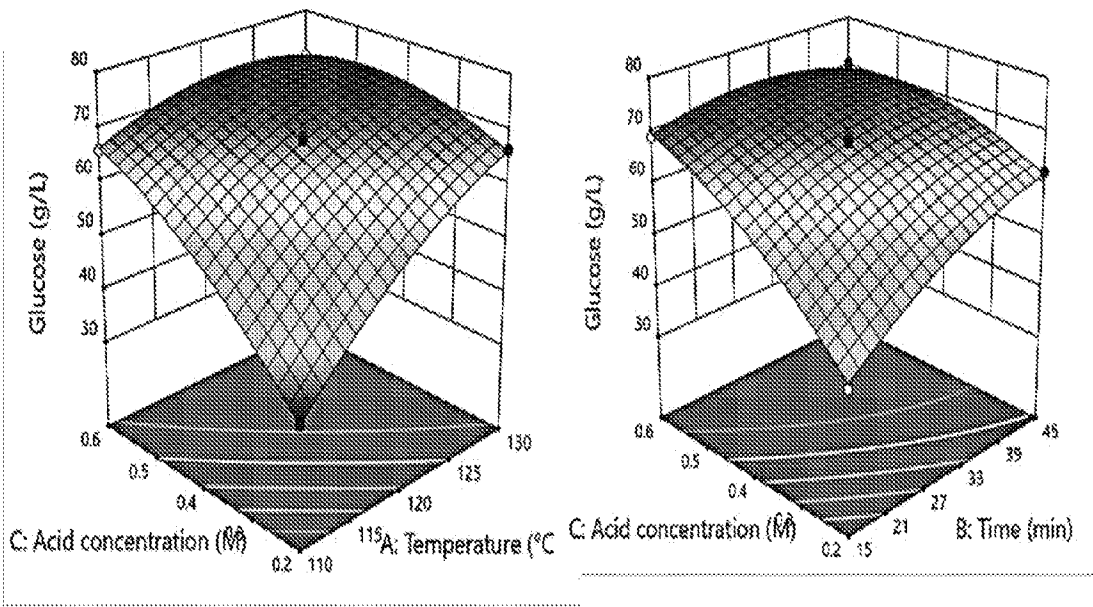


FIG. 3E

FIG. 3F

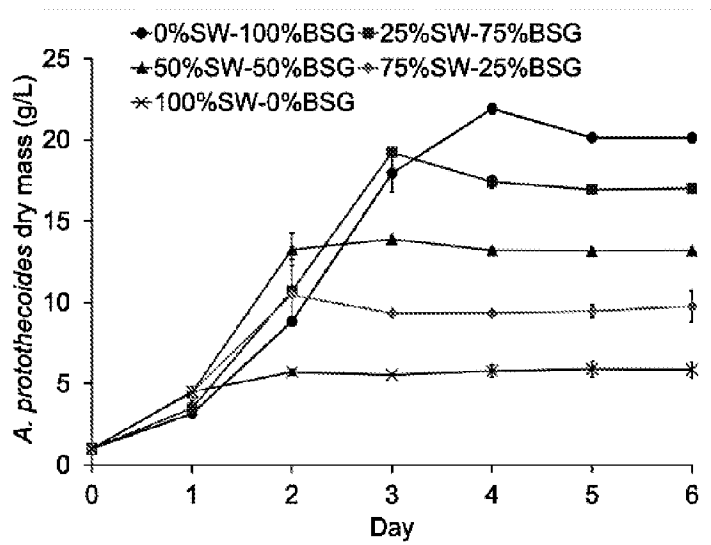


FIG. 4A

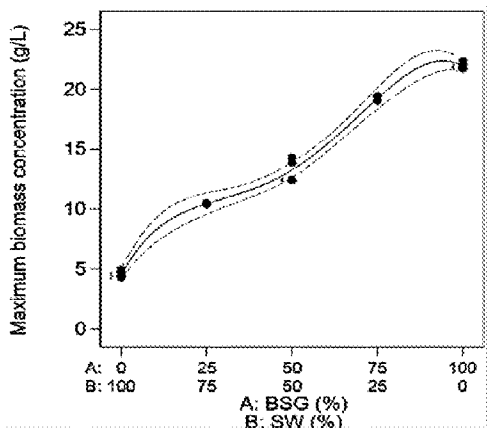


FIG. 4B

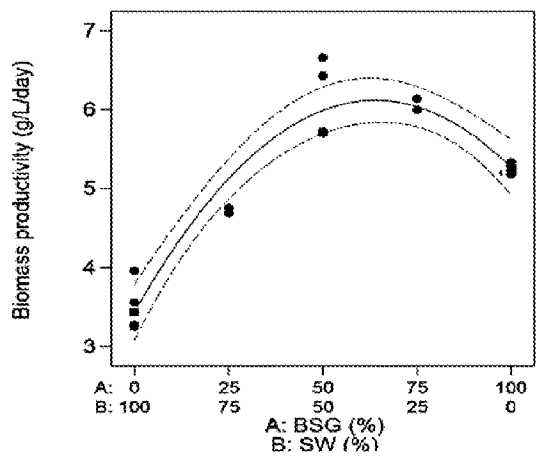


FIG. 4C

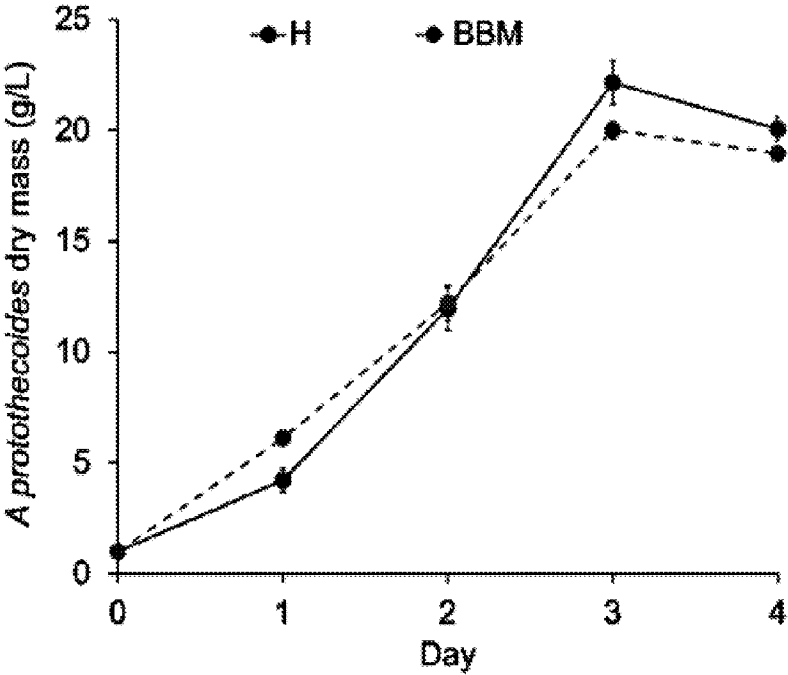


FIG. 5A

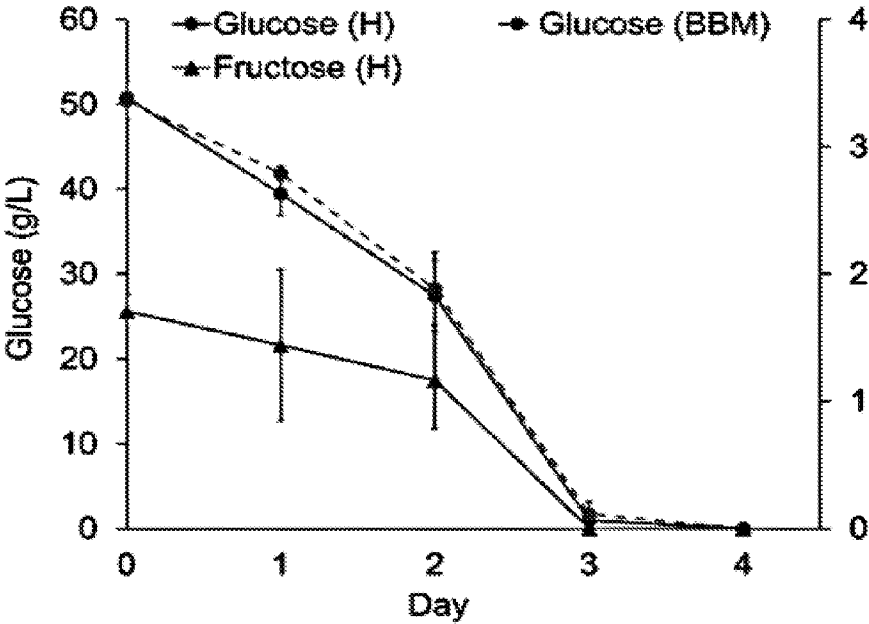


FIG. 5B

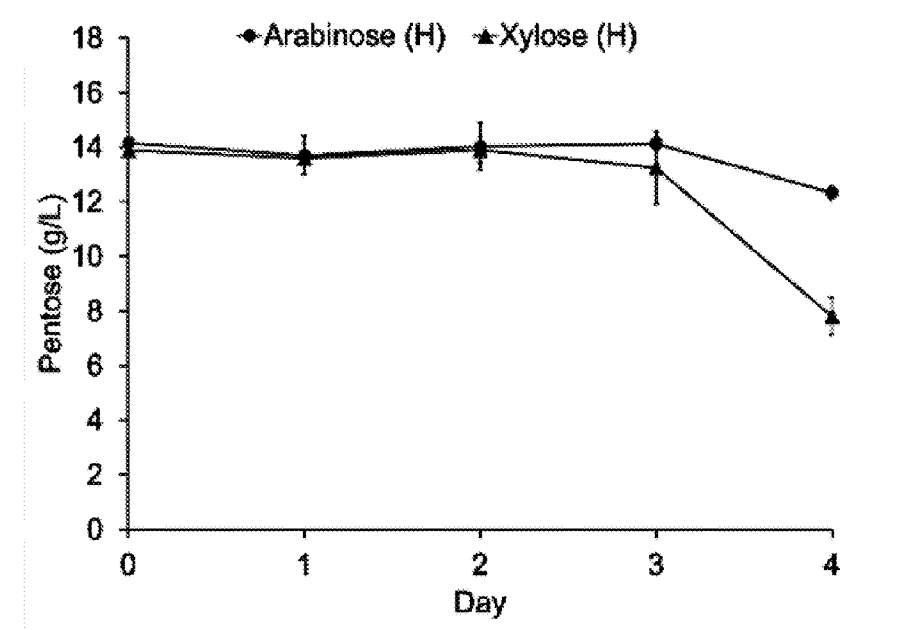


FIG. 5C

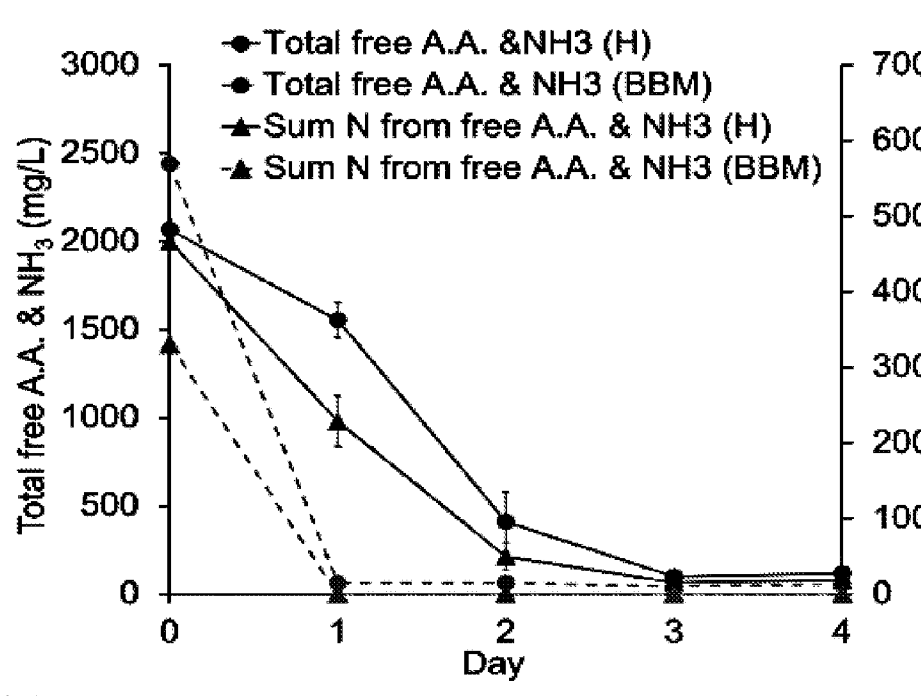


FIG. 5D

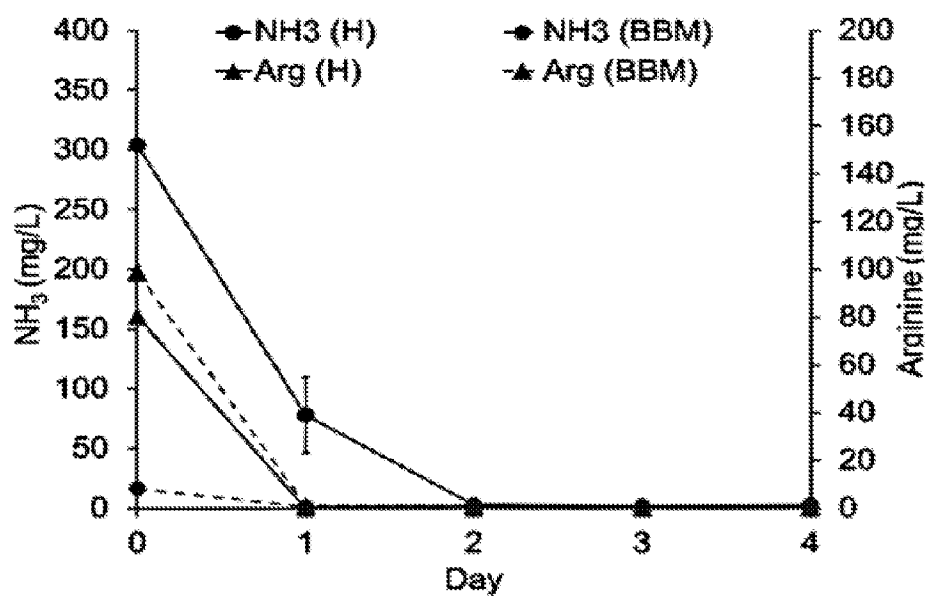


FIG. 5E

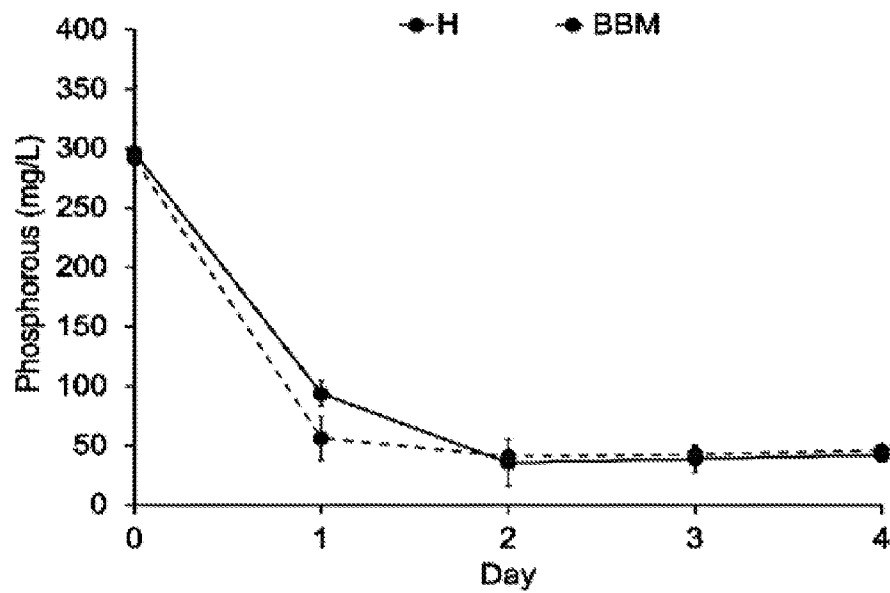


FIG. 5F

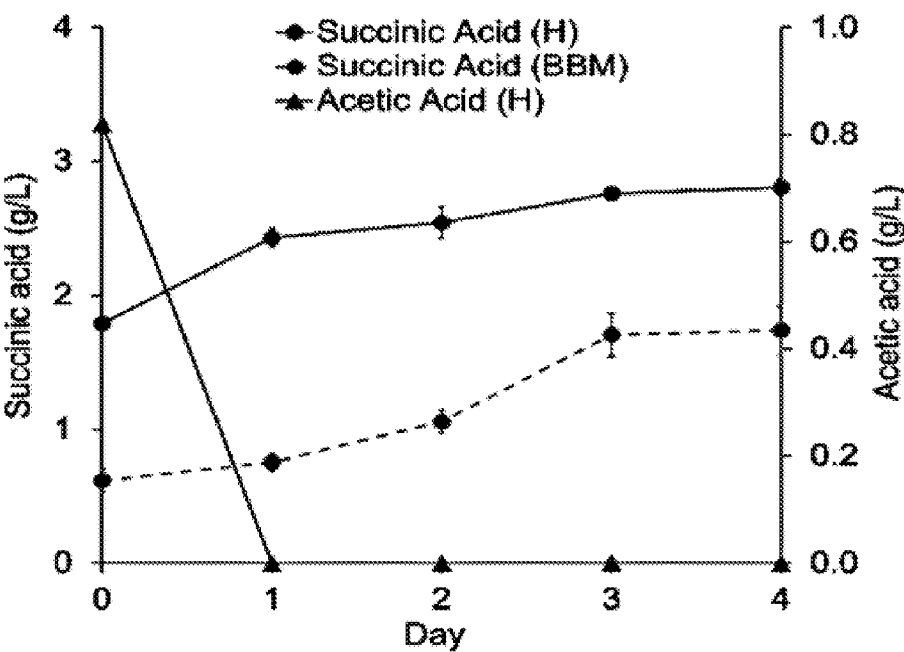


FIG. 5G

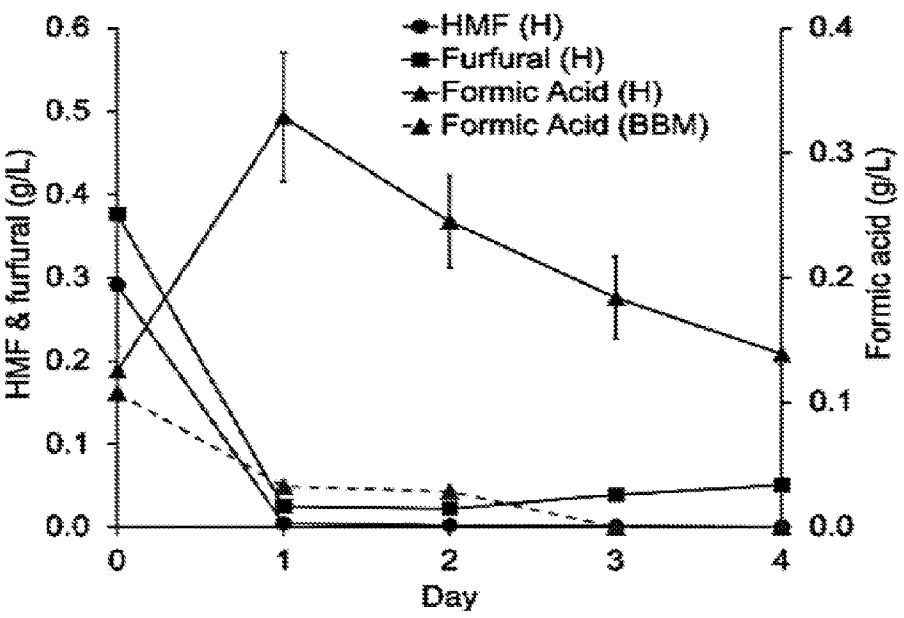


FIG. 5H

