

METHOD OF ENHANCING VITAMIN B₁₂ PRODUCTION IN PLANT-BASED MEDIA

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of priority of Singapore application No. 10202300430Q filed 20 February 2023, the contents of it being hereby incorporated by reference in its entirety for all purposes.

TECHNICAL FIELD

[0002] The present disclosure generally relates to a method of enhancing vitamin B₁₂ production in a plant-based medium. In particular, the present disclosure relates to a method of enhancing vitamin B₁₂ production in a plant-based medium supplemented with a reducing agent.

BACKGROUND

[0003] Vitamin B₁₂ is a vitamin that is used by the body to keep blood and nerve cells healthy and plays a part in DNA formation. A recommended dietary allowance of 2.4 mcg of vitamin B₁₂ is suggested for people aged 18 and older. Bacteria such as *Propionibacterium freudenreichii* and *Bacillus megaterium* have been industrially utilized to produce costly vitamin B₁₂. The resulting vitamin B₁₂ is used in various forms, both as a supplement and as an additive in food products.

[0004] Plant-based diets, in various forms, have gained increasing popularity in the West. This trend has seen significant growth due to rising concerns about the consumption of animal products and their adverse effects on health and the environment. With a strong worldwide interest in plant-based foods, there has been an increase in vegans from 4 million in 2014 to nearly 20 million in 2017 in the United States of America alone. The adoption of a plant-based diet has been shown to have beneficial effects, including a lower incidence of colon cancer and type 2 diabetes mellitus. Yet, there are concerns about deficiencies in vitamin B₁₂ and other

minerals even when a diverse range of plant-based products is consumed (*Clin. Nutr.* 2021, May 40(5), 3503–3521). Plant foods do not naturally contain vitamin B₁₂. To address the deficiencies, vegans and vegetarians often turn to vitamin B₁₂ supplements or synthetically fortified foods. However, deficiencies may still exist if vegans and vegetarians do not take the supplements on a regular basis. Additionally, improper administration of vitamin B₁₂ supplements carries risk of an overdose or elevated B₁₂ levels, which may lead to side effects such as skin rash, fatigue, etc. Furthermore, excessive consumption of supplements may be linked to a negative social stigma.

[0005] Hence, an alternative is to explore the creation of healthy plant-based beverages or food products with naturally produced vitamin B₁₂. This could aid in increasing the vitamin B₁₂ intake among vegans and vegetarians, and enriching plant-based meat products with vitamin B₁₂ to meet nutritional requirements.

[0006] It is therefore desirable to provide a method of enhancing vitamin B₁₂ production in plant-based media which seeks to address at least one of the problems described hereinabove, or at least to provide an alternative.

SUMMARY

[0007] In one aspect of the present disclosure a method of enhancing vitamin B₁₂ production in a plant-based medium is provided. The method comprises inoculating a plant-based medium supplemented with a reducing agent with a bifidobacterium; and subjecting the plant-based medium supplemented with the reducing agent to conditions conducive to anaerobic fermentation of the plant-based medium for enhancing vitamin B₁₂ production in the plant-based medium.

[0008] In some embodiments, the reducing agent is selected from cysteine, cysteine-HCl and ascorbic acid, and combinations thereof.

[0009] In some embodiments, the bifidobacterium is selected from the species *Bifidobacterium animalis* subsp. *lactis* and *Bifidobacterium longum* subsp. *longum*.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] Various embodiments of the present disclosure are described hereinbelow in the detailed description with reference to the following drawings:

FIG. 1 shows the changes of (a) cell count, and (b) pH of *Bifidobacterium animalis* subsp. *lactis* BI-04 (BI-04) or *Bifidobacterium animalis* subsp. *lactis* LATFI B94 (B94) fermented soy whey, supplemented with glucose (G), with glucose and cysteine (GC), with glucose, cysteine and yeast extract (GCY), and with no supplement (as a control) (CN).

FIG. 2 shows the changes of (a) cell count, and (b) pH of *Bifidobacterium longum* subsp. *longum* BB536 (BB536) fermented soy whey, supplemented with glucose (G), with glucose and cysteine (GC), with glucose, cysteine and yeast extract (GCY), and with no supplement (as a control) (CN).

