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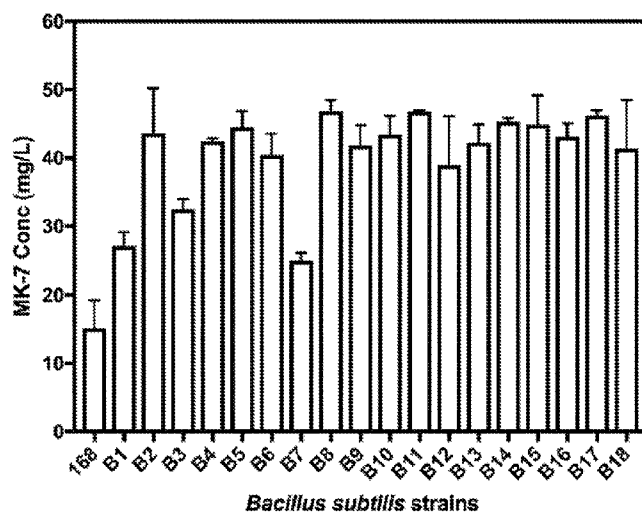


FIGURE 1

(57) Abstract: The present disclosure relates to compositions and processes for producing vitamin K and in particular to compositions and processes for producing menaquinone-7. According to one embodiment there is provided a process for producing vitamin K the process comprising: combining a first carbon source, a first nitrogen source and a fermentative bacterium to provide a fermentation mixture; an incubation phase wherein the fermentation mixture is incubated such that the fermentative bacterium consumes the first carbon source and thereby produces vitamin K; and a feeding phase wherein a second carbon source and a second nitrogen source are fed into the fermentation mixture such that the fermentative bacterium consumes the second carbon source and thereby produces vitamin K.



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COMPOSITIONS AND PROCESSES FOR PRODUCING VITAMIN K

Field of the disclosure

[0001] The present disclosure relates to compositions and processes for producing vitamin K and in particular to compositions and processes for producing menaquinone-7.

Background

[0002] Any discussion of the prior art throughout the specification should in no way be considered as an admission that such prior art is widely known or forms part of the common general knowledge in the field.

[0003] Vitamins are typically organic compounds which organisms require for proper metabolism, but which organisms are unable to synthesise themselves. Menaquinone (vitamin K₂) is a sub-class of vitamin K and comprises a 2-methyl-1,4-naphthquinone head and isoprenoid tails of varying length (Bentley and Meganathan. *Microbiol. Rev.* 1982. 46(3): 241-280). Menaquinone plays important roles in blood coagulation, and in the prevention of osteoporosis and cardiovascular calcification (Knapen *et al. Osteopor. Int.* 2015. 24(9): 2499-2507). Menaquinone-7 (MK-7), a subcategory of vitamin K₂, has a longer half-life in the human body than its counterparts phyloquinone and menaquinone-4, and thus offers unique therapeutic benefits.

[0004] Despite these health benefits, many people do not receive sufficient vitamin K as part of their normal diet. This is due, in part, to the large volume of processed food and the small amount of green vegetables that are consumed in a modern diet. To account for this shortfall, many people consume vitamin K supplements.

[0005] Demand for vitamin K is strong, but its product cost remains high, largely due to high substrate costs and low yielding production techniques. In this context, there is a need for compositions and processes for producing vitamin K, and in particular, menaquinone-7.

Summary of the disclosure

[0006] In a first aspect, the present disclosure provides a process for producing vitamin K the process comprising:

combining a first carbon source, a first nitrogen source and a fermentative bacterium to provide a fermentation mixture;

an incubation phase wherein the fermentation mixture is incubated such that the fermentative bacterium consumes the first carbon source and thereby produces vitamin K; and

a feeding phase wherein a second carbon source and a second nitrogen source are fed into the fermentation mixture such that the fermentative bacterium consumes the second carbon source and thereby produces vitamin K.

[0007] The fermentative bacterium is *Bacillus subtilis*. In certain embodiments, the fermentative bacterium is *Bacillus subtilis natto*.

[0008] The vitamin K is preferably menaquinone-7.

[0009] In certain embodiments, the second carbon source is fed into the fermentation mixture as a plurality of pulsed doses.

[0010] The second carbon source may be fed into the fermentation mixture at a pulse rate of one dose every about 5 to 120 minutes. In some embodiments, the second carbon source is fed into the fermentation mixture at a pulse rate of one dose every about 5 to 60 minutes. In certain embodiments, the second carbon source is fed into the fermentation mixture at a pulse rate of one dose every about 5 to 30 minutes.

[0011] In some embodiments, each pulsed dose of the second carbon source lasts for a period of between about 2 minutes and 15 minutes.

[0012] In certain embodiments, the second nitrogen source is fed into the fermentation mixture as a plurality of pulsed doses.

[0013] The second nitrogen source may be fed into the fermentation mixture at a pulse rate of one dose every about 10 to 120 minutes. In some embodiments, the second nitrogen source is fed into the fermentation mixture at a pulse rate of one dose every about 30 to 90 minutes. In certain embodiments, the second nitrogen

source is fed into the fermentation mixture at a pulse rate of one dose every about 60 minutes.

[0014] In some embodiments, each pulsed dose of the second nitrogen source lasts for a period of between about 5 minutes and 15 minutes.

[0015] The second carbon source may be fed into the fermentation mixture at a feeding rate of between about 1 g/L/h and 10 g/L/h. Preferably, the second carbon source is fed into the fermentation mixture at a feeding rate of between about 2 g/L/h and 5 g/L/h.

[0016] The second nitrogen source may be fed into the fermentation mixture at a feeding rate of between about 1 g/L/h and 5 g/L/h. Preferably, the second nitrogen source is fed into the fermentation mixture at a feeding rate of between about 1 g/L/h and 3 g/L/h.

[0017] In certain embodiments, the first carbon source and the first nitrogen source are combined such that the first carbon source is present in the fermentation mixture at a concentration that does not exceed about 50 g/L. Preferably, the first carbon source and the first nitrogen source are combined such that the first carbon source is present in the fermentation mixture at a concentration that does not exceed about 25 g/L.

[0018] In certain embodiments, the first carbon source and the first nitrogen source are combined such that the first nitrogen source is present in the fermentation mixture at a concentration that does not exceed about 50 g/L. Preferably, the first carbon source and the first nitrogen source are combined such that the first nitrogen source is present in the fermentation mixture at a concentration that does not exceed about 25 g/L.

[0019] In some embodiments, the incubation phase lasts for a period of between about 3 hours and 10 hours.

[0020] The feeding phase may be triggered when the fermentation mixture reaches a predetermined pH. In certain embodiments, the feeding phase is triggered when the pH of the fermentation mixture rises above about 7.

[0021] In some embodiments, the feeding phase lasts for a period of at least about 20 hours. Preferably, the feeding phase lasts for a period of at least about 40 hours.

[0022] In certain embodiments, dissolved oxygen remains below about 50% in the fermentation mixture during the feeding phase for a period of at least about 10 hours. Preferably, dissolved oxygen remains below about 50% in the fermentation mixture during the feeding phase for a period of at least about 20 hours.

[0023] In some embodiments, the first carbon source and the second carbon source together remain at a concentration of less than about 10 g/L in the fermentation mixture for a period of at least about 10 hours during the feeding phase. The first carbon source and the second carbon source together may remain at a concentration of less than about 5 g/L in the fermentation mixture for a period of at least about 10 hours during the feeding phase. Preferably, the first carbon source and the second carbon source together remain at a concentration of less than about 5 g/L in the fermentation mixture for a period of at least about 20 hours during the feeding phase.

[0024] In certain embodiments, the fermentation mixture maintains a pH of between about 6 and 8 for at least about 10 hours during the feeding phase without addition of an acid or a base to the fermentation mixture. Preferably, the fermentation mixture maintains a pH of between about 6 and 8 for at least about 20 hours during the feeding phase without addition of an acid or a base to the fermentation mixture.

[0025] In some embodiments, the first carbon source and the second carbon source are independently selected from the group consisting of corn syrup, sucrose, inulin, maltodextrin, glycerol, fructose and glucose. The first carbon source and the second carbon source may be independently selected from inulin and glycerol. Preferably, the first carbon source and the second carbon source are glycerol.

[0026] In certain embodiments, the first nitrogen source and the second nitrogen source comprise proteins, peptides or amino acids.

[0027] The first nitrogen source and the second nitrogen source may be independently obtained from a plant or an animal. In some embodiments, the first nitrogen source and the second nitrogen source are independently obtained from soybean, pea, almond, peanut, wheat, rice, sorghum or milk. The first nitrogen source and the second nitrogen source may be obtained from a plant. In certain embodiments, the first nitrogen source and the second nitrogen source are independently obtained from a legume, a nut or a cereal. The first nitrogen source

and the second nitrogen source may be obtained from a legume. The legume is preferably a soybean plant or a pea plant.

[0028] In certain embodiments, the first nitrogen source and the second nitrogen source comprise less than about 300 mg of aromatic amino acids per gram of total nitrogen.

[0029] In some embodiments, the fermentation mixture further comprises an antifoam. The antifoam may be a silicone-based antifoam or an oil-based antifoam.

[0030] The process may produce menaquinone-7 at a concentration of at least about 100 mg/L. Preferably, the process produces menaquinone-7 at a concentration of at least about 110 mg/L.

[0031] In certain embodiments, the process produces menaquinone-7 at a rate of at least about 2 mg/L/h.

[0032] In a second aspect, the present disclosure provides vitamin K when produced by the process of the first aspect.

[0033] In a third aspect, the present disclosure provides a food or drink product comprising the vitamin K of the second aspect.

[0034] In a fourth aspect, the present disclosure provides a dietary supplement comprising the vitamin K of the second aspect.

[0035] In a fifth aspect, the present invention provides a process for producing vitamin K the process comprising:

combining a carbon source, a nitrogen source and a fermentative bacterium to provide a fermentation mixture, wherein the fermentation mixture comprises a higher concentration of the nitrogen source than the carbon source; and

incubating the fermentation mixture such that the fermentative bacterium consumes the carbon source and thereby produces vitamin K.

[0036] In certain embodiments, the fermentative bacterium is *Bacillus subtilis*. Preferably, the fermentative bacterium is *Bacillus subtilis natto*.

[0037] The vitamin K is preferably menaquinone-7.

[0038] Preferably, the concentration of the nitrogen source is at least twice the concentration of the carbon source in the fermentation mixture.

[0039] The fermentation mixture may comprise the carbon source at a concentration of between about 25 g/L and 80 g/L. Preferably, the fermentation mixture comprises the carbon source at a concentration of between about 40 g/L and 60 g/L.