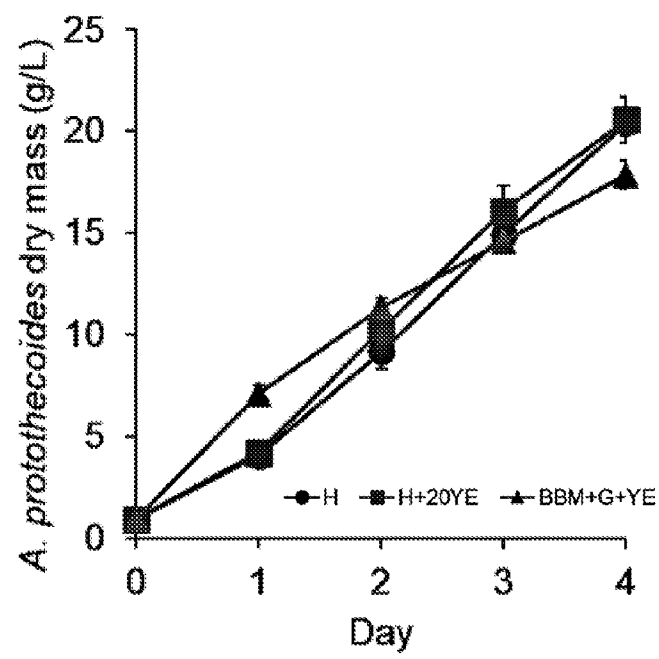


FIG. 6A

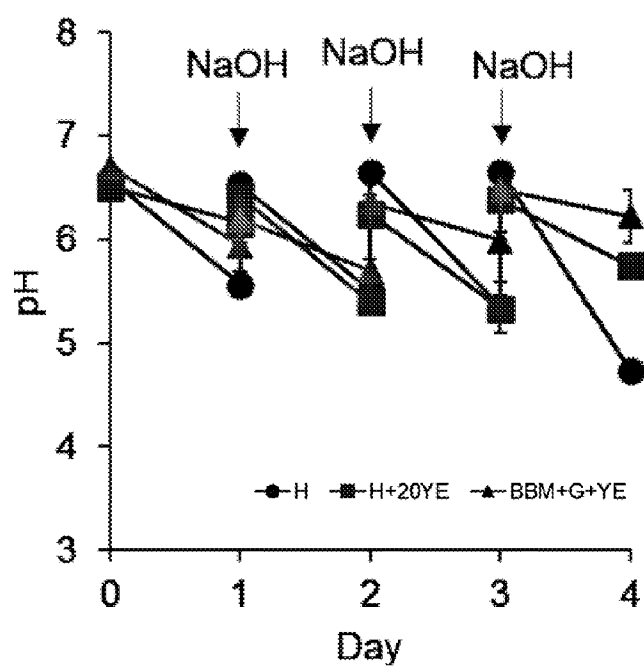


FIG. 6B

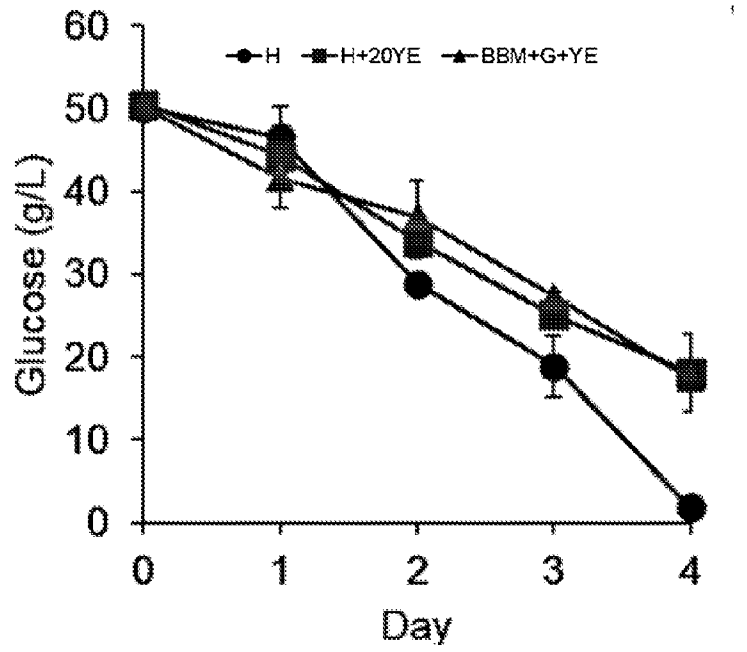


FIG. 6C

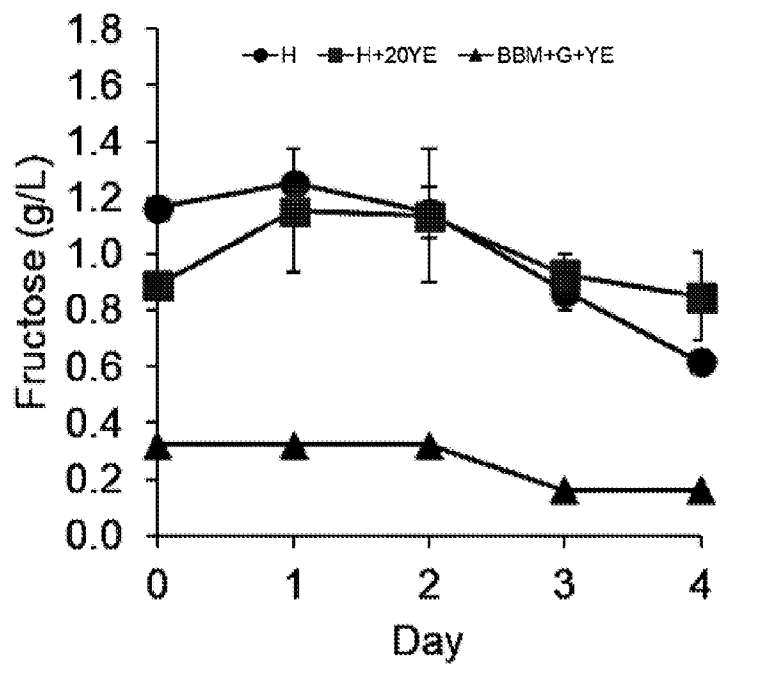


FIG. 6D

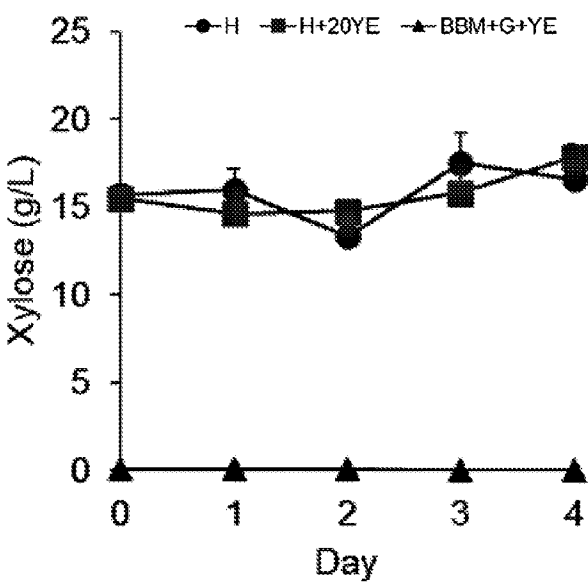


FIG. 6E

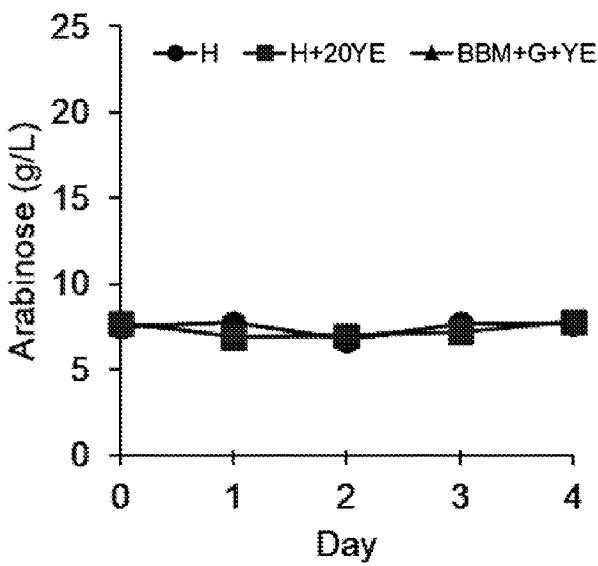


FIG. 6F

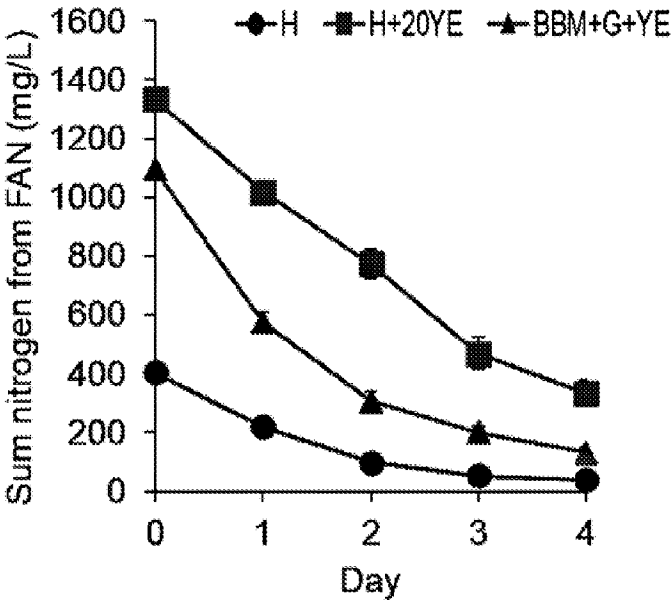


FIG. 6G

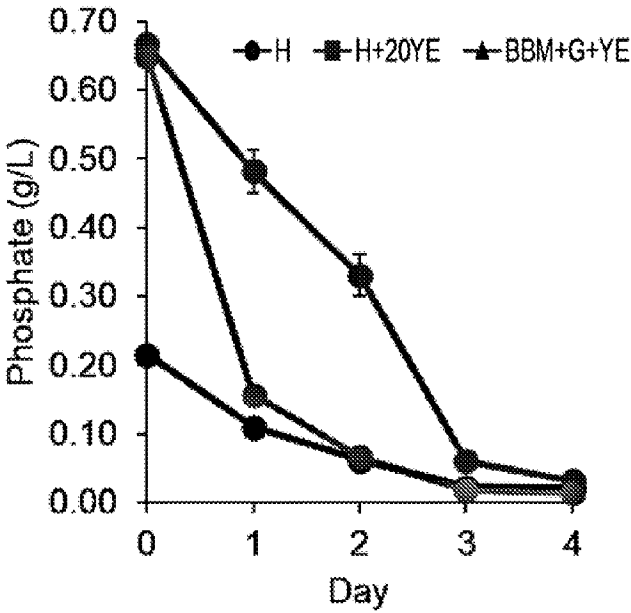


FIG. 6H

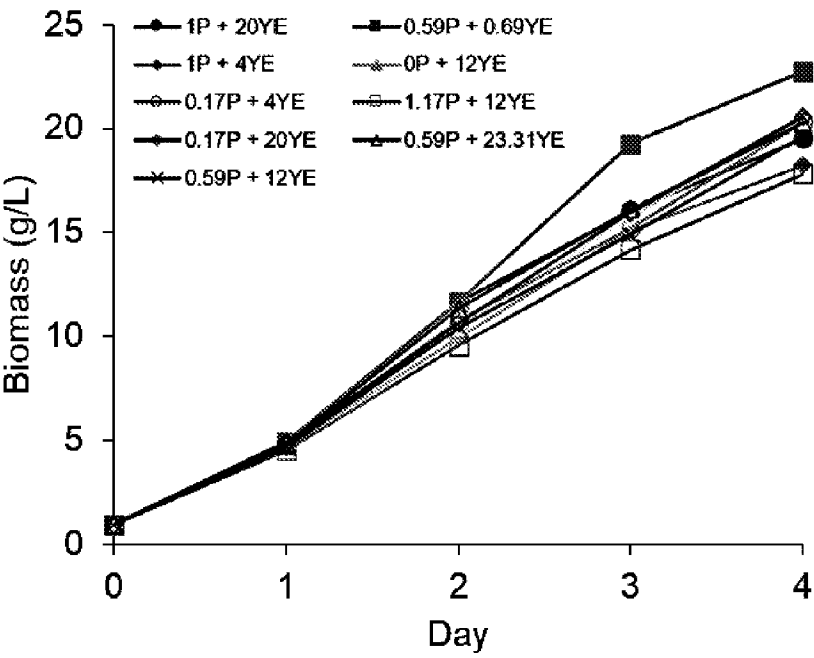


FIG. 7A

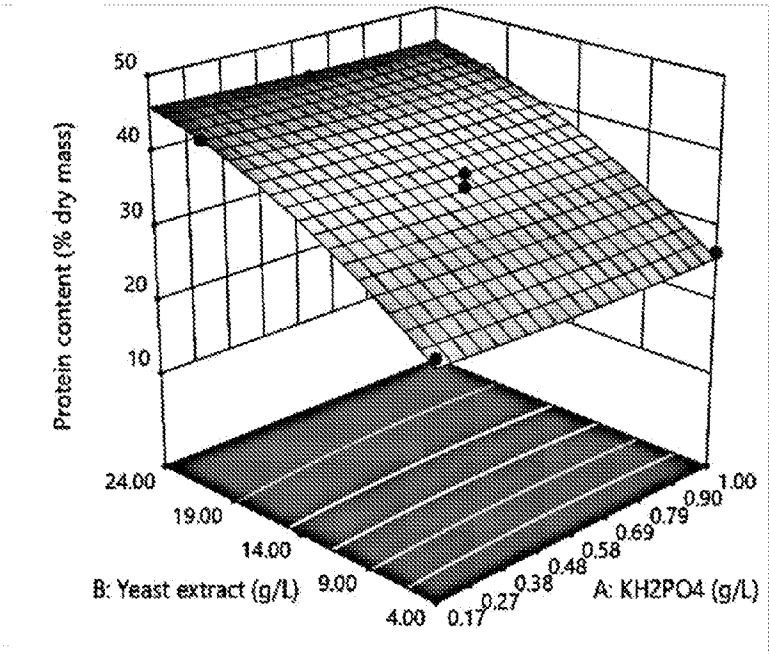


FIG. 7B

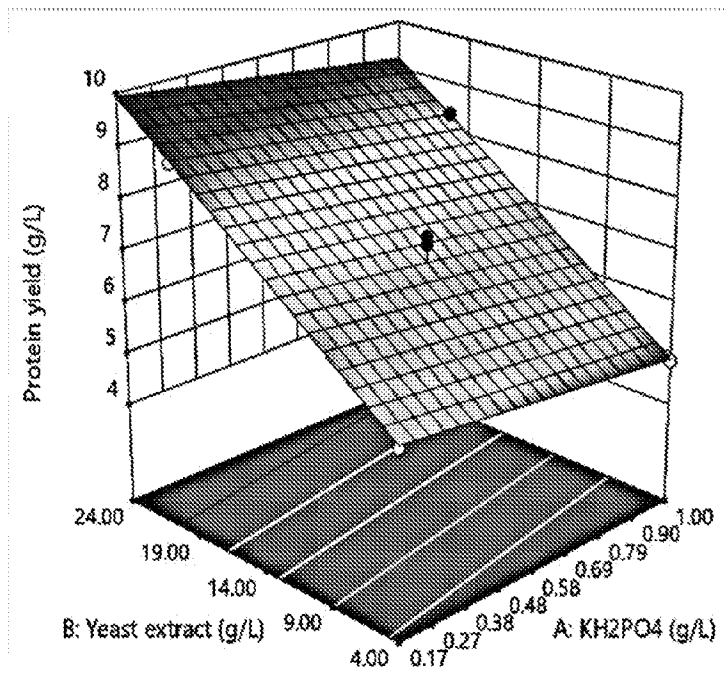


FIG. 7C

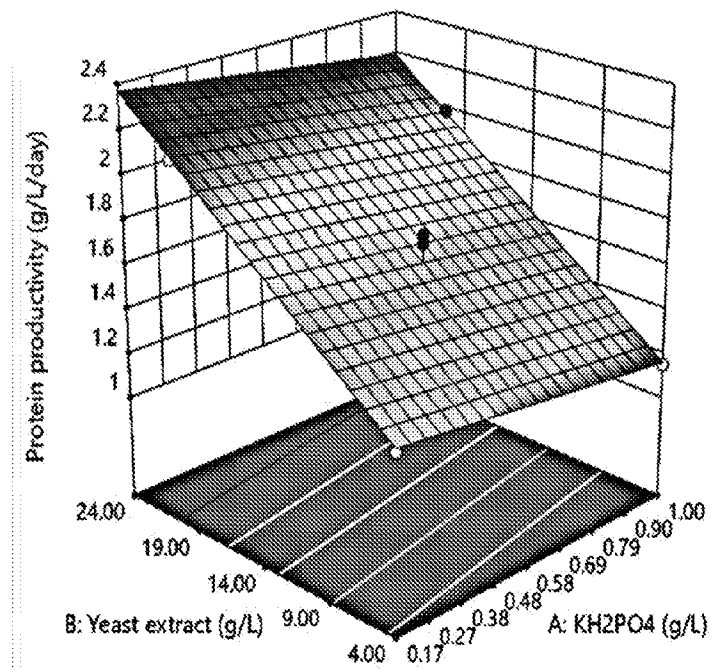


FIG. 7D

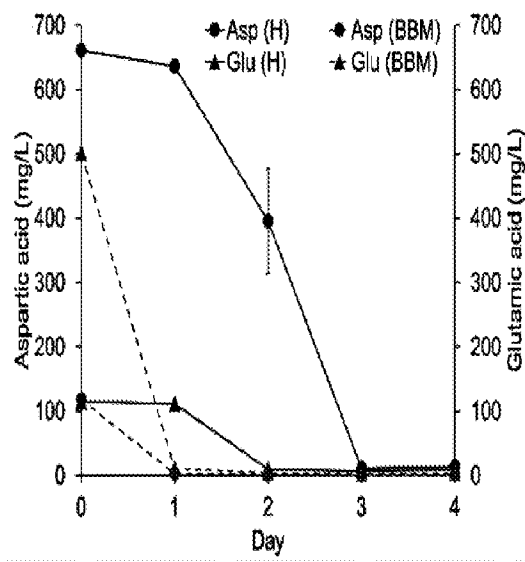