FIG. 3 shows the vitamin B₁₂ amount in different supplemented soy whey media fermented with strains B94, BI-04, and BB536, respectively, or unfermented (UT). The control (CN) is unsupplemented. Error bars represent the mean $\pm$ SD, and significant differences between treatments within supplements are determined by one way ANOVA where ****: $P \leq 0.0001$.

FIG. 4 is a graph showing the vitamin B₁₂ concentration ($\mu\text{g/L}$) produced by strain BI-04 in soy whey against different reducing agent supplementation dose at 48-hour fermentation. The reducing agents used are ascorbic acid (AA), cysteine (Cy) and cysteine-HCl (CyHCl). The line is the best-of-fit for the different reducing agents.

DESCRIPTION

[0011] The following description sets forth exemplary methods, parameters, and the like. The embodiments are described in sufficient detail to enable those skilled in the art to practise the invention. Other embodiments may be utilized, and structural and logical changes may be made without departing from the scope of the invention. The various embodiments are not

necessarily mutually exclusive, as some embodiments can be combined with one or more other embodiments to form new embodiments.

[0012] Features that are described in the context of an embodiment may correspondingly be applicable to the same or similar features in the other embodiments. Features that are described in the context of an embodiment may correspondingly be applicable to the other embodiments, even if not explicitly described in these other embodiments. Furthermore, 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.

[0013] In the context of various embodiments, the articles “a”, “an” and “the” as used regarding a feature or element include a reference to one or more of the features or elements.

[0014] 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, e.g. within 20% of the specified value.

[0015] As used herein, the term “and/or” includes any and all combinations of one or more of the associated listed items.

[0016] By “comprising” it is meant 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.

[0017] By “consisting of” is meant including, and limited to, whatever follows the phrase “consisting of”. Thus, the phrase “consisting of” indicates that the listed elements are required or mandatory, and that no other elements may be present.

[0018] The present disclosure relates to a method of enhancing vitamin B₁₂ production in a plant-based medium. The method comprises inoculating a plant-based medium supplemented with a reducing agent with a bifidobacterium; and subjecting the plant-based medium supplemented with the reducing agent to conditions conducive to anaerobic fermentation of the plant-based medium for enhancing vitamin B₁₂ production in the plant-based medium.

[0019] The term “plant-based medium” as used herein refers to a medium derived from plants. The medium can be derived from any parts of a plant including the root, leaf, fruit, stem, and seeds. The medium can also include manufacturing by-products of plant-based food including, but not limited to, whey, wastewater, bran, and press cakes. The plant-based medium is used as a fermentation substrate for enrichment with vitamin B₁₂.

[0020] Suitable plant-based medium that may be employed includes, but is not limited to, soy whey, carrot, and pear. In one embodiment, the plant-based medium is soy whey.

[0021] In some embodiments, the plant-based medium may be further processed to suitable form prior to supplementing the plant-based medium with the reducing agent or prior to inoculating with the bifidobacterium. For example, the plant-based medium may be processed from solid to liquid form before supplementing the plant-based medium with the reducing agent or before inoculating with the bifidobacterium.

[0022] The term “reducing agent” as used herein refers to a food-grade agent that is capable of creating a reducing environment that is conducive for anaerobic fermentation. In some embodiments, the reducing agent is selected from cysteine, cysteine-HCl and ascorbic acid, and combinations thereof. One skilled in the art will deduce that other food-grade reducing agents or combinations of natural reducing agents natively/naturally present in plant sources may be employed, so long as the reducing agent is of food grade and is capable of creating a reducing environment suitable for anaerobic fermentation.

[0023] In some embodiments, the plant-based medium is supplemented with glucose and cysteine (or cysteine-HCl) (GC), with glucose, cysteine (or cysteine-HCl) and yeast extract (GCY), with cysteine (or cysteine-HCl) or with ascorbic acid. Glucose is added to provide an additional carbon and energy source, while yeast extract is added to provide extra nitrogen and micronutrient sources for growth. Either cysteine or cysteine-HCl can be used depending on the specific applications and consideration such as solubility.

[0024] In some embodiments, the weight ratio of glucose to cysteine (or cysteine-HCl) ranges from 20:1 to 50:1 (w/w), depending on the plant-based medium that is used. In some embodiments, the weight ratio of glucose to cysteine (or cysteine-HCl) to yeast extract ranges from 20:1:5 to 50:1:15 (w/w/w) based on the natural carbon and nitrogen sources present in the plant-based medium.