[0040] The fermentation mixture may comprise the nitrogen source at a concentration of between about 40 g/L and 150 g/L. Preferably, the fermentation mixture comprises the nitrogen source at a concentration of between about 80 g/L and 125 g/L.

[0041] The carbon source may be selected from the group consisting of corn syrup, sucrose, inulin, maltodextrin, glycerol, fructose and glucose. In certain embodiments, the carbon source is selected from inulin and glycerol. Preferably, the carbon source is glycerol.

[0042] In certain embodiments, the nitrogen source comprises proteins, peptides or amino acids.

[0043] The nitrogen source may be obtained from a plant or an animal. In certain embodiments, the nitrogen source is obtained from soybean, pea, almond, peanut, wheat, rice, sorghum or milk. In some embodiments, the nitrogen source is obtained from a plant. The nitrogen source is obtained from a legume, a nut or a cereal. Preferably, the nitrogen source is obtained from a legume. The legume may be a soybean plant or a pea plant.

[0044] In certain embodiments, the nitrogen source comprises less than about 300 mg of aromatic amino acids per gram of total nitrogen.

[0045] The fermentation mixture may further comprise an antifoam. The antifoam may be a silicone-based antifoam or an oil-based antifoam.

[0046] In a sixth aspect, the present disclosure provides vitamin K when produced by the process of the fifth aspect.

[0047] In a seventh aspect, the present disclosure provides a food or drink product comprising the vitamin K of the sixth aspect.

[0048] In an eighth aspect, the present disclosure provides a dietary supplement comprising the vitamin K the sixth aspect.

Brief description of the drawings

[0049] **Figure 1.** MK-7 production by the domesticated lab strain of *Bacillus subtilis* (168) and 18 different isolates of *Bacillus subtilis natto* taken from commercial natto products.

[0050] **Figure 2.** MK-7 production using different carbon and nitrogen sources derived from food and agricultural products.

[0051] **Figure 3.** Effect of initial glycerol concentration on: (A) MK-7 production (black) and viable cell counts (clear); and (B) residual glycerol concentration (black) and final pH (clear).

[0052] **Figure 4.** Effect of initial soy protein concentration on: (A) MK-7 production (black) and viable cell counts (clear); and (B) residual glycerol concentration (black) and final pH (grey).

[0053] **Figure 5.** Fed-batch fermentation in a 2 L bioreactor using glycerol feeding rates of (A) 1.3 g/L/h, (B) 2.4 g/L/h and (C) 3 g/L/h. $\blacktriangle$: MK-7 titre, $\blacklozenge$: viable cell counts, $\blacksquare$: residue glycerol, dotted line: pH, solid line: dissolved oxygen.

Detailed description

Definitions

[0054] The term "about" is understood to refer to a range of +/- 10%, preferably +/- 5% or +/- 1% or, more preferably, +/- 0.1%.

[0055] The terms "comprise", "comprises", "comprised" or "comprising", "including" or "having" and the like in the present specification and claims are used in an inclusive sense, *ie*, to specify the presence of the stated features but not preclude the presence of additional or further features.

[0056] As used herein, the term "fermentative bacterium" refers to a bacterium that is capable of fermenting a substrate such as a carbon source to produce vitamin K, and preferably to produce menaquinone-7. A fermentative bacterium may include, for

example, *Lactococcus lactis*, *Leuconostoc lactis*, *Brochonthrix thermosphacta*, *Staphylococcus xylosus*, *Staphylococcus equorum*, *Arthrobacter nicotinae* *Bacillus amyloliquefaciens*, *Bacillus licheniformis* or *Bacillus subtilis*.

[0057] The term "milk" includes dairy and non-dairy milk. Dairy milk includes, *inter alia*, cow milk, goat milk and camel milk. Non-dairy milk includes, *inter alia*, soy milk, almond milk and rice milk.

Processes

[0058] The present disclosure provides a process for producing vitamin K the process comprising: combining a first carbon source, a first nitrogen source and a fermentative bacterium to provide a fermentation mixture; an incubation phase wherein the fermentation mixture is incubated such that the fermentative bacterium consumes the first carbon source and thereby produces vitamin K; and a feeding phase wherein a second carbon source and a second nitrogen source are fed into the fermentation mixture such that the fermentative bacterium consumes the second carbon source and thereby produces vitamin K. The fermentative bacterium may be *Bacillus subtilis*, such as *Bacillus subtilis natto*. The vitamin K is preferably menaquinone-7.

[0059] The incubation phase enables at least some of the first carbon source to be consumed by the fermentative bacterium. The duration of the incubation phase may therefore depend, in part, on the concentration of the first carbon source and the first nitrogen source in the fermentation mixture. As the first carbon source and the first nitrogen source are consumed by the fermentative bacterium, its concentration in the fermentation mixture drops. The feeding phase is then initiated to provide a new source of carbon and nitrogen, ie, the second carbon source and the second nitrogen. Those skilled in the art will understand that the incubation phase may last for a matter of seconds or minutes such as at least about 1 minute, at least about 2 minutes, at least about 5 minutes, at least about 10 minutes, at least about 20 minutes at least about 30 minutes, at least about 45 minutes or at least about 60 minutes, for example, when the concentration of the first carbon source is low. Alternatively, the incubation phase may last for at least about 2 hours, at least about 3 hours, at least about 4 hours, at least about 5 hours, at least about 6 hours, at least about 7 hours, at least about 8 hours, at least about 9 hours, at least about 10 hours, at least about 15 hours or at least about 20

hours, for example, when the concentration of the first carbon source is higher. Other factors may also influence the duration of the incubation phase, for example, factors that affect the rate of fermentation such as temperature, aeration and pH.

[0060] In preferred embodiments, the feeding phase will commence when the second carbon source and the second nitrogen source are fed into the fermentation mixture. However, it will be understood that the feeding phase may be initiated when either the second carbon source or the second nitrogen source is fed into the fermentation mixture. For example, in embodiments where the first nitrogen source and the second nitrogen source are the same (eg, soybean protein), that nitrogen source may be combined with the first carbon source by a feeding mechanism (eg, as a continuous flow or a plurality of pulsed doses) in order to provide the fermentation mixture. The feeding may continue through the incubation phase and into the feeding phase. In such embodiments, the feeding phase will commence when the second carbon source is fed into the fermentation mixture. In other embodiments, if the first carbon source and the second carbon source are the same (eg, glycerol), that carbon source may be combined with the first nitrogen source by a feeding mechanism (eg, as a continuous flow or a plurality of pulsed doses) in order to provide the fermentation mixture. The feeding may continue through the incubation phase and into the feeding phase. In such embodiments, the feeding phase will commence when the second nitrogen source is fed into the fermentation mixture.

[0061] The second carbon source and the second nitrogen source may be fed into the fermentation mixture at different rates with respect to each other. Alternatively, the second carbon source and the second nitrogen source may be fed into the fermentation mixture at substantially the same rate.

[0062] The feeding phase may last for a period of at least about 5 hours, such as at least about 10 hours, at least about 15 hours, at least about 20 hours, at least about 25 hours, at least about 30 hours, at least about 35 hours, at least about 40 hours, at least about 45 hours, at least about 50 hours, at least about 55 hours, or at least about 60 hours. In certain embodiments, dissolved oxygen remains below about 80% such as below about 50%, such as below about 45%, in the fermentation mixture during the feeding phase for a period of at least about 5 hours, at least about 10 hours, at least about 15 hours, at least about 20 hours, at least about 25 hours, at least about 30 hours,

at least about 35 hours, at least about 40 hours, at least about 45 hours, or at least about 50 hours during the feeding phase.

[0063] In some embodiments, the first carbon source and the second carbon source together remain at a concentration of less than about 10 g/L, such as less than about 9 g/L, or less than about 7 g/L, or less than about 6 g/L, or less than about 5 g/L, or less than about 4 g/L in the fermentation mixture for a period of at least about 5 hours, at least about 10 hours, at least about 15 hours, at least about 20 hours, at least about 25 hours, at least about 30 hours, at least about 35 hours, at least about 40 hours, at least about 45 hours, or at least about 50 hours during the feeding phase.

[0064] In certain embodiments, the fermentation mixture maintains a pH of between about 6 and 8 for at least about 5 hours, at least about 10 hours, at least about 15 hours, at least about 20 hours, at least about 25 hours, at least about 30 hours, at least about 35 hours, at least about 40 hours, at least about 45 hours, or at least about 50 hours during the feeding phase without addition of an acid or a base to the fermentation mixture.

[0065] In certain embodiments, the second carbon source and the second nitrogen source are fed into the fermentation mixture as a plurality of pulsed doses. However, it will be understood that the second carbon source or the second nitrogen source may be fed into the fermentation mixture as a continuous flow. Accordingly, in some embodiments, the present disclosure provides a process for producing vitamin K the process comprising: combining a first carbon source, a first nitrogen source and a fermentative bacterium to provide a fermentation mixture; an incubation phase wherein the fermentation mixture is incubated such that the fermentative bacterium consumes the first carbon source and thereby produces vitamin K; and a feeding phase wherein a second carbon source and a second nitrogen source are fed into the fermentation mixture such that the fermentative bacterium consumes the second carbon source and thereby produces vitamin K, wherein the second carbon source is fed into the fermentation mixture as a continuous flow, and wherein the second nitrogen source is fed into the fermentation mixture as a plurality of pulsed doses. In other embodiments, the present disclosure provides a process for producing vitamin K the process comprising: combining a first carbon source, a first nitrogen source and a fermentative bacterium to provide a fermentation mixture; an incubation phase wherein the fermentation mixture is incubated such that the fermentative

bacterium consumes the first carbon source and thereby produces vitamin K; and a feeding phase wherein a second carbon source and a second nitrogen source are fed into the fermentation mixture such that the fermentative bacterium consumes the second carbon source and thereby produces vitamin K, wherein the second nitrogen source is fed into the fermentation mixture as a continuous flow, and wherein the second carbon source is fed into the fermentation mixture as a plurality of pulsed doses.

[0066] In certain embodiments, the second carbon source is fed into the fermentation mixture at a pulse rate of one dose every about 5 to 120 minutes. For example, the second carbon source may be fed into the fermentation mixture at a pulse rate of one dose every about 5 to 100 minutes, or one dose every about 10 to 90 minutes, or one dose every about 10 to 80 minutes, or one dose every about 10 to 60 minutes, or one dose every about 10 to 45 minutes, or one dose every about 10 to 30 minutes, or one dose every about 15 to 30 minutes.