FIG. 8A

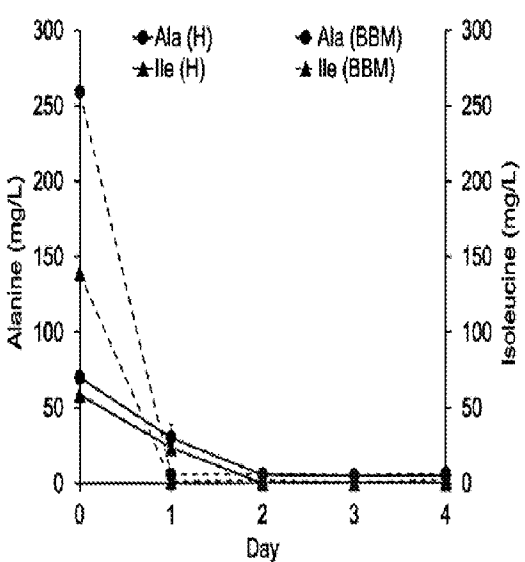


FIG. 8B

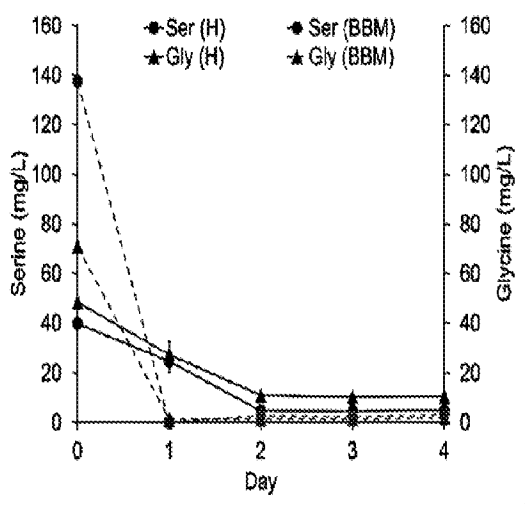


FIG. 8C

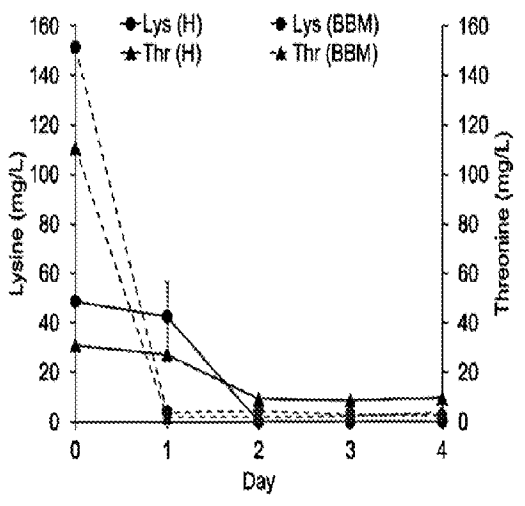


FIG. 8D

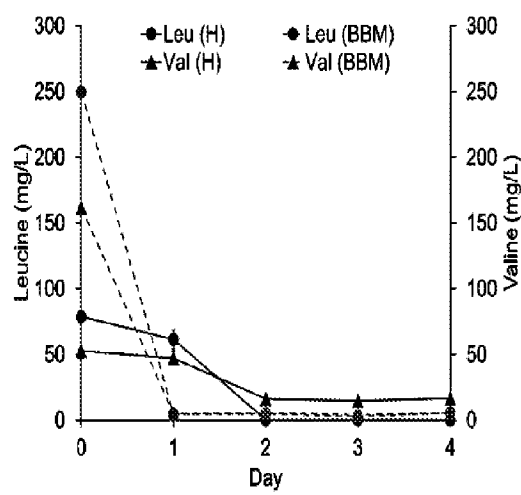


FIG. 8E

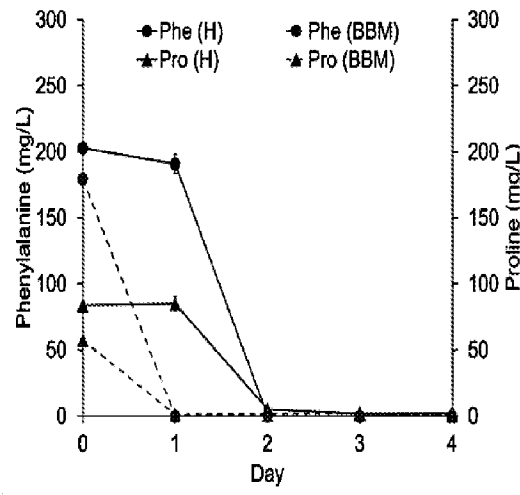


FIG. 8F

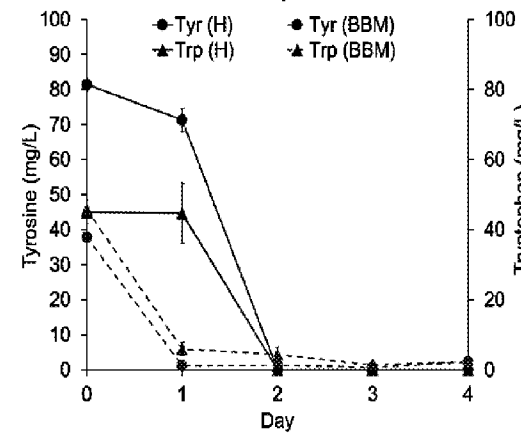


FIG. 8G

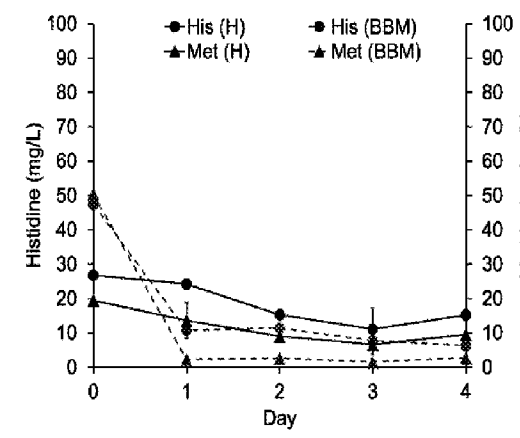


FIG. 8H

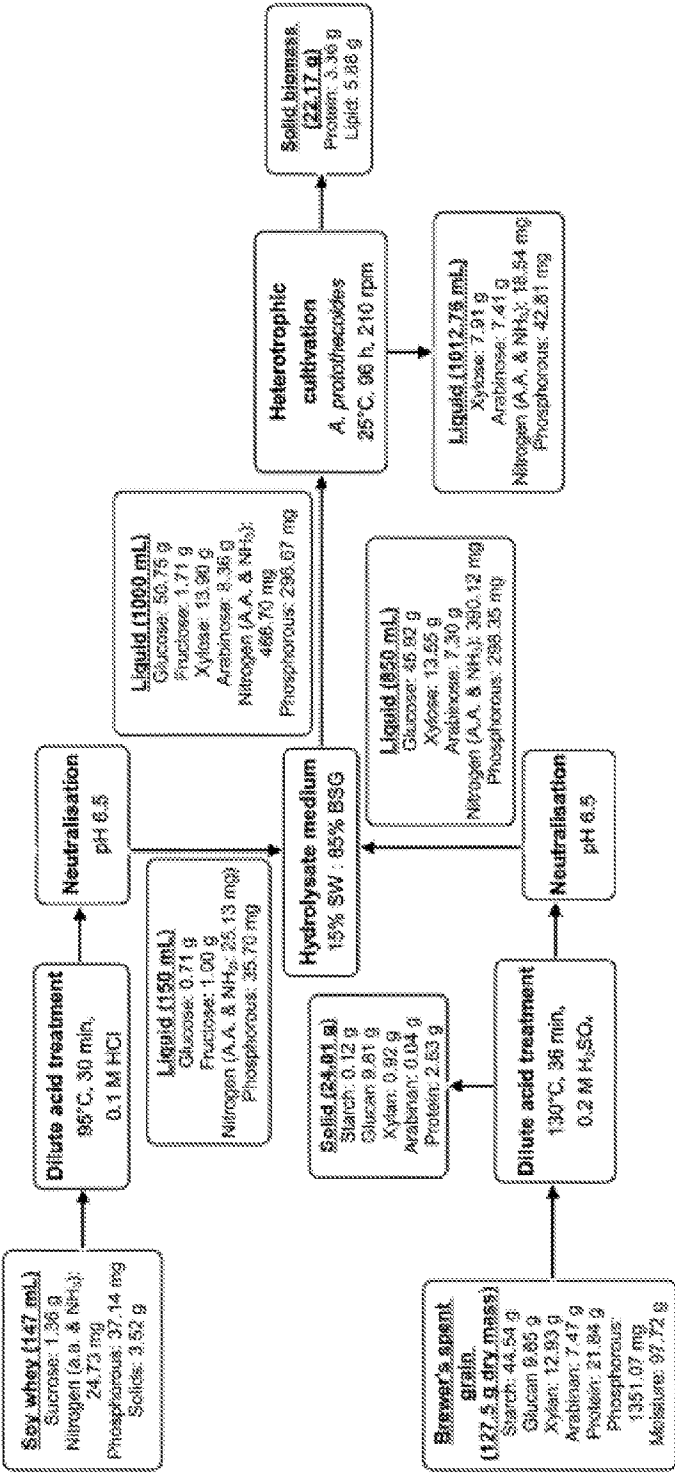


FIG. 9

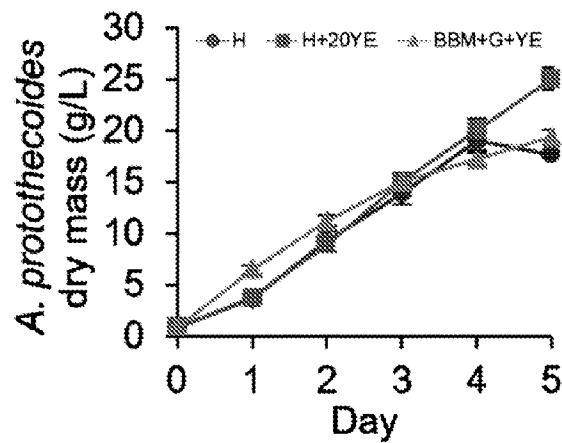


FIG. 10A

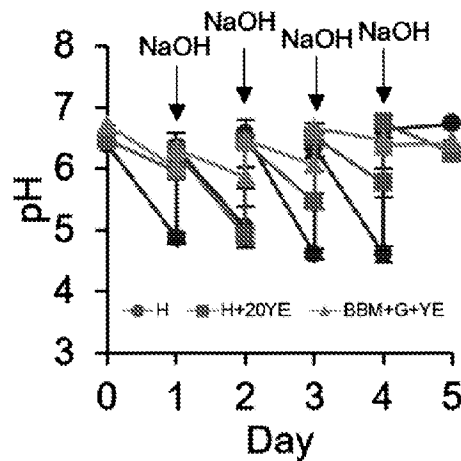


FIG. 10B

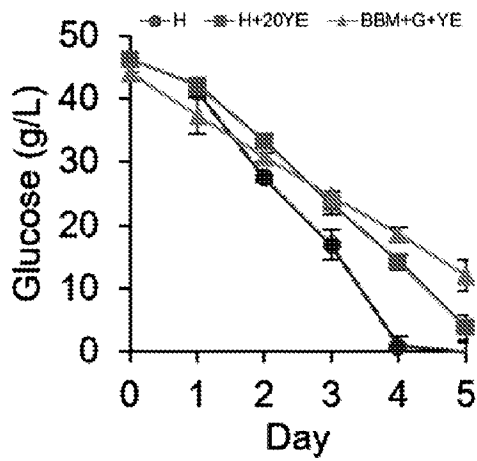


FIG. 10C

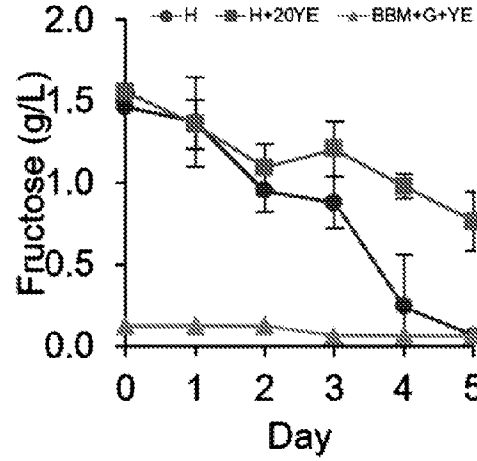