[0025] In some embodiments, the plant-based medium is supplemented with the reducing agent by adding the reducing agent to the plant-based medium in liquid form and subjecting the plant-based medium with the reducing agent to pasteurization. In some embodiments, the pasteurization process is carried out at a temperature ranging from 85 °C to 95 °C, from 89 °C to 92 °C or at about 90 °C for 5 to 60 minutes. In some embodiments, the pasteurization process is carried out at a higher temperature for a shorter duration. In some exemplary embodiments, the pasteurization process is carried out at temperature ranging from 120 °C to 150 °C or 138 °C to 150 °C, for several seconds or for 1 to 2 seconds. Other methods of pasteurization may be employed depending on the type of plant-based medium and reducing agent that are used. Supplementing a plant-based medium with a reducing agent creates a reducing environment for anaerobic fermentation which is needed for enhancing vitamin B₁₂ production in the plant-based medium.

[0026] The plant-based medium supplemented with the reducing agent is inoculated with the bifidobacterium. In some embodiments, the conditions conducive to anaerobic fermentation of the plant-based medium include inoculating the plant-based medium supplemented with the reducing agent with the bifidobacterium at an inoculation ratio ranging from 0.5% to 5.0% (v/v) to attain an initial cell count of at least 5 or 6 log CFU/mL of bifidobacterium. The cell count should preferably be at least 5 or 6 log CFU/mL to help establish the conditions necessary for efficient fermentation. In some embodiments, the amount of the bifidobacterium used for the inoculation is about 1% (v/v) based on the overall volume of the plant-based medium.

[0027] The conditions conducive to anaerobic fermentation of the plant-based medium further includes adjusting the pH of the plant-based medium supplemented with the reducing agent to within 5 to 8, 6 to 7, or 6.5. The pH of the plant-based medium supplemented with the reducing agent can be adjusted before or after the plant-based medium supplemented with the reducing agent comes into contact with the bifidobacterium. In some preferred embodiments, the pH of the plant-based medium supplemented with the reducing agent is adjusted before the plant-based medium supplemented with the reducing agent comes into contact with the bifidobacterium.

[0028] The conditions conducive to anaerobic fermentation of the plant-based medium may also include incubating the plant-based medium supplemented with the reducing agent with the bifidobacterium at a temperature ranging from 30 °C to 40 °C, from 36 °C to 38 °C, or at about 37 °C for a duration ranging from 12 to 72 hours, from 36 to 60 hours, from 46 to 50 hours or for about 48 hours for fermentation to take place.

[0029] In some embodiments, the bifidobacterium is selected from the species *Bifidobacterium animalis* subsp. *lactis*, and *Bifidobacterium longum* subsp. *longum*. In further embodiments, the bifidobacterium is a strain selected from *Bifidobacterium animalis* subsp. *lactis* B1-04 (B1-04), *Bifidobacterium animalis* subsp. *lactis* LATFI B94 (B94) and *Bifidobacterium longum* subsp. *longum* BB536 (BB536). All three strains of *Bifidobacterium* are publicly available. Selected species can encompass other species including, but are not limited to, *Bifidobacterium breve*, *Bifidobacterium infantis*, and *Bifidobacterium bifidum*.

[0030] In some embodiments, the bifidobacterium is used in its provided or original form. In some embodiments, the bifidobacterium may be further processed prior to use. In some embodiments, the bifidobacterium may be further processed by supplementing the bifidobacterium with cysteine-HCl for a duration ranging from 20 to 30 hours, 24 to 30 hours, or for about 24 hours, and at a temperature ranging from 30 °C to 40 °C, from 36 °C to 38 °C, or at about 37 °C to obtain cell pellets. This is followed by washing the cell pellets and

resuspending the cell pellets in unsupplemented plant-based medium. The resulting mixture serves as a pre-culture for use in anaerobic fermentation.

[0031] The method of the present disclosure has several advantages. One of the advantages is that the method can be applied to producing beverages comprising plant-based medium enriched with vitamin B₁₂. It can also be applied to produce supplements or ingredients for use in producing plant-based meat products with an increased amount of vitamin B₁₂. The inventors have surprisingly found that supplementing a plant-based medium with a reducing agent can enhance production of vitamin B₁₂ in the plant-based medium. The inventors have also surprisingly found that certain bifidobacteria exhibit excellent growth in plant-based medium that is supplemented with a reducing agent. The increase in vitamin B₁₂ from supplementation using a reducing agent makes it possible for the development of beverages or functional ingredients to supplement dietary choices that may lack these nutrients. For example, for vegetarians and vegans, vitamin B₁₂ intake is often deficient. Having plant-based beverages and/or plant-based meat products, enriched with vitamin B₁₂, can serve as a source of vitamin B₁₂ for vegetarians and vegans, instead of relying on chemically synthesized vitamin B₁₂.