[0067] The second carbon source may be fed into the fermentation mixture at a feeding rate of less than about 20 g/L/h, such as less than about 15 g/L/h, such as less than about 10 g/L/h, such as less than about 9 g/L/h, such as less than about 8 g/L/h, such as less than about 7 g/L/h, such as less than about 6 g/L/h, such as less than about 5 g/L/h, such as less than about 4 g/L/h or less than about 3 g/L/h. In other embodiments, the second carbon source is fed into the fermentation mixture at a feeding rate of between about 1 g/L/h and 20 g/L/h, such as between about 1 g/L/h and 15 g/L/h, such as between about 1 g/L/h and 10 g/L/h, such as between about 2 g/L/h and 10 g/L/h, such as between about 2 g/L/h and 7 g/L/h, such as between about 2 g/L/h and 5 g/L/h.

[0068] By way of illustrative example, in embodiments where the second carbon source is fed into the fermentation mixture at a pulse rate of one dose every 60 minutes and with a feeding rate of 5 g/L/h, then it will be understood that each dose adds approximately 5 g of the second carbon source per litre of fermentation mixture. Alternatively, if the second carbon source is fed into the fermentation mixture at a pulse rate of one dose every 30 minutes and with a feeding rate of 5 g/L/h, then each dose adds approximately 2.5 g of the second carbon source per litre of fermentation mixture.

[0069] In certain embodiments, the second nitrogen source is fed into to the fermentation mixture at a pulse rate of one dose every about 5 to 120 minutes. For example, the second nitrogen source may be fed into the fermentation mixture at a pulse rate of one dose every about 5 to 100 minutes, or one dose every about 10 to 90 minutes, or one dose every about 10 to 80 minutes, or one dose every about 20 to 80 minutes, or one dose every about 30 to 80 minutes, or one dose every about 45 to 80 minutes, or one dose every about 50 to 70 minutes.

[0070] The second nitrogen source may be fed into the fermentation mixture at a feeding rate of less than about 20 g/L/h, such as less than about 15 g/L/h, such as less than about 10 g/L/h, such as less than about 9 g/L/h, such as less than about 8 g/L/h, such as less than about 7 g/L/h, such as less than about 6 g/L/h, such as less than about 5 g/L/h, such as less than about 4 g/L/h, such as less than about 3 g/L/h, or less than about 2 g/L/h. In other embodiments, the second nitrogen source is fed into the fermentation mixture at a feeding rate of between about 1 g/L/h and 20 g/L/h, such as between about 1 g/L/h and 15 g/L/h, such as between about 1 g/L/h and 10 g/L/h, such as between about 1 g/L/h and 5 g/L/h, such as between about 1 g/L/h and 4 g/L/h, such as between about 1 g/L/h and 3 g/L/h.

[0071] By way of illustrative example, in embodiments where the second nitrogen source is fed into the fermentation mixture at a pulse rate of one dose every 60 minutes and with a feeding rate of 5 g/L/h, then it will be understood that each dose adds approximately 5 g of the second nitrogen source per litre of fermentation mixture. Alternatively, if the second nitrogen source is fed into the fermentation mixture at a pulse rate of one dose 30 minutes and with a feeding rate of 5 g/L/h, then each dose adds approximately 2.5 g of the second nitrogen source per litre of fermentation mixture.

[0072] Each pulsed dose of the second carbon source may be administered over a short period of time such as over a period of about 1 second or less, or gradually such as over a period of about 10 minutes or more. In certain embodiments, each pulsed dose of the second carbon source lasts for at least about 10 seconds, such as at least about 20 seconds or at least about 30 seconds or at least about 1 minute or at least about 2 minutes or at least about 5 minutes or at least about 10 minutes or at least about 15 minutes or at least about 20 minutes. In some embodiments, each pulsed dose of the second carbon source lasts for a period of between about 1

minutes and 15 minutes, such as for a for a period of between about 2 minutes and 15 minutes, such as for a period of between about 2 minutes and 10 minutes, such as for a period of between about 5 minutes and 15 minutes. Each pulsed dose is separated by an interval wherein the second carbon source is not added to the fermentation mixture. During that interval, the second carbon source is consumed by the fermentative bacterium, causing its concentration to drop, before the next dose is pulsed into the fermentation mixture.

[0073] Each pulsed dose of the second nitrogen source may be administered over a short period of time such as over a period of 1 second or less, or gradually such as over a period of 10 minutes or more. In certain embodiments, each pulsed dose of the second nitrogen source lasts for at least about 10 seconds, such as at least about 20 seconds or at least about 30 seconds or at least about 1 minute or at least about 2 minutes or at least about 5 minutes or at least about 10 minutes or at least about 15 minutes or at least about 20 minutes. In some embodiments, each pulsed dose of the second nitrogen source lasts for a period of between about 1 minutes and 15 minutes, such as for a for a period of between about 2 minutes and 15 minutes, such as for a period of between about 5 minutes and 15 minutes. Each pulsed dose is separated by an interval wherein the second nitrogen source is not added to the fermentation mixture. During that interval, the second nitrogen source is consumed by the fermentative bacterium, causing its concentration to drop, before the next dose is pulsed into the fermentation mixture.

[0074] In certain embodiments, there is provided a process for producing menaquinone-7 the process comprising:

- combining a first carbon source, a first nitrogen source and *Bacillus subtilis* to provide a fermentation mixture;

- an incubation phase wherein the fermentation mixture is incubated such that the *Bacillus subtilis* consumes the first carbon source and thereby produces menaquinone-7; and

- a feeding phase wherein a second carbon source and a second nitrogen source are fed into the fermentation mixture such that the *Bacillus subtilis* consumes the second carbon source and thereby produces menaquinone-7,

- wherein the second carbon source and the second nitrogen source are fed into the fermentation mixture as a plurality of pulsed doses.

[0075] In some embodiments, there is provided a process for producing menaquinone-7 the process comprising:

combining a first carbon source, a first nitrogen source and *Bacillus subtilis* to provide a fermentation mixture;

an incubation phase wherein the fermentation mixture is incubated such that the *Bacillus subtilis* consumes the first carbon source and thereby produces menaquinone-7; and

a feeding phase wherein a second carbon source and a second nitrogen source are fed into the fermentation mixture such that the *Bacillus subtilis* consumes the second carbon source and thereby produces menaquinone-7,

wherein the second carbon source and the second nitrogen source are fed into the fermentation mixture as a plurality of pulsed doses,

and wherein the second carbon source is fed into the fermentation mixture at a pulse rate of one dose every about 5 to 30 minutes,

and wherein the second nitrogen source is fed into the fermentation mixture at a pulse rate of one dose every about 30 to 90 minutes.

[0076] In some embodiments, there is provided a process for producing menaquinone-7 the process comprising:

combining a first carbon source, a first nitrogen source and *Bacillus subtilis* to provide a fermentation mixture;

an incubation phase wherein the fermentation mixture is incubated such that the *Bacillus subtilis* consumes the first carbon source and thereby produces menaquinone-7; and

a feeding phase wherein a second carbon source and a second nitrogen source are fed into the fermentation mixture such that the *Bacillus subtilis* consumes the second carbon source and thereby produces menaquinone-7,

wherein the second carbon source and the second nitrogen source are fed into the fermentation mixture as a plurality of pulsed doses,

and wherein the second carbon source is fed into the fermentation mixture at a feeding rate of less than about 5 g/L/h and at a pulse rate of one dose every about 5 to 30 minutes,

and wherein the second nitrogen source is fed into the fermentation mixture at a feeding rate of less than about 2 g/L/h and at a pulse rate of one dose every about 30 to 90 minutes.

[0077] In some embodiments, there is provided a process for producing menaquinone-7 the process comprising:

combining a first carbon source, a first nitrogen source and *Bacillus subtilis* to provide a fermentation mixture;

an incubation phase wherein the fermentation mixture is incubated such that the *Bacillus subtilis* consumes the first carbon source and thereby produces menaquinone-7; and

a feeding phase wherein a second carbon source and a second nitrogen source are fed into the fermentation mixture such that the *Bacillus subtilis* consumes the second carbon source and thereby produces menaquinone-7,

wherein the second carbon source and the second nitrogen source are fed into the fermentation mixture as a plurality of pulsed doses,

and wherein the second carbon source is fed into the fermentation mixture at a feeding rate of less than about 5 g/L/h and at a pulse rate of one dose every about 5 to 30 minutes,

and wherein the second nitrogen source is fed into the fermentation mixture at a feeding rate of less than about 2 g/L/h and at a pulse rate of one dose every about 30 to 90 minutes,

and wherein each pulsed dose of the second carbon source lasts for a period of between about 2 minutes and 15 minutes,

and wherein each pulsed dose of the second nitrogen source lasts for a period of between about 5 minutes and 15 minutes.

[0078] The first carbon source and the second carbon source may be different or the same. The first carbon source and the second carbon source may be, for example, a simple sugar or a complex sugar. Preferably, the first carbon source and the second carbon source are complex sugars. In certain embodiments, the first carbon source and/or the second carbon source are a food or drink product, or the by-product of a food or drink manufacturing process. For example, the first carbon source and/or the second carbon source may be obtained from a plant or an animal. In certain embodiments, the first carbon source and the second carbon source are independently selected from the group consisting of corn syrup, oil such as sunflower or canola oil, starch, dextrin, amylose, amylopectin, malt oligo sugar, cyclodextrin, pullulan, sucrose, inulin, maltodextrin, maltose, sucrose, molasses, treacle, demerara, turbinado, golden syrup, maple syrup, honey, jaggery, glycerol,

fructose, fruit products, cereal products, sorbitol, piconillo, galactose and glucose. In preferred embodiments, the first carbon source and the second carbon source are independently selected from inulin and glycerol. In certain embodiments, the first carbon source and the second carbon source are glycerol. Those skilled in the art will be aware that several other carbon sources may be used in the processes of the present disclosure.

[0079] The first nitrogen source and the second nitrogen source may be different or the same. In certain embodiments, the first nitrogen source and/or the second nitrogen source are a food or drink product, or the by-product of a food or drink manufacturing process. The first nitrogen source and the second nitrogen source preferably comprise organic nitrogen compounds such as proteins, peptides or amino acids. The first nitrogen source and the second nitrogen source may be independently obtained from a plant or an animal. For example, the first nitrogen source and the second nitrogen source may be independently obtained from soybean (eg, soy protein isolate, soy milk, soybean flour, soybean paste or tofu), almond (eg, almond paste, almond milk, almond protein isolate and almond powder), milk (eg, cow milk, goat milk, camel milk, almond milk, rice milk, soy milk, milk powder and milk protein isolate), peanut (eg, peanut paste and peanut powder), wheat (including processed wheat such as bread, flour, breakfast cereal, biscuits, wheat protein isolate and Weet-Bix), sorghum (including processed sorghum such as bread, flour, breakfast cereal, sorghum protein isolate and biscuits), rice (including processed rice such as breakfast cereal, biscuits, rice protein isolate and rice milk), pea (eg, pea protein isolate, pea flour and pea paste), chia (including processed chia such as breakfast cereals, biscuits, flour, chia protein isolate and bread), sesame (including processed sesame, such as breakfast cereals, biscuits, flour, and sesame protein isolate) etc. In preferred embodiments, the first nitrogen source and the second nitrogen source are obtained from a plant such as a legume, a nut or a cereal. Preferably, the first nitrogen source and the second nitrogen source are obtained from a legume such as a soybean plant or a pea plant. Those skilled in the art will be aware that several other nitrogen sources may be used in the processes of the present disclosure.