FIG. 10D

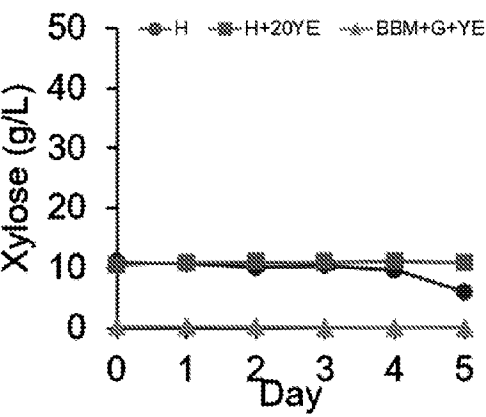


FIG. 10E

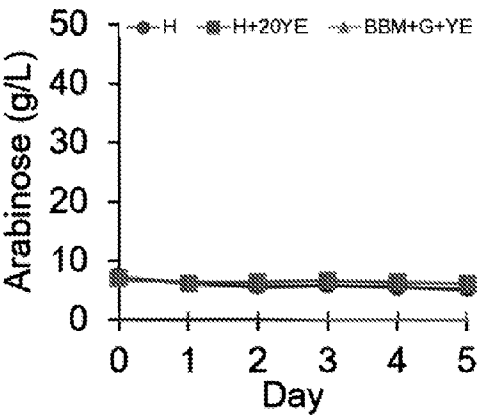


FIG. 10F

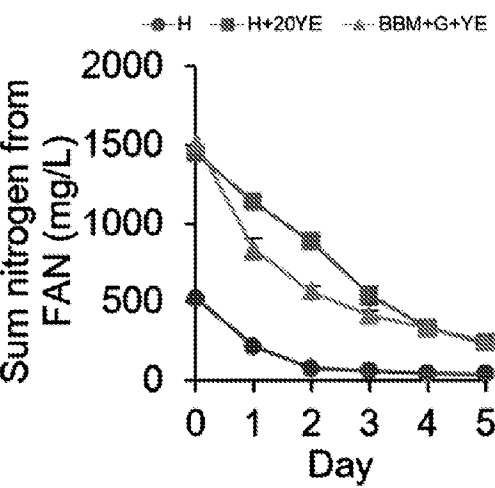


FIG. 10G

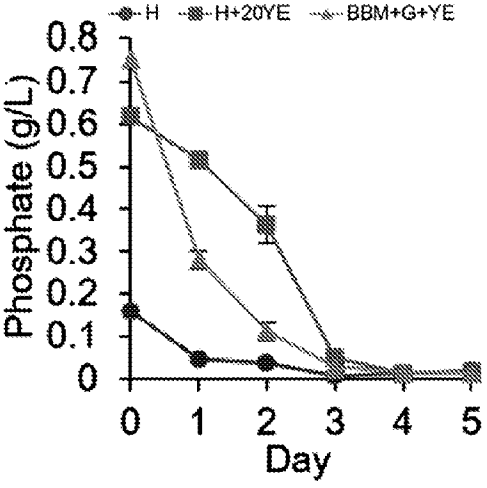


FIG. 10H

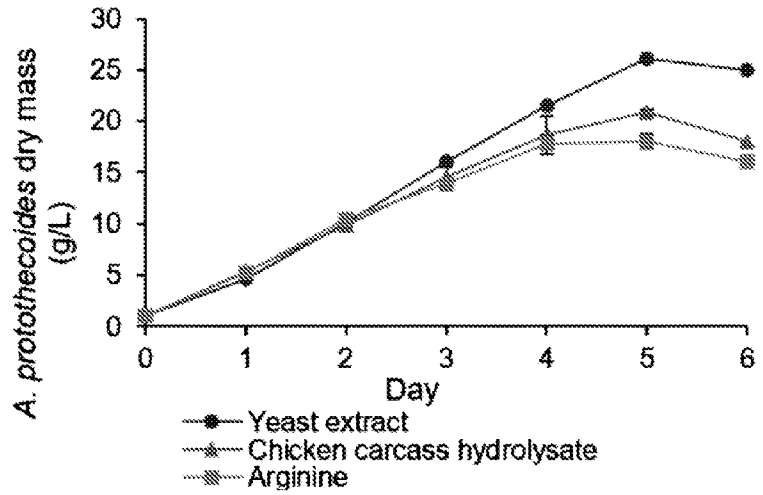


FIG. 11A

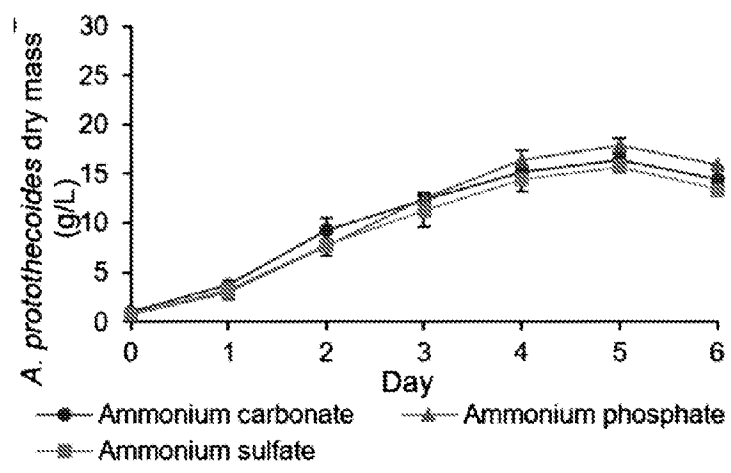


FIG. 11B

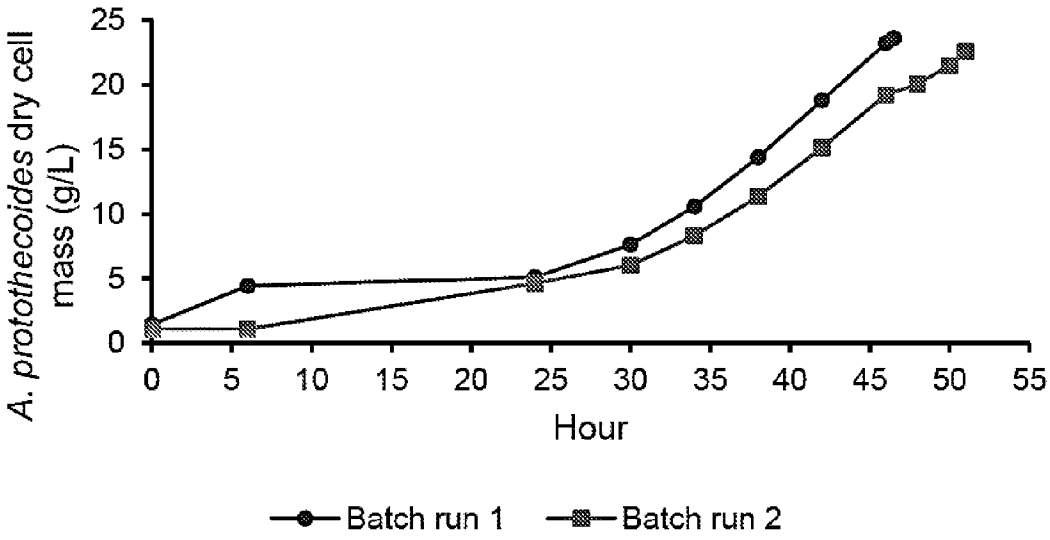


FIG. 12

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2024/050373

A. CLASSIFICATION OF SUBJECT MATTER**C12N 1/12 (2006.01) C12P 19/02 (2006.01) C12R 1/89 (2006.01) A23J 1/00 (2006.01)**

According to International Patent Classification (IPC)