[0032] Furthermore, in the exemplary embodiment where soy whey is used as the plant-based medium, the fermentation process fully utilizes the soy whey generated from the tofu and soy protein making process. This helps to reduce waste from food manufacture.

[0033] To facilitate a better understanding of the present disclosure, the following examples of specific embodiments are given. In no way should the following examples be read to limit or define the entire scope of the disclosure. One skilled in the art will recognize that the examples set out below are not an exhaustive list of the embodiments of this disclosure.

EXAMPLES

Example 1

[0034] Preparation of Bifidobacterial Cultures

[0035] Pure frozen cultures of *Bifidobacterium longum* subsp. *longum* BB536 (BB536) (Morinaga, Tokyo, Japan), *Bifidobacterium animalis* subsp. *lactis* BI-04 (BI-04) (Danisco, Copenhagen, Denmark), and *Bifidobacterium animalis* subsp. *lactis* LATFI B94 (B94) (Lallemand, Montreal, Canada) were twice sub-cultured statically in Man, Rogosa and Sharpe (MRS) (Oxoid Ltd., Hampshire, United Kingdom) broth, supplemented with 0.05% cysteine HCl (sigma) (MRSC) for 24 hours at 37 °C. Following the sub-culturing, the cell pellets were washed twice with 0.85% (w/v) saline solution before being resuspended in unsupplemented soy whey, serving as pre-cultures for fermentation.

[0036] **Preparation of Soy (Tofu) Whey**

[0037] Soy whey was prepared in a food processing facility. To begin, soybeans were soaked for 16 h in 1:6 (w/v) deionized water ratio. Soybeans were then strained and blended using a blender with deionized water in 1:6 dry weight to volume ratio to obtain a slurry. Soymilk was filtered through a cheesecloth to isolate the okara. Deionized water at 1:2 (w/v) was used to wash the okara and the flow-through was pooled together with the soymilk. The soymilk was then brought to a boil and held for 5 min. The soymilk was cooled to 87 °C before 2% (soybean dry weight, w/v) gypsum (calcium sulfate, Home Brew Ohio Sandusky, United States) suspended in water in 10% ratio (w/v) was added to it. The soymilk and gypsum mixture was stirred well and left to curdle for 15 min before being hydraulically pressed to separate the soy whey from the tofu.

[0038] **Soy Whey Supplementation**

[0039] Soy whey was split into four equal portions, of which one was left as unsupplemented for use as a control (CN). The others were supplemented with 2% (w/v) glucose (G); 2% (w/v) glucose and 0.05% (w/v) cysteine-HCl (GC); and 2% (w/v) glucose, 0.05% (w/v) cysteine-HCl and 0.25% yeast extract (GCY) (Oxoid Ltd., Hampshire, United Kingdom), respectively. The soy whey was pasteurized at 90°C for 30 min and potato dextrose agar was used to verify the effectiveness of the pasteurization.

Example 2**[0040] Fermentation**

[0041] Duplicate laboratory scale fermentations were conducted twice. The pre-cultures of strains BI-04, B94 and BB536 prepared in Example 1 were inoculated into respective soy whey at 1% (v/v) inoculation ratio to obtain an initial cell count of about 5 log CFU/mL. Aliquots of 40 mL or 10 mL were then transferred to 50 mL or 15 mL centrifuge tubes, respectively. Incubation was carried out at 37 °C for the 48-hour fermentation period before the 15 mL tubes were transferred to 4 °C or 25 °C for storage following the completion of the 48-hour fermentation. Cell count measurements were conducted at 0 h, 6 h, 12 h, 24 h, 36 h, and 48 h using the 40 mL aliquots and at week 1, week 2, week 3, and week 4 using the 10 mL aliquots. Cell enumeration was carried out with pour plating on MRSC agar incubated for 48 hours at 37 °C in an anaerobic jar with AnaeroGen™ (Oxoid Ltd., Hampshire, United Kingdom). pH measurements were performed with a pH meter (827 pH Lab, Metrohm, Herisau, Switzerland). The results are shown in FIG. 1 (for BI-04 and B94 strains) and FIG. 2 (for BB536 strain).