[0080] In certain embodiments, there is provided a process for producing menaquinone-7 the process comprising:

combining a first carbon source, a first nitrogen source and *Bacillus subtilis* to provide a fermentation mixture, wherein the first carbon source is glycerol or inulin and the first nitrogen source is obtained from a legume;

an incubation phase wherein the fermentation mixture is incubated such that the *Bacillus subtilis* consumes the first carbon source and thereby produces menaquinone-7; and

a feeding phase wherein a second carbon source and a second nitrogen source are fed into the fermentation mixture such that the *Bacillus subtilis* consumes the second carbon source and thereby produces menaquinone-7, wherein the second carbon source is glycerol or inulin and the second nitrogen source is obtained from a legume.

[0081] In certain embodiments, there is provided a process for producing menaquinone-7 the process comprising:

combining a first carbon source, a first nitrogen source and *Bacillus subtilis* to provide a fermentation mixture, wherein the first carbon source is glycerol or inulin and the first nitrogen source is obtained from a legume;

an incubation phase wherein the fermentation mixture is incubated such that the *Bacillus subtilis* consumes the first carbon source and thereby produces menaquinone-7; and

a feeding phase wherein a second carbon source and a second nitrogen source are fed into the fermentation mixture such that the *Bacillus subtilis* consumes the second carbon source and thereby produces menaquinone-7, wherein the second carbon source is glycerol or inulin and the second nitrogen source is obtained from a legume,

and wherein the second carbon source and the second nitrogen source are fed into the fermentation mixture as a plurality of pulsed doses.

[0082] In certain embodiments, there is provided a process for producing menaquinone-7 the process comprising:

combining a first carbon source, a first nitrogen source and *Bacillus subtilis* to provide a fermentation mixture, wherein the first carbon source is glycerol and the first nitrogen source is obtained from soybean or pea;

an incubation phase wherein the fermentation mixture is incubated such that the *Bacillus subtilis* consumes the first carbon source and thereby produces menaquinone-7; and

a feeding phase wherein a second carbon source and a second nitrogen source are fed into the fermentation mixture such that the *Bacillus subtilis* consumes the second carbon source and thereby produces menaquinone-7, wherein the second carbon source is glycerol and the second nitrogen source is obtained from soybean or pea,

and wherein the second carbon source and the second nitrogen source are fed into the fermentation mixture as a plurality of pulsed doses.

[0083] In certain embodiments, there is provided a process for producing menaquinone-7 the process comprising:

combining a first carbon source, a first nitrogen source and *Bacillus subtilis* to provide a fermentation mixture, wherein the first carbon source is glycerol and the first nitrogen source is obtained from soybean or pea;

an incubation phase wherein the fermentation mixture is incubated such that the *Bacillus subtilis* consumes the first carbon source and thereby produces menaquinone-7; and

a feeding phase wherein a second carbon source and a second nitrogen source are fed into the fermentation mixture such that the *Bacillus subtilis* consumes the second carbon source and thereby produces menaquinone-7, wherein the second carbon source is glycerol and the second nitrogen source is obtained from soybean or pea,

and wherein the second carbon source and the second nitrogen source are fed into the fermentation mixture as a plurality of pulsed doses,

and wherein the second carbon source is fed into the fermentation mixture at a pulse rate of one dose every about 5 to 30 minutes,

and wherein the second nitrogen source is fed into the fermentation mixture at a pulse rate of one dose every about 30 to 90 minutes.

[0084] The processes disclosed herein produce high yields of menaquinone-7 with relatively little foaming. Notwithstanding, foaming can be further reduced by the addition of an antifoam such as a silicone-based antifoam or an oil-based antifoam. Those skilled in the art will be aware of several other antifoams that may be used in the processes of the present disclosure.

[0085] In certain embodiments, the processes of the present disclosure produce menaquinone-7 at a concentration of at least about 50 mg/L, such as at least about 60

mg/L, or least about 70 mg/L, or at least about 80 mg/L, or at least about 90 mg/L, or at least about 100 mg/L or least about 110 mg/L. The processes of the present disclosure may produce menaquinone-7 at a rate of at least about 1 mg/L/h, such as at least about 1.1 mg/L/h, or at least about 1.2 mg/L/h, or at least about 1.3 mg/L/h, or at least about 1.4 mg/L/h, or at least about 1.5 mg/L/h, or at least about 1.6 mg/L/h, or at least about 1.7 mg/L/h, or at least about 1.8 mg/L/h, or at least about 1.9 mg/L/h or at least about 2 mg/L/h.

[0086] The present disclosure also provides a process for producing vitamin K the process comprising: combining a carbon source, a nitrogen source and a fermentative bacterium to provide a fermentation mixture, wherein the fermentation mixture comprises a higher concentration of the nitrogen source than the carbon source; and incubating the fermentation mixture such that the fermentative bacterium consumes the carbon source and thereby produces vitamin K. The fermentative bacterium may be *Bacillus subtilis*, such as *Bacillus subtilis natto*. The vitamin K is preferably menaquinone-7. In such embodiments, the nitrogen source is preferably at least twice the concentration of the carbon source in the fermentation mixture. The fermentation mixture may comprise the carbon source at a concentration of between about 15 g/L and 100 g/L, such as about 15 g/L, or about 20 g/L, or about 25 g/L, or about 30 g/L, or about 35 g/L, or about 40 g/L or about 45 g/L, or about 50 g/L, or about 55 g/L, or about 60 g/L, or about 65 g/L, or about 70 g/L, or about 75 g/L or about 80 g/L, or about 90 g/L, or about 95 g/L or about 100 g/L. The fermentation mixture may comprise the nitrogen source at a concentration of between about 40 g/L and 170 g/L, such as about 40 g/L, or about 45 g/L, or about 50 g/L, or about 55 g/L, or about 60 g/L, or about 65 g/L, or about 70 g/L, or about 75 g/L, or about 80 g/L, or about 85 g/L, or about 90 g/L, or about 95 g/L, or about 100 g/L, or about 105 g/L, or about 110 g/L, or about 115 g/L, or about 120 g/L, or about 125 g/L, or about 130 g/L, or about 135 g/L, or about 140 g/L, or about 145 g/L, or about 150 g/L, or about 155 g/L, or about 160 g/L, or about 165 g/L or about 170 g/L.

[0087] The carbon source may be a simple sugar or a complex sugar. Preferably, the carbon source is a complex sugar. In certain embodiments, the carbon source is a food or drink product, or the by-product of a food or drink manufacturing process. For example, the carbon source may be obtained from a plant or an animal. In certain embodiments, the carbon source is selected from the group

consisting of corn syrup, oil such as sunflower or canola oil, starch, dextrin, amylose, amylopectin, malt oligo sugar, cyclodextrin, pullulan, sucrose, inulin, maltodextrin, maltose, sucrose, molasses, treacle, demerara, turbinado, golden syrup, maple syrup, honey, jaggery, glycerol, fructose, fruit products, cereal products, sorbitol, piloncillo, galactose and glucose. In preferred embodiments, the carbon source is inulin or glycerol. In certain embodiments, the carbon source is glycerol. Those skilled in the art will be aware that several other carbon sources may be used in the processes of the present disclosure.

[0088] The nitrogen source may be a food or drink product, or the by-product of a food or drink manufacturing process. The nitrogen source preferably comprises organic nitrogen compounds such as proteins, peptides or amino acids. The nitrogen source may be obtained from a plant or an animal. For example, the nitrogen source may be obtained from soybean (eg, soy protein isolate, soy milk, soybean flour, soybean paste or tofu), almond (eg, almond paste, almond milk, almond protein isolate and almond powder), milk (eg, cow milk, goat milk, camel milk, almond milk, rice milk, soy milk, milk powder and milk protein isolate), peanut (eg, peanut paste and peanut powder), wheat (including processed wheat such as bread, flour, breakfast cereal, biscuits, wheat protein isolate and Weet-Bix), sorghum (including processed sorghum such as bread, flour, breakfast cereal, sorghum protein isolate and biscuits), rice (including processed rice such as breakfast cereal, biscuits, rice protein isolate and rice milk), pea (eg, pea protein isolate, pea flour and pea paste), chia (including processed chia such as breakfast cereals, biscuits, flour, chia protein isolate and bread), sesame (including processed sesame, such as breakfast cereals, biscuits, flour, and sesame protein isolate) etc. In preferred embodiments, the nitrogen source is obtained from a plant such as a legume, a nut or a cereal. Preferably, the nitrogen source is obtained from a legume such as a soybean plant or a pea plant. Those skilled in the art will be aware that several other nitrogen sources may be used in the processes of the present disclosure.

[0089] In some embodiments, the present disclosure provides a process for producing menaquinone-7 the process comprising:

combining a carbon source, a nitrogen source and *Bacillus subtilis* to provide a fermentation mixture, wherein the fermentation mixture comprises a higher concentration of the nitrogen source than the carbon source; and

incubating the fermentation mixture such that the *Bacillus subtilis* consumes the carbon source and thereby produces menaquinone-7.

[0090] In some embodiments, the present disclosure provides a process for producing menaquinone-7 the process comprising:

combining a carbon source, a nitrogen source and *Bacillus subtilis* to provide a fermentation mixture, wherein the concentration of the nitrogen source is at least twice the concentration of the carbon source in the fermentation mixture; and

incubating the fermentation mixture such that the *Bacillus subtilis* consumes the carbon source and thereby produces menaquinone-7.

[0091] In some embodiments, the present disclosure provides a process for producing menaquinone-7 the process comprising:

combining a carbon source, a nitrogen source and *Bacillus subtilis* to provide a fermentation mixture, wherein the fermentation mixture comprises the carbon source at a concentration of between about 25 g/L and 80 g/L and the nitrogen source at a concentration of between about 60 g/L and 150 g/L; and

incubating the fermentation mixture such that the *Bacillus subtilis* consumes the carbon source and thereby produces menaquinone-7.

[0092] In some embodiments, the present disclosure provides a process for producing menaquinone-7 the process comprising:

combining a carbon source, a nitrogen source and *Bacillus subtilis* to provide a fermentation mixture, wherein the fermentation mixture comprises the carbon source at a concentration of between about 40 g/L and 60 g/L and the nitrogen source at a concentration of between about 80 g/L and 125 g/L; and

incubating the fermentation mixture such that the *Bacillus subtilis* consumes the carbon source and thereby produces menaquinone-7.