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N C12P C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

FAMPAT/MEDLINE/EMBASE/BIOSIS: cultivation medium, hydrolysate, soy whey, brewer's spent grain, microalgae, and related terms.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MATA T.M. ET AL., Sustainability Evaluation of Biodiesel Produced from Microalgae <i>Chlamydomonas</i> sp Grown in Brewery Wastewater. <i>Chemical Engineering Transactions</i> , 20 June 2014, Vol. 37, pages 823-828 [Retrieved on 2024-08-01] <DOI: 10.3303/CET1437138> whole document	1-30
A	YAP, S.M. ET AL., Enhancement of protein production using synthetic brewery wastewater by <i>Haematococcus pluvialis</i> . <i>Journal of Biotechnology</i> , 21 March 2022, Vol. 350, pages 1-10 [Retrieved on 2024-08-01] <DOI: 10.1016/J.JBIOTEC.2022.03.008> whole document	-
A	DIDAK LJUBAS, B. ET AL., Production of Different Biochemicals by <i>Paenibacillus polymyxa</i> DSM 742 From Pretreated Brewers' Spent Grains. <i>Frontiers in Microbiology</i> , 4 March 2022, Vol. 13, pages e812457:1-16 [Retrieved on 2024-08-01] <DOI: 10.3389/FMICB.2022.812457> whole document	-

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

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Date of the actual completion of the international search

13/08/2024

(day/month/year)

Date of mailing of the international search report

15/08/2024

(day/month/year)

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2024/050373

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CN 115678785 A (ZHUHAI GUANGZAO LIFE SCIENCE CO LTD) 3 February 2023 whole document of the original non-English language document (a machine translation is enclosed only for your reference)	-
A	WANG, S.K. ET AL., Tofu whey wastewater is a promising basal medium for microalgae culture. <i>Bioresource Technology</i> , 4 January 2018, Vol. 253, pages 79-84 [Retrieved on 2024-08-01] <DOI: 10.1016/J.BIORTECH.2018.01.012> whole document	-
A	WONG, M., <i>Chlorella</i> cultivation in the barley and soybean residues. <i>Journal of Environmental Science and Health. Part A: Environmental Science and Engineering</i> , 31 December 1977, Vol. 12, No. 10, pages 567-574 [Retrieved on 2024-08-01] <DOI: 10.1080/10934527709374782> abstract	-

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/SG2024/050373

Note: This Annex lists known patent family members relating to the patent documents cited in this International Search Report. This Authority is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CN 115678785 A	03/02/2023	NONE	