[0042] Bifidobacterial Growth and pH Changes

[0043] FIG. 1 shows the cell count and pH changes of soy whey over 48-hour fermentation with *B. lactis* strains BI-04 and B94. Strain BI-04 was able to grow in non-supplemented and supplemented soy whey, while strain B94 was able to grow in GC and GCY supplemented soy whey. Supplementation led to a rapid increase in cell count after 24 hours of fermentation for both BI-04 and B94 strains by about 1.58 (BI-04 in GC), 2.48 (BI-04 in GCY), 2.52 (B94 in GC), and 2.04 log CFU/mL (B94 in GCY).

[0044] CN and G soy whey were only able to sustain growth for strain BI-04 by 2.24 (CN) and 2.39 log CFU/mL (G) but not for strain B94. As shown in FIG. 1(b), the trends in pH changes were similar between B94 and BI-04 samples where the cell counts increased. B94 fermented CN and G samples did not show pH decreases due to their inability to support growth.

[0045] FIG. 2 shows the cell count and pH changes of soy whey over 48-hour fermentation with strain BB536. The cell counts in GC and GCY increased from 24 hours onwards (FIG. 2(a)), while the cell counts decreased continuously in CN and G soy whey. The lack of growth of strain BB536 in CN and G soy whey indicates the incompatibility of strain BB536 to those supplements. The increase in cell counts in GC and GCY could be attributed to strain BB536 having a longer lag phase in GC and GCY soy whey. GCY supplementation showed promising results to support bifidobacterial growth with the highest cell count of 7.7 log CFU/mL. The cysteine and yeast extract supplementation enabled strain BB536 to grow in soy whey. The cysteine would have provided the media with lower redox potential and hence a better anaerobic condition for bifidobacteria. Yeast extract would have provided the media with nutrients such as amino acids and B-vitamins which are known to be an excellent growth promoter. The soy whey pH stayed constant throughout the 48-hour fermentation for CN and G supplementation, while GC showed a slight decrease at 48-hour with GCY supplementation having the greatest pH decrease (FIG. 2(b)). The pH results aligned well with the growth kinetic curves shown in FIG. 2(a).

Example 3

[0046] Vitamin B₁₂ Analysis

[0047] Vitamin B₁₂ analysis follows the methodology from a previous study (*Journal of AOAC International*, 2008, 94(4), 786-793; *Journal of AOAC International*, 2014, 97(5), 1397-1402; *Journal of Chromatography A*, 2006, 1101(1-2), 63-68).

[0048] Briefly, 6 mL of the sample was transferred to a 15-mL centrifuge tube along with 0.1 mL of 1% potassium cyanide (KCN) solution and 2.5 mL of 0.4 M sodium acetate (pH 4) solution. The samples were then vortexed and autoclaved at 100°C for 30 min before being cooled in an ice bath. Prior to extraction, samples were centrifuged at 11,000 × g at 4 °C. Oasis HLB SPE columns (3-cc, 60-mg bed mass, Waters, MA, USA) were equilibrated with 2 mL of methanol, followed by equilibration with 2 mL of distilled water. Sample was then loaded into

the cartridge, followed by a 2 mL of a 5% (v/v) methanol wash and a 1 mL of a 90% methanol elution. The extracted samples were then filtered through a 0.22 µm filter and stored in a 2 mL amber vial before analysis. The sample analysis was conducted using HPLC (Shimadzu, Kyoto, Japan) coupled with a PDA detector. A Zorbax Eclipse Plus C18 column (150 x 4.6 mm; Agilent Santa Clara, CA, USA) was used for separation with gradient elution with a flow rate of 0.25 mL/min and column temperature at 40 °C with PDA set at 550 nm.

[0049] Vitamin B₁₂ results are presented in FIG. 3.