[0093] In some embodiments, the present disclosure provides a process for producing menaquinone-7 the process comprising:

combining a carbon source selected from glycerol and inulin, a nitrogen source obtained from a legume, and *Bacillus subtilis* to provide a fermentation mixture, wherein the fermentation mixture comprises the carbon source at a concentration of between about 40 g/L and 60 g/L and the nitrogen source at a concentration of between about 80 g/L and 125 g/L; and

incubating the fermentation mixture such that the *Bacillus subtilis* consumes the carbon source and thereby produces menaquinone-7.

Compositions

[0094] The present disclosure also provides vitamin K when produced by the processes described herein. Although the presently described processes are suitable for producing vitamin K, such as menaquinone-7, those skilled in the art will understand that the processes described herein may be used to produce compounds other than vitamin K. For example, *Bacillus subtilis* may be employed in the processes of the present disclosure to produce secondary metabolites such as lipopeptides, iturin A, surfactin and fengycin which could be used in biosurfactants, antibiotics and antiviral agents.

[0095] Also provided herein are food and drink products comprising vitamin K which has been produced by the processes of the present disclosure. Suitable food products may include breakfast cereals, bread, biscuits, yoghurt, cheese, muesli bars, spreads, snack bars, soup, cake, processed meat products, soybean products such as tofu, processed potato or cereal products, noodles, vermicelli, macaroni, spaghetti, jam, oils and fats such as butter or margarine, confectionary etc. Suitable drink products may include dairy and non-dairy milk-based products, breakfast drinks, infant drinks, probiotics, juices, malt-based beverages, teas etc.

[0096] In preferred embodiments, the food and drink products comprise ingredients from which the carbon or nitrogen source used in the fermentation mixture have been obtained. For example, in embodiments where the nitrogen source is obtained from a cereal, the food product comprising the vitamin K may be a breakfast cereal, a muesli bar, a biscuit, bread, etc. Likewise, in embodiments where the nitrogen source is obtained from soybean, the drink product may be a soy milk-based drink. Those skilled in the art will understand that the vitamin K produced by the processes of the present disclosure may be used in a variety of other food and drink products.

[0097] The present disclosure also provides a dietary supplement comprising vitamin K which has been prepared by the processes of the present disclosure. Suitable dietary supplements may be in the form of a drink, a probiotic, a pill, a tablet, a dragee, a capsule or a powder. Those skilled in the art will understand that the dietary supplement may take a variety of other forms.

Examples

Chemicals

[0098] Soy protein isolate was obtained from a commercial supplier (Redox). Antifoam A (A6582) was purchased from Sigma Aldrich. HPLC standard menaquinone-7 (sc-218691) and menaquinone-9 (sc-211788) were purchased from Santa Cruz Biotechnology.

Isolation of microorganisms

[0099] Menaquinone-7-producing strains were isolated from commercially available natto. Samples (typically one soy bean) were streaked onto tryptic soy agar plates (tryptone 17g/L, soytone 3g/L, NaCl 5g/L, K₂HPO₄ 2.5g/L, agar 15 g/L) and grown at 30°C for 24 hours. Single colonies from these plates were isolated and grown in 5 mL tryptic soy broth at 37°C overnight. Glycerol stocks were prepared by combining sterile glycerol with overnight cultures to 10% (v/v), and storing at -80°C. Strain types were confirmed by performing 16s rRNA sequence analysis. Genomic DNA was extracted following a FastPrep purification protocol (Yeates and Gillings. *Lett. in Appl. Micro.* 1998. 27(1): 49-53) and was used as a template for the PCR amplification of corresponding 16S rRNA fragments. The amplified fragments were sequenced using the Sanger sequencing service provided by the Australian Genome Research Facility, NSW Node, and analysed using Basic Local Alignment Search Tool (BLAST).

Culture conditions

[00100] Culture media for screening high menaquinone-7-producing strains comprised 18.9% (w/v) soy peptone, 5% (w/v) yeast extract, 5% (w/v) glycerol and 0.6% (w/v) K₂HPO₄. Culture media for screening nitrogen and carbon sources comprised 10% (w/v) nitrogen sources and 5% (w/v) carbon sources with supplementation of 0.6% K₂HPO₄. Optimisation experiments were performed using glycerol concentrations of between 2 and 15% (w/v), and soy protein concentrations of between 3.7 and 13.7% (w/v), while the concentration of K₂HPO₄ and yeast extract was fixed at 0.6% (w/v) and 0.5% (w/v) respectively.

[00101] Screening of *Bacillus subtilis natto* strains was carried out in 5 mL culture in 25 mL amber vials at 40°C for 144 hours without shaking. Selection of nitrogen and carbon

source was carried out in 10 mL culture in 25 mL amber vials at 40°C for 144 hours without shaking. Shake flask experiments were carried out in 20 mL medium in 100 mL flasks at 40°C for 144 hours with shaking (120 rpm). *Bacillus subtilis natto* spore solution (4×10^8 CFU/mL) was used to inoculate the flask media at 2% (v/v).

[00102] A 2 L bench scale bioreactor (New Brunswick™ BioFlo®/ CelliGen® 115) was used to carry out the fed-batch experiments with an initial working volume of 800 mL. The inoculation size was 2% (v/v) with an overnight culture of seed media of the same composition as the initial culture. The incubation temperature was controlled at 40°C and the dissolved oxygen level was maintained at 20 - 50% by adjusting the aeration rate between 1 - 5 vvm and the agitation rate between 500 - 1000 rpm. The feeding rates of glycerol and soy protein solution for the fed-batch cultures were controlled via speed-adjustable pumps installed in the bioreactor. A built-in foam sensor was used to prevent foam formation by adding antifoam A to the culture as appropriate.

Viable cell counts

[00103] Viable cell counts were determined by performing serial dilutions of the homogenised fermented samples with sterile phosphate buffer solution. Serial dilutions were performed to a final count of 30-300 colonies per plate (typical samples were diluted by 4-8 orders of magnitude). The number of colonies was counted after 24 hours incubation at 30°C and expressed as colony forming units (CFU) per mL sample.

Measurement of glycerol and menaquinone-7 concentrations

[00104] The concentration of glycerol was assayed with a free glycerol determination kit from Sigma Aldrich (USA). To determine the amount of glycerol which remained unconsumed in the culture media, 10 µL of water, glycerol standard (0.26 g/L, G7793, Sigma Aldrich) and sample were added to 800 µL free glycerol reagent (F6428, Sigma Aldrich) and incubated at 37°C for 5 minutes. The UV absorbance of water, standard and sample at 540 nm were recorded as A_{blank} , A_{standard} and A_{sample} . The glycerol concentration was calculated as follows:

$$\text{glycerol concentration} = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{standard}} - A_{\text{blank}}} \times \text{standard concentration}$$

[00105] MK-7 was extracted from the fermentation media using organic solvent 2-propanol and *n*-hexane mixture in 1:2:5 ratio (culture medium: 2-propanol: *n*-hexane). A

predetermined amount (to a final concentration of 50 mg/L) of internal standard menaquinone-9 was spiked into the fermentation media before extraction. The mixture was vigorously vortexed after addition of each solvent and then centrifuged to facilitate phase separation. The organic phase was then separated and evaporated under vacuum to recover the extracted MK-7.

[00106] An Agilent HPLC HP1050 (Hewlett-Packard, USA) equipped with a single channel UV detector and a Gemini C₁₈ 110A column (150 mm × 4.60 mm × 5 µm, Phenomenex, USA) was used at 40°C for the analysis of MK-7 concentration. Methanol:dichloromethane (9:1, (v/v)) was used as the mobile phase with a flow rate of 1 mL/min. A single channel UV wavelength of 248 nm was used for calibration and analysis. The calibration curve was determined by performing linear regression to 5 standard points between 5 mg/L and 100 mg/L ($R^2 = 0.9999$). The MK-7 titres were also confirmed by analysis of a separate HPLC system (Shimadzu Prominence-i LC-2030) using a C₁₈ column (150 mm × 2.0 mm × 3 µm particle size, 12 nm pore size, YMC-Pack ODS-AM), a post-column zinc column and a fluorescence detector (Shimadzu RF-20Axs, 35°C cell temperature, excitation wavelength 243 nm and emission wavelength 430 nm). The mobile phase contained methanol:dichloromethane (9:1, v/v), ZnCl₂ 1.37 g/L, sodium acetate 0.41 g/L and glacial acetic acid 0.30 g/L, and ran at a flowrate of 0.3 mL/min and a temperature of 40°C. The calibration curve was determined between five standard concentrations between 156.25 µg/L and 2500 µg/L (linear regression, $R^2 = 0.9999$).

Example 1: screening *Bacillus subtilis* strains

[00107] Pure *Bacillus subtilis natto* colonies were isolated from 18 different commercial natto products. Final MK-7 titres varied from 25 mg/L to 50 mg/L. Referring to Figure 1, isolate B11 produced the highest amount of MK-7 with the least variation within the three replicates, and was therefore chosen for future media optimisation and scale-up. However, as shown in Figure 1, several isolates produced MK-7 in amounts that were comparable to B11.

Example 2: media development

[00108] A range of food and drink products, and carbon sources including simple sugars, oligosaccharide mixtures such as inulin and corn syrup were screened as

substrates for MK-7 fermentation. Different substrates were paired to identify the most effective combinations. Of the carbon sources tested, glycerol was found to support the highest rate of MK-7 production (Figure 2). Surprisingly, a large amount of MK-7 was produced by *Bacillus subtilis* (24 mg/L) when inulin was used as the carbon source, and paired with soy protein. In comparison, when fructose was used as the carbon source, the MK-7 concentration was 12 mg/L. This is an interesting finding since inulin is essentially the polymer form of fructose.

[00109] With regard to the food proteins tested, soy protein was found to yield the highest MK-7 titre when paired with glycerol (Figure 2). Another legume protein, yellow pea protein, was also tested, and was found to yield 25 mg/L MK-7, a comparable amount to soy protein at 27 mg/L. Pea protein may therefore serve as an allergen-free alternative to soy protein as a nitrogen source for MK-7 production.

[00110] Despite its high protein content, milk protein isolate supported less than 5 mg/L MK-7 production across the range of carbon sources studied (Figure 2). This may be due to the amino acid profile of milk protein powder, which is rich in aromatic tyrosine (311 mg/g total nitrogen) compared to soybean protein and pea protein (196 mg/g total nitrogen and 170 mg/g total nitrogen respectively) (FAO, 1970).

Example 3: glycerol concentration

[00111] Various initial concentrations of glycerol were tested, from 20 g/L to 150 g/L. It was found that 50 g/L glycerol supported the highest MK-7 production (30 mg/L), as well as the highest cell density (1.5×10^9 CFU/mL) (Figure 3A). At the end of the incubation period, the residual glycerol concentration in the media fell to below 0.5 g/L, indicating that most glycerol was consumed. The final pH of the culture media correlated with the residual glycerol concentration (Figure 3B). On the basis of these results, an initial glycerol concentration of 50 g/L was used to determine the optimum concentration of soy protein.

Example 4: soy protein concentration

[00112] Four initial soy protein concentrations were tested, from 37 g/L to 137 g/L, with a constant glycerol concentration of 50 g/L. 5 g/L of yeast extract was added to the culture media to provide metal elements and vitamins for cell growth and metabolite production.

[00113] It was found that 100 g/L soy protein supported the highest MK-7 production at 30 mg/L (Figure 4A). A slightly higher cell density (2.1×10^9) was achieved when 74 g/L soy protein was used in the media compared to 100 g/L protein (1.5×10^9). The residual glycerol concentrations in all conditions tested were below 0.5 g/L, indicating that most of the glycerol had been exhausted (Figure 4B). The pH of the fermentation mixture remained at about 7.

Example 5: dual feeding fermentation process

[00114] As menquinone-7 plays a role in electron transport (Bentley and Meganathan. *Microbiol. Rev.* 1982. 46(3): 241-280), its production may be supported by highly aerobic conditions. However, high protein concentrations together with high rates of aeration and agitation, can lead to unwanted foaming. Antifoam can reduce foaming; however, excessive use of silicone-based antifoam may decrease the oxygen transfer rate and therefore compromise efficient MK-7 production. Vegetable oil-based antifoam can also reduce foaming as well as provide another carbon source; however, large quantities are often required to substantially reduce foaming, and the addition of oils can also result in separation of the fermentation mixture into two phases.

[00115] The present inventors developed a dual feeding strategy to address the foaming issue and meet substrate demands for achieving efficient MK-7 production. A fermentation mixture was prepared by combining 20 g/L of soy protein and 20 g/L of glycerol along with *Bacillus subtilis* in an initial working volume of 800 mL. The fermentation mixture was incubated, and as the culture entered the exponential growth phase, the dissolved oxygen began to decrease (Figure 5). The pH initially dropped from a starting pH of 7.8 to a pH of 6.5 as glycerol was consumed, before rising again when the glycerol was exhausted and the cells started to metabolise soy protein as a carbon source. A feeding phase, in which glycerol and soy protein were fed into the fermentation mixture, was triggered when the pH rose above 7. This generally occurred after an initial fermentation phase of about 5 to 6 hours. The dual feeding process enabled the pH of the fermentation mixture to be maintained at a suitable level without the addition of an acid or base.

[00116] During the feeding phase, glycerol was fed into the fermentation mixture as a plurality of pulsed doses at three different feeding rates; namely, 1.3 g/L/h, 2.4 g/L/h and 3 g/L/h. When glycerol was fed at a feeding rate of 1.3 g/L/h, it was pulsed at a rate of

one dose every 60 minutes. Each pulsed dose lasted about 10 minutes and was followed by a 50 minute interval in which no glycerol was added. When glycerol was fed at a feeding rate of 2.4 g/L/h, it was pulsed at a rate of one dose every 30 minutes. Each pulsed dose lasted about 10 minutes and was followed by a 20 minute interval in which no glycerol was added. When glycerol was fed at a feeding rate of 3 g/L/h, it was pulsed at a rate of one dose every 25 minutes. Each pulsed dose lasted about 5 minutes and was followed by a 20 minute interval in which no glycerol was added. In each experiment, soy protein was fed into the fermentation mixture as a plurality of pulsed doses, at a rate of between 1.3 g/L/h and 1.6 g/L/h. The soy protein was fed at a pulse rate of one dose every 60 minutes. Each pulsed dose lasted about 10 minutes and was followed by a 50 minute interval in which no soy protein was added.

[00117] Under each condition, cell densities reached a maximum after about 12 hours. Cell density then remained relatively constant as the cells entered the stationary phase. Substantial MK-7 was produced by cells in the stationary phase, which may be related to sporulation induced by nutrient exhaustion. The MK-7 concentration increased from 20 mg/L at 24 h to 58 mg/L at 48 h when glycerol was fed at 2.4 g/L/h (Figure 5B); and from 34 mg/L at 24 h to 112 mg/L at 56 h when glycerol was fed at a rate of 3 g/L/h (Figure 5C).

[00118] The residual glycerol concentration decreased rapidly in the first 5 to 6 hours of fermentation, before more glycerol was fed into the fermentation mixture. Although glycerol was fed at three different rates, its residual concentration remained at about 2 g/L. Dissolved oxygen also rapidly decreased as the carbon source was consumed in the first 5 to 6 hours of fermentation. Because the glycerol was fed into the fermentation mixture as a plurality of pulsed doses, the dissolved oxygen level served as an indication of the residual glycerol level in the fermentation mixture. The dissolved oxygen rose as glycerol was consumed, and dropped as another pulse of glycerol was fed into the fermentation mixture.

[00119] Although the invention has been described with reference to specific embodiments, it will be appreciated by those skilled in the art that the invention may be embodied in many other forms.

Claims

1. A process for producing vitamin K the process comprising:
combining a first carbon source, a first nitrogen source and a fermentative bacterium to provide a fermentation mixture;
an incubation phase wherein the fermentation mixture is incubated such that the fermentative bacterium consumes the first carbon source and thereby produces vitamin K; and
a feeding phase wherein a second carbon source and a second nitrogen source are fed into the fermentation mixture such that the fermentative bacterium consumes the second carbon source and thereby produces vitamin K.
2. The process of claim 1 wherein the fermentative bacterium is *Bacillus subtilis*.
3. The process of claim 2 wherein the fermentative bacterium is *Bacillus subtilis natto*.
4. The process of any one of claims 1 to 3 wherein the vitamin K is menaquinone-7.
5. The process of any one of claims 1 to 4 wherein the second carbon source is fed into the fermentation mixture as a plurality of pulsed doses.
6. The process of claim 5 wherein the second carbon source is fed into the fermentation mixture at a pulse rate of one dose every about 5 to 120 minutes.
7. The process of claim 5 wherein the second carbon source is fed into the fermentation mixture at a pulse rate of one dose every about 5 to 60 minutes.
8. The process of claim 5 wherein the second carbon source is fed into the fermentation mixture at a pulse rate of one dose every about 5 to 30 minutes.
9. The process of any one of claims 5 to 8 wherein each pulsed dose of the second carbon source lasts for a period of between about 2 minutes and 15 minutes.

10. The process of any one of claims 1 to 9 wherein the second nitrogen source is fed into the fermentation mixture as a plurality of pulsed doses.
11. The process of claim 10 wherein the second nitrogen source is fed into the fermentation mixture at a pulse rate of one dose every about 10 to 120 minutes.
12. The process of claim 10 wherein the second nitrogen source is fed into the fermentation mixture at a pulse rate of one dose every about 30 to 90 minutes.
13. The process of claim 10 wherein the second nitrogen source is fed into the fermentation mixture at a pulse rate of one dose every about 60 minutes.
14. The process of any one of claims 10 to 13 wherein each pulsed dose of the second nitrogen source lasts for a period of between about 5 minutes and 15 minutes.
15. The process of any one of claims 1 to 14 wherein the second carbon source is fed into the fermentation mixture at a feeding rate of between about 1 g/L/h and 10 g/L/h.
16. The process of claim 15 wherein the second carbon source is fed into the fermentation mixture at a feeding rate of between about 2 g/L/h and 5 g/L/h.
17. The process of any one of claims 1 to 16 wherein the second nitrogen source is fed into the fermentation mixture at a feeding rate of between about 1 g/L/h and 5 g/L/h.
18. The process of claim 17 wherein the second nitrogen source is fed into the fermentation mixture at a feeding rate of between about 1 g/L/h and 3 g/L/h.
19. The process of any one of claims 1 to 18 wherein the first carbon source and the first nitrogen source are combined such that the first carbon source is present in the fermentation mixture at a concentration that does not exceed about 50 g/L.
20. The process of claim 19 wherein the first carbon source and the first nitrogen source are combined such that the first carbon source is present in the fermentation mixture at a concentration that does not exceed about 25 g/L.

21. The process of any one of claims 1 to 20 wherein the first carbon source and the first nitrogen source are combined such that the first nitrogen source is present in the fermentation mixture at a concentration that does not exceed about 50 g/L.
22. The process of claim 21 wherein the first carbon source and the first nitrogen source are combined such that the first nitrogen source is present in the fermentation mixture at a concentration that does not exceed about 25 g/L.
23. The process of any one of claims 1 to 22 wherein the incubation phase lasts for a period of between about 3 hours and 10 hours.
24. The process of any one of claims 1 to 23 wherein the feeding phase is triggered when the fermentation mixture reaches a predetermined pH.
25. The process of claim 24 wherein the feeding phase is triggered when the pH of the fermentation mixture rises above about 7.
26. The process of any one of claims 1 to 25 wherein the feeding phase lasts for a period of at least about 20 hours.
27. The process of claim 26 wherein the feeding phase lasts for a period of at least about 40 hours.
28. The process of any one of claims 1 to 27 wherein dissolved oxygen remains below about 80% in the fermentation mixture during the feeding phase for a period of at least about 10 hours.
29. The process of claim 28 wherein dissolved oxygen remains below about 50% in the fermentation mixture during the feeding phase for a period of at least about 10 hours.
30. The process of any one of claims 1 to 29 wherein the first carbon source and the second carbon source together remain at a concentration of less than about 10

g/L in the fermentation mixture for a period of at least about 10 hours during the feeding phase.

31. The process of claim 30 wherein the first carbon source and the second carbon source together remain at a concentration of less than about 5 g/L in the fermentation mixture for a period of at least about 10 hours during the feeding phase.

32. The process of claim 30 wherein the first carbon source and the second carbon source together remain at a concentration of less than about 5 g/L in the fermentation mixture for a period of at least about 20 hours during the feeding phase.

33. The process of any one of claims 1 to 32 wherein the fermentation mixture maintains a pH of between about 6 and 8 for at least about 10 hours during the feeding phase without addition of an acid or a base to the fermentation mixture.

34. The process of claim 33 wherein the fermentation mixture maintains a pH of between about 6 and 8 for at least about 20 hours during the feeding phase without addition of an acid or a base to the fermentation mixture.

35. The process of any one of claims 1 to 34 wherein the first carbon source and the second carbon source are independently selected from the group consisting of corn syrup, sucrose, inulin, maltodextrin, glycerol, fructose and glucose.

36. The process of claim 35 wherein the first carbon source and the second carbon source are independently selected from inulin and glycerol.