[0050] FIG. 3 shows that GCY supplementation led to vitamin B₁₂ production by all the examined bifidobacterial strains, B94, BI-04 and BB536, while GC supplementation led to vitamin B₁₂ production by *B. lactis* strains B94 and BI-04. Strain BI-04 produced 3.01 and 4.56 µg/L of vitamin B₁₂ in GC and GCY soy whey, respectively. Strain B94 produced 2.06 µg/L of vitamin B₁₂ in GC and GCY soy whey. Strain BB536 produced 2.09 µg/L of vitamin B₁₂ in GCY soy whey. Strain BI-04 was capable of growing in the control (CN) and glucose (G) supplemented soy whey (without cysteine addition), however, this did not lead to the production of vitamin B₁₂, which led us to believe that a reducing environment created by cysteine (the reducing agent) may be necessary for the B₁₂ production. Strain BB536 did not produce vitamin B₁₂ in a reducing environment created by GC possibly due to the minimal growth.

[0051] Vitamin B₁₂ production in bacteria can occur through two distinct pathways, aerobic and anaerobic. The aerobic pathway starts with oxygen being a requirement in corrin ring synthesis and cobalt chelation happening late in the pathway. The anaerobic pathway is differentiated by an early cobalt chelation to precorrin-2. *Bacillus megaterium* and *Propionibacterium freudenreichii*, the industrial producers of vitamin B₁₂, produce it through the aerobic pathway. Interestingly, both microbes are able to produce vitamin B₁₂ when grown in aerobic and anaerobic condition (*Appl. Microbiol. Biotechnol.* 2018, 102, 515–538). This is not the case for strain BI-04. Strain BI-04 was able to grow in soy whey supplemented with and without cysteine. However, Vitamin B₁₂ was only detected in fermented soy whey

supplemented with cysteine, which led us to believe that production of vitamin B₁₂ by *B. lactis* strains can only happen in a strictly anaerobic condition.

Example 4

[0052] Dose-response Analysis

[0053] Duplicate laboratory scale fermentations were conducted once. Firstly, soy whey was supplemented with ascorbic acid at dosages of 0, 0.1, 0.25, 0.5, 0.75 and 1.0 g/L or with cysteine at dosages 0, 0.1, 0.25, 0.5, 0.75 and 1.0 g/L or with 0.5 g/L cysteine-HCl. The pH of the supplemented soy whey was adjusted to 6.5 and inoculated with 1% (v/v) BI-04 strain to attain a cell count of about 6 log CFU/mL. Incubation was carried out at 37 °C for a duration of 48 hours for fermentation. Aliquots of 40 mL were then transferred to 50-mL centrifuge tubes respectively. The results are tabulated and shown in FIG. 4.

[0054] The results show that supplementation of ascorbic acid or cysteine at certain levels is necessary for vitamin B₁₂ production in plant-based medium by strain BI-04. FIG. 4 shows the production of vitamin B₁₂ by strain BI-04 against the different doses of added reducing agents to soy whey. Dosage of a reducing agent shows a positive correlation with vitamin B₁₂ concentration in soy whey. FIG. 4 shows that at 0 g/L dose of cysteine (Cy) and ascorbic acid (AA), no B₁₂ was detected. At 0.1 g/L of cysteine (Cy) and ascorbic acid (AA), no B₁₂ was detected. However, at 0.25 g/L, vitamin B₁₂ was detected in soy whey supplemented with ascorbic acid (AA) but not cysteine (Cy). At 0.5 g/L, 0.75 g/L and 1.0 g/L of cysteine (Cy) or ascorbic acid (AA), vitamin B₁₂ was detected in all samples. From the results, it appears that there is a dose-response relationship in vitamin B₁₂ production with increasing reducing agent supplementation. From these trials, it is evident that the level of B₁₂ production increases with the addition of more cysteine. The same can be said for ascorbic acid barring the dip of B₁₂ concentration at 0.5 g/L dose.

Example 5

[0055] Creating Anaerobic Environment using AnaeroGen™

[0056] Duplicate laboratory scale fermentations were conducted once. Volumes of 1,000 mL of soy whey were used for each treatment where 500 mL were used for each replicate. Treatments were either 0.5 g/L cysteine supplement, AnaeroGen™, or control (no cysteine supplement, no AnaeroGen™). Soy whey was adjusted to pH 6.5 and inoculated with 1% (v/v) BI-04 strain to attain a cell count of about 6 log CFU/mL. Incubation was carried out at 37°C for a duration of 48 hours. Samples were taken after 48 hours of fermentation. Vitamin B₁₂ content and pH of the soy whey were taken, and the readings are shown in Table 1.

[0057] AnaeroGen™ is an anaerobic atmosphere generation system placed in a sealed jar. The oxygen present is rapidly absorbed while carbon dioxide is generated. Within 30 minutes, oxygen level in the jar will be below 1% and carbon dioxide levels will be between 9% and 13%. This test uncovered that an anaerobic condition is necessary for *B. lactis* strain to produce vitamin B₁₂ in soy whey. Table 1 shows that 0.24 µg/L vitamin B₁₂ was detected in anarogen treated soy whey and 0.35 µg/L vitamin B₁₂ in cysteine supplemented soy whey, while no B₁₂ was detected in the control. This further strengthens the necessity of an anaerobic environment for B₁₂ production, and that reducing agent supplementation is sufficient.

Table 1 - Vitamin B₁₂ content and pH of soy whey after 48-hour fermentation.

Treatment	pH	Vitamin B ₁₂ (µg/L)
Control	4.12 ± 0.03	0.00 ± 0.00
AnaeroGen™	4.39 ± 0.07	0.24 ± 0.01
Cysteine	4.01 ± 0.01	0.35 ± 0.04

Example 6

[0058] Volume Size and Anaerobic Environment

[0059] Duplicate laboratory scale fermentations were conducted once. Volumes of 250 mL, 500 mL, and 1,000 mL of soy whey were aliquoted to volume-specific blue cap bottles. Soy whey was adjusted to pH 6.5 and inoculated with 1% (v/v) BI-04 strain to attain a cell count of

about 6 log CFU/mL. Incubation was carried out at 37 °C for a duration of 48 hours. Samples were taken after 48 hours of fermentation. Vitamin B₁₂ content and pH of the soy whey were taken, and the readings are shown in Table 2.

[0060] Fermentation volume is hypothesized to be able to create an anaerobic environment to produce vitamin B₁₂. The largest volume tested is 1,000 mL, which did not lead to any vitamin B₁₂ production (Table 2). This could be due to volumes up to several hundred liters needed to achieve an anaerobic environment. This shows that different volumes of fermentation could lead to different fermentation outcomes. For example, the higher the volume (or height), the less liquid is exposed to air compared to a smaller fermentation volume. This can lead to a difference in oxygen content at the top and bottom of the fermentation vessel. This Example shows that for a fermentation volume up to 1000mL, anaerobic conditions were not achieved, as shown by the lack of vitamin B₁₂ (see Table 2).

Table 2 - Vitamin B₁₂ content and pH of soy whey after 48-hour fermentation

Vessel volume (mL)	Vessel height (cm)	pH	Vitamin B ₁₂ (µg/L)
250	8	4.09 ± 0.01	0
500	11	4.12 ± 0.03	0
1000	15.5	4.11 ± 0.03	0

Example 7

[0061] Other Plant-Based Media as Fermentation Substrates for Vitamin B₁₂ Enrichment

[0062] Different plant-based media were investigated for vitamin B₁₂ production by bifidobacterial fermentation.

[0063] Duplicate laboratory scale fermentations were conducted on pear and carrot. The pear and carrot were purchased from a local supermarket. Individual ingredients were diced and blended using in a blender and the juices were strained through a strainer and collected. Each

type of juice was either supplemented with 0.5 g/L cysteine or not supplemented, followed by pH adjustment to 6.5 and pasteurization at 90°C for 30 min. Juices were inoculated with 1% (v/v) to attain about 6 log CFU/mL of B1-04 strain and monitored for 48 hours for pH change and vitamin B₁₂ production. Aliquots of 12 mL were then transferred to 15-mL centrifuge tubes respectively. Incubation was done at 37 °C for the 48-hour fermentation period. The vitamin B₁₂ content and pH of the different plant-based media were measured, and the results are shown in Table 3.

[0064] As shown in Table 3, reducing agent supplementation is needed as vitamin B₁₂ is only detected in pear and carrot juice supplemented with cysteine.