37. The process of claim 36 wherein the first carbon source and the second carbon source are glycerol.

38. The process of any one of claims 1 to 37 wherein the first nitrogen source and the second nitrogen source comprise proteins, peptides or amino acids.

39. The process of any one of claims 1 to 38 wherein the first nitrogen source and the second nitrogen source are independently obtained from a plant or an animal.
40. The process of claim 39 wherein the first nitrogen source and the second nitrogen source are independently obtained from soybean, pea, almond, peanut, wheat, rice, sorghum or milk.
41. The process of claim 39 wherein the first nitrogen source and the second nitrogen source are obtained from a plant.
42. The process of claim 41 wherein the first nitrogen source and the second nitrogen source are independently obtained from a legume, a nut or a cereal.
43. The process of claim 42 wherein the first nitrogen source and the second nitrogen source are obtained from a legume.
44. The process of claim 43 wherein the legume is a soybean plant or a pea plant.
45. The process of any one of claims 1 to 44 wherein the first nitrogen source and the second nitrogen source comprise less than about 300 mg of aromatic amino acids per gram of total nitrogen.
46. The process of any one of claims 1 to 45 wherein the fermentation mixture further comprises an antifoam.
47. The process of claim 46 wherein the antifoam is a silicone-based antifoam or an oil-based antifoam.
48. The process of any one of claims 1 to 47 wherein the process produces menaquinone-7 at a concentration of at least about 100 mg/L.
49. The process of claim 48 wherein the process produces menaquinone-7 at a concentration of at least about 110 mg/L.

50. The process of any one of claims 1 to 49 wherein the process produces menaquinone-7 at a rate of at least about 2 mg/L/h.
51. Vitamin K when produced by the process of any one of claims 1 to 50.
52. A food or drink product comprising the vitamin K of claim 51.
53. A dietary supplement comprising the vitamin K of claim 51.
54. A process for producing vitamin K the process comprising:
combining a carbon source, a nitrogen source and a fermentative bacterium to provide a fermentation mixture, wherein the fermentation mixture comprises a higher concentration of the nitrogen source than the carbon source; and
incubating the fermentation mixture such that the fermentative bacterium consumes the carbon source and thereby produces vitamin K.
55. The process of claim 54 wherein the fermentative bacterium is *Bacillus subtilis*.
56. The process of claim 55 wherein the fermentative bacterium is *Bacillus subtilis natto*.
57. The process of any one of claims 54 to 56 wherein the vitamin K is menaquinone-7.
58. The process of any one of claims 54 to 57 wherein the concentration of the nitrogen source is at least twice the concentration of the carbon source in the fermentation mixture.
59. The process of any one of claims 54 to 58 wherein the fermentation mixture comprises the carbon source at a concentration of between about 25 g/L and 80 g/L.

60. The process of claim 59 wherein the fermentation mixture comprises the carbon source at a concentration of between about 40 g/L and 60 g/L.

61. The process of any one of claims 54 to 60 wherein the fermentation mixture comprises the nitrogen source at a concentration of between about 40 g/L and 150 g/L.

62. The process of claim 61 wherein the fermentation mixture comprises the nitrogen source at a concentration of between about 80 g/L and 125 g/L.

63. The process of any one of claims 54 to 62 wherein the carbon source is selected from the group consisting of corn syrup, sucrose, inulin, maltodextrin, glycerol, fructose and glucose.

64. The process of claim 63 wherein the carbon source is selected from inulin and glycerol.

65. The process of claim 64 wherein the carbon source is glycerol.

66. The process of any one of claims 54 to 65 wherein the nitrogen source comprises proteins, peptides or amino acids.

67. The process of any one of claims 54 to 66 wherein the nitrogen source is obtained from a plant or an animal.

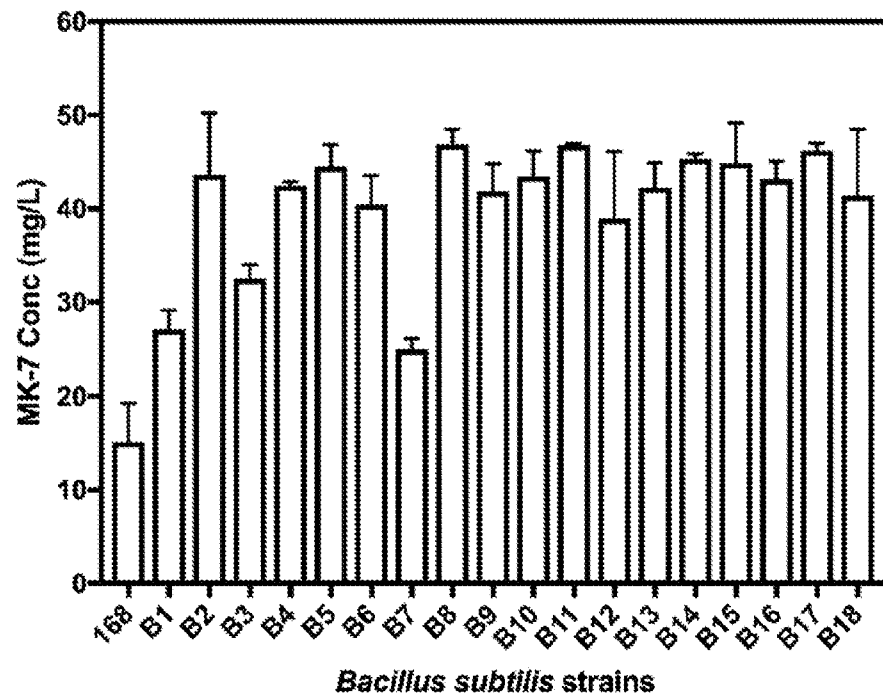
68. The process of claim 67 wherein the nitrogen source is obtained from soybean, pea, almond, peanut, wheat, rice, sorghum or milk.

69. The process of claim 67 wherein the nitrogen source is obtained from a plant.

70. The process of claim 69 wherein the nitrogen source is obtained from a legume, a nut or a cereal.

71. The process of claim 70 wherein the nitrogen source is obtained from a legume.

72. The process of claim 71 wherein the legume is a soybean plant or a pea plant.
73. The process of any one of claims 54 to 72 wherein the nitrogen source comprises less than about 300 mg of aromatic amino acids per gram of total nitrogen.
74. The process of any one of claims 54 to 73 wherein the fermentation mixture further comprises an antifoam.
75. The process of claim 74 wherein the antifoam is a silicone-based antifoam or an oil-based antifoam.
76. Vitamin K when produced by the process of any one of claims 54 to 75.
77. A food or drink product comprising the vitamin K of claim 76.
78. A dietary supplement comprising the vitamin K of claim 76.