Table 3 - Vitamin B₁₂ content and pH of different plant-based media after 48-hour fermentation

Plant-based medium	pH	Vitamin B ₁₂ (µg/L)
Pear	5.61 ± 0.09	0.00 ± 0.00
Pear – with cysteine	4.54 ± 0.03	3.51 ± 0.35
Carrot	3.79 ± 0.01	0.00 ± 0.00
Carrot – with cysteine	3.77 ± 0.00	1.28 ± 0.35

[0065] Conclusions

[0066] The Examples and results demonstrated the viability of utilizing bifidobacteria to produce vitamin B₁₂ in plant-based media supplemented with a reducing agent such as cysteine, cysteine-HCl or ascorbic acid to create anaerobic conditions. Both *B. lactis* B1-04 and B94 strains exhibited excellent growth in GC and GCY supplemented soy whey, while BB536 strain exhibited excellent growth in GCY supplemented soy whey and with lesser growth in GC supplemented soy whey. Metabolic activities led to a decrease in pH. Vitamin B₁₂ was only synthesized in the presence of a reducing agent. The reducing agent dose shows a positive correlation with the vitamin B₁₂ concentration. Anaerobic environment needed for vitamin B₁₂ production can be achieved through reducing agent supplementation. Other plant-based media, such as pear and carrot juice, can also be used with bifidobacteria to produce vitamin B₁₂ in the

plant-based media when the plant-based media is supplemented with a reducing agent. In conclusion, reducing agent supplementation is one feasible way for vitamin B₁₂ production in plant-based media using bifidobacteria.

[0067] Although embodiments of the invention have been shown and described, the invention is not limited to the described embodiments. Instead, it would be appreciated by those skilled in the art that various modifications and variations can be made to the embodiments of the invention without departing from the scope of the invention, the scope of which is set forth in the following claims.

Claims

1. A method of enhancing vitamin B₁₂ production in a plant-based medium, the method comprising:
inoculating a plant-based medium supplemented with a reducing agent with a bifidobacterium; and
subjecting the plant-based medium supplemented with the reducing agent to conditions conducive to anaerobic fermentation of the plant-based medium for enhancing vitamin B₁₂ production in the plant-based medium.
2. The method of claim 1, wherein the reducing agent is selected from the group consisting of cysteine, cysteine-HCl and ascorbic acid, and combinations thereof.
3. The method of claim 1, wherein the plant-based medium is supplemented with cysteine, with ascorbic acid, with glucose and cysteine (or cysteine-HCl), or with glucose, cysteine (or cysteine-HCl) and yeast extract.
4. The method of claim 1 or 3, wherein the bifidobacterium is a species selected from the group consisting of *Bifidobacterium animalis* subsp. *lactis* and *Bifidobacterium longum* subsp. *longum*.
5. The method of claim 1 or 4, wherein the bifidobacterium is a strain selected from the group consisting of *Bifidobacterium animalis* subsp. *lactis* B1-04 (B1-04), *Bifidobacterium animalis* subsp. *lactis* LATF1 B94 (B94) and *Bifidobacterium longum* subsp. *longum* BB536 (BB536).

6. The method of claim 1, wherein the plant-based medium is selected from the group consisting of soy whey, carrot and pear.
7. The method of claim 6, wherein the plant-based medium is soy whey.
8. The method of claim 3, wherein the plant-based medium is supplemented with glucose and cysteine (or cysteine-HCl), wherein the weight ratio of glucose to cysteine (or cysteine-HCl) ranges from 20:1 to 50:1 (w/w).
9. The method of claim 3, wherein the plant-based medium is supplemented with glucose, cysteine (or cysteine-HCl) and yeast extract, wherein the weight ratio of glucose to cysteine (or cysteine-HCl) to yeast extract ranges from 20:1:5 to 50:1:15 (w/w/w).
10. The method of any one of claims 1 to 9, wherein the conditions conducive to anaerobic fermentation include adjusting pH of the plant-based medium supplemented with the reducing agent to 6 to 7.
11. The method of any one of claims 1 to 10, wherein the conditions conducive to anaerobic fermentation include maintaining the temperature of anaerobic fermentation at a temperature ranging from 36 °C to 38 °C.
12. The method of any one of claims 1 to 11, wherein the conditions conducive to anaerobic fermentation include inoculating the plant-based medium supplemented with the reducing agent with the bifidobacterium at an inoculation ratio of 0.5% to 5% (v/v) to achieve an initial cell count of at least 5 log CFU/mL.

13. The method of any one of claims 1 to 12, wherein the conditions conducive to anaerobic fermentation include incubating the plant-based medium supplemented with the reducing agent with the bifidobacterium at a temperature ranging from 36 °C to 38 °C for a duration ranging from 46 to 50 hours.

14. The method of claim 1, further comprising:
subjecting the plant-based medium supplemented with the reducing agent to pasteurization prior to inoculating the plant-based medium supplemented with the reducing agent with the bifidobacterium.