**FIGURE 1**

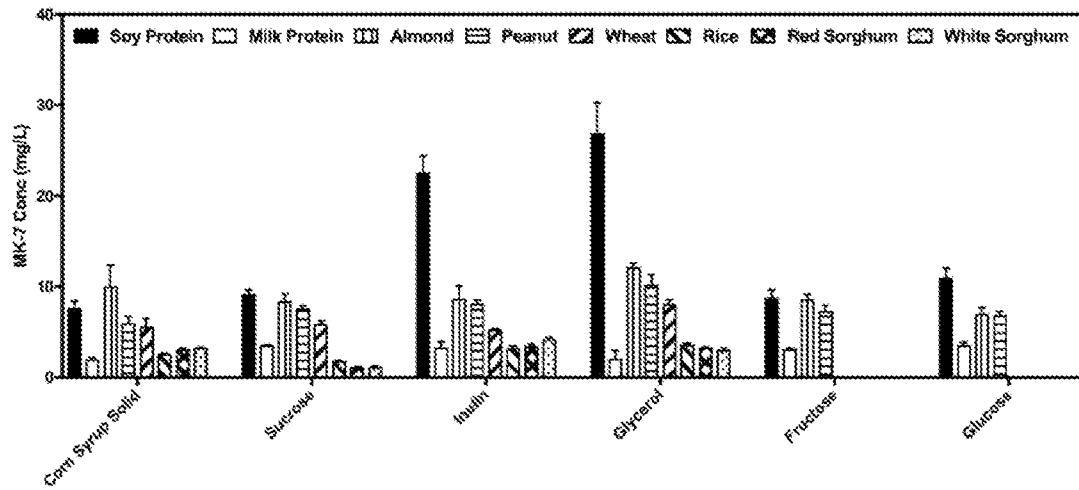


FIGURE 2

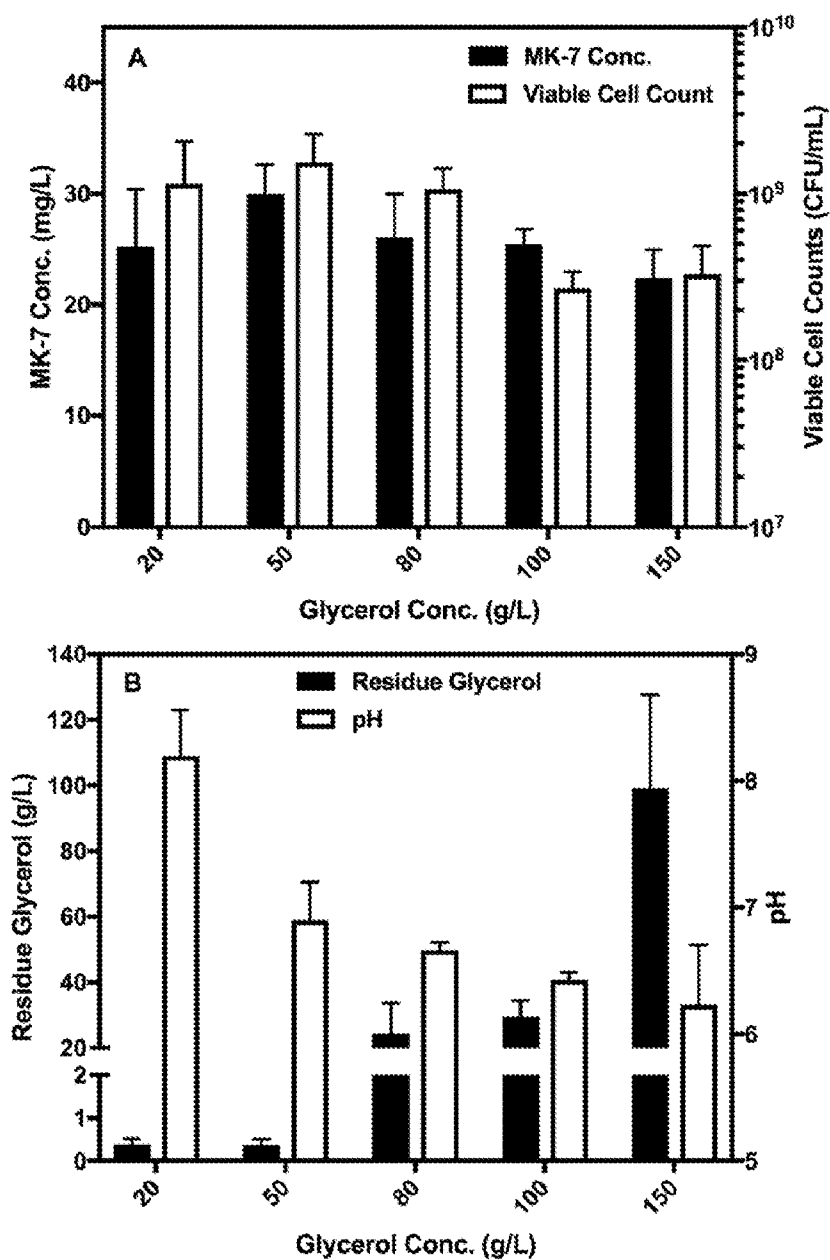


FIGURE 3

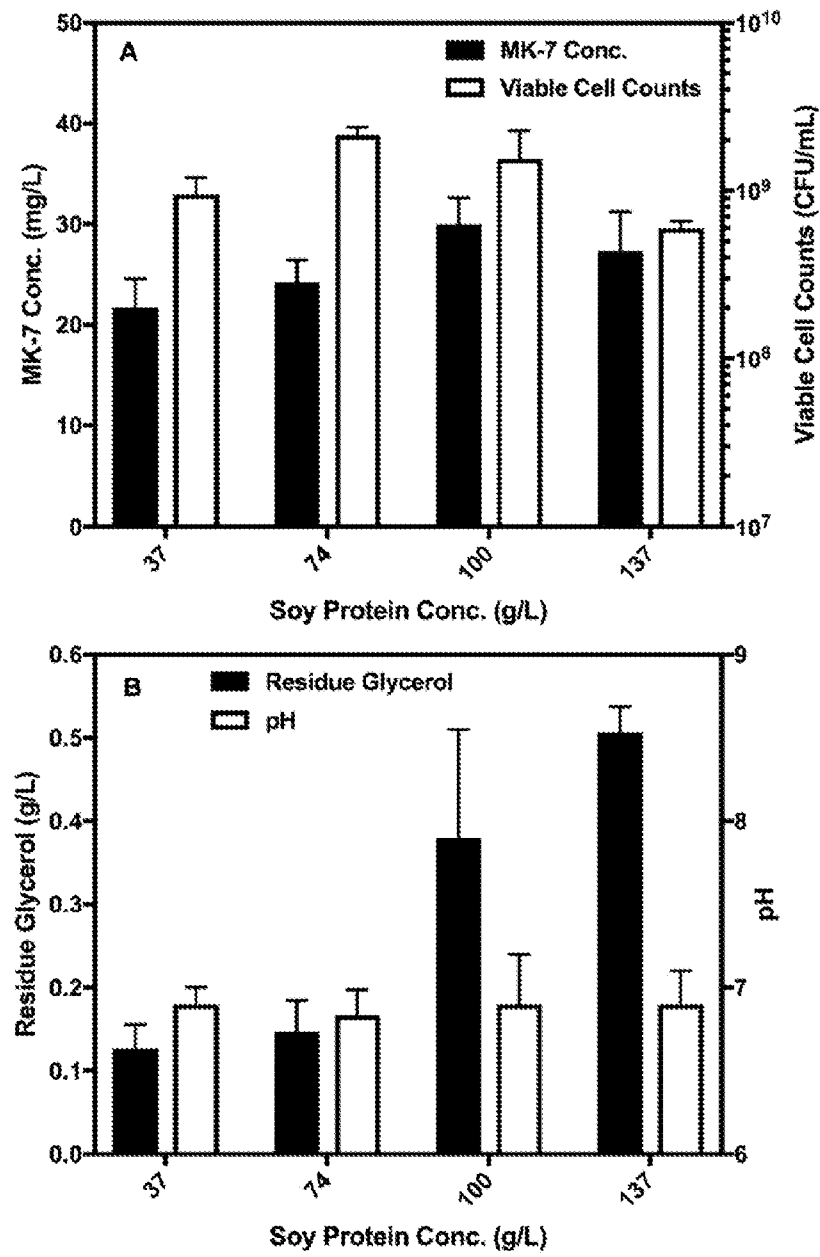


FIGURE 4

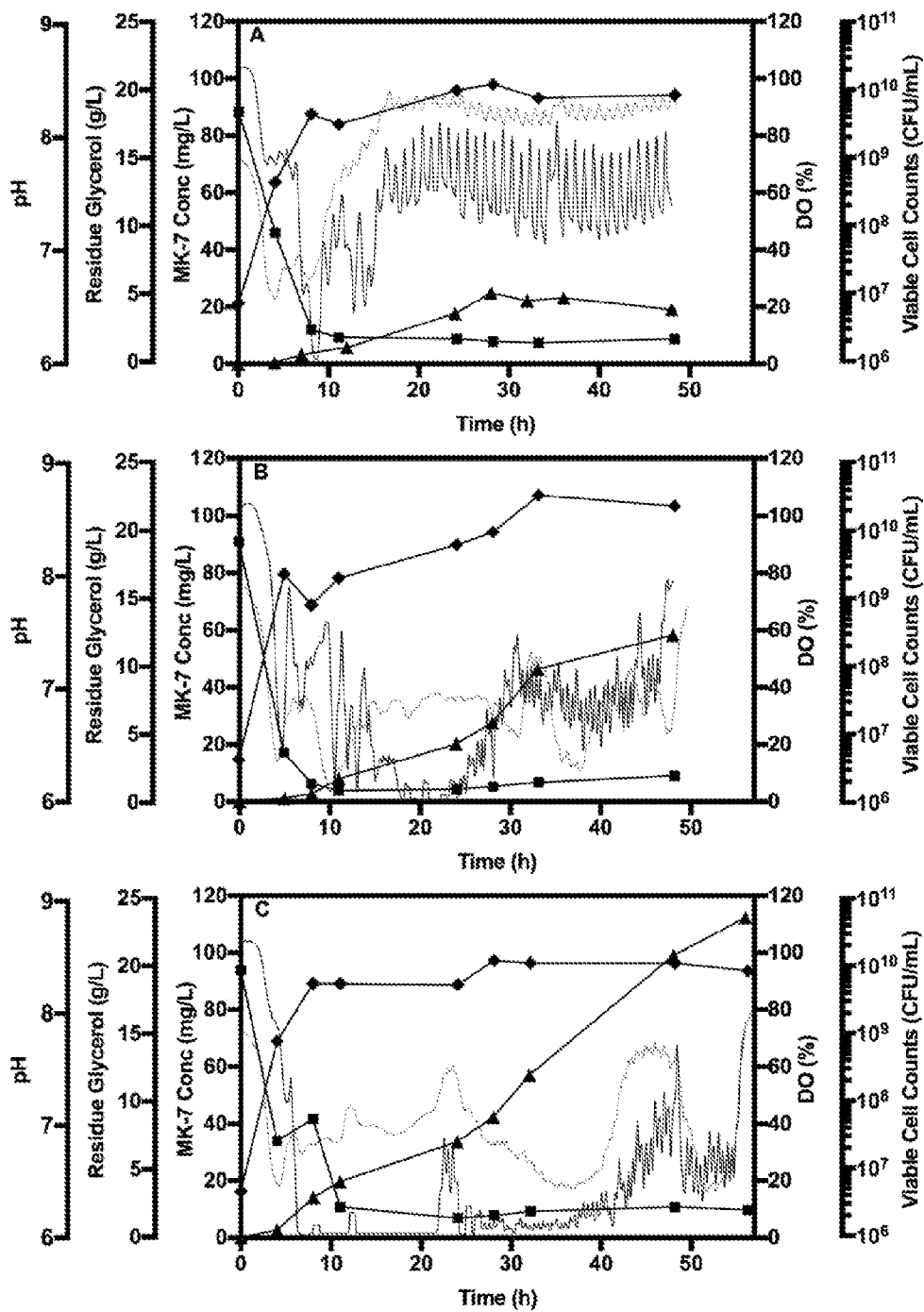


FIGURE 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2019/050917

A. CLASSIFICATION OF SUBJECT MATTER		
C12P 7/66 (2006.01) C12N 1/20 (2006.01) C12R 1/125 (2006.01) A23L 33/15 (2016.01)		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
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)		
Databases: Google/Google Scholar/Google Patents, Espace, PatenW, CAplus, Biosis, Embase, Medline, FSTA		
IPC/CPC symbols: C12P7/66; C12R 1/125; C12N 1/20; A23L 33/15; A23V2250/7144; A23V2200/30		
Key words: fermentation; Vitamin K; menaquinone-7, MK-7, Bacillus, subtilus, natto, inulin, sucrose, corn syrup, maltodextrin, glycerol, fructose, glucose, sugar, legume, pulse, fabaceae, nut, cereal, grain, soybean, pea, almond, peanut, wheat, rice, millet, maize, sorghum, barley, rye, Pulse feed, pulse dose, dual feeding, dual dose, fed-batch culture		
Applicant/Inventor: UNIVERSITY OF SYDNEY; MA, Yanwei; MCCLURE, Dale; KAVANAGH, John; DEGHANI, Fariba		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	
☒ Further documents are listed in the continuation of Box C ☒ See patent family annex		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "D" document cited by the applicant in the international application "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family	
Date of the actual completion of the international search 14 November 2019	Date of mailing of the international search report 14 November 2019	
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustalia.gov.au	Authorised officer Luke Burow AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61262104091	

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/AU2019/050917
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KR 20180015417 A (MIN BYOUNG GYU) 13 February 2018 See whole of document and especially: abstract; pages 1-8; examples 3-6; figures	1-78
X	WO 2014/131084 A1 (The University of Sydney; Agricure Pty Ltd) 04 September 2014 See whole of document and especially: abstract; paragraphs 10, 13, 32-33, 58, 78, 80, 88, 108; figure 3	1-5, 10, 26-32, 35-44, 46-53, 76-78
X	Mahdinia, E., et al. 'Production and application of menaquinone-7 (vitamin K2): a new perspective', World J Microbiol Biotechnol (2017) 33:2, pg 1-7. See whole of document and especially: page 3, right column; page 4, right column	51-53, 76-78

Form PCT/ISA/210 (fifth sheet) (July 2019)

INTERNATIONAL SEARCH REPORT Information on patent family members		International application No. PCT/AU2019/050917	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
KR 20180015417 A	13 February 2018	KR 20180015417 A	13 Feb 2018
		KR 101875502 B1	09 Jul 2018
WO 2014/131084 A1	04 September 2014	WO 2014131084 A1	04 Sep 2014
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2019)			