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(57) Abstract :
The present invention is related to an economical and eco friendly process of supercritical fluid extraction, for the extraction and purification of Vitamin K2-7 from the microbial fermentation broth, involving the sequential steps of (a) culturing and harvesting B. subtilis natto; (b) concentrating the broth containing K2-7 cells by cross flow procedure; (c) mixing the concentrate with an adsorbent; (d) subjecting mixture to supercritical fluid extraction (SFE) and (e) separation by expansion of gas/solute mixture.

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COMPLETE SPECIFICATION

(See Section 10, Rule 13)

TITLE

"A NOVEL, ECONOMIC AND ECO-FRIENDLY PROCESS FOR EXTRACTION OF MENAQUINONE-7 (VITAMIN K_2-7)"

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The following specification describes the invention

A novel, economic and eco-friendly process for extraction of Menaquinone-7 (Vitamin K_2-7)

Field of the Invention

The present invention provides a novel, economic and eco-friendly method for extraction and purification of Menaquinone-7 (Vitamin K_2-7) using supercritical fluid extraction.

Background

Vitamin K is a fat-soluble vitamin known to play a role in the process of coagulation of blood. It is also known as an "Antihemorrhagic vitamin" as its deficiency causes excessive bleeding. US6677141 disclose the role of vitamin K in the prevention of osteoporosis. There are two types of naturally occurring forms of Vitamin K, phylloquinone (also known as Vitamin K1) and menaquinone (Vitamin K2). The menaquinones are generally denoted as MK-n, where n stands for the number of unsaturated isoprenoid residues, which may vary between 1 and 14. Phylloquinone is found in plants and the most common forms of menaquinone found in food are MK-4, MK-7, MK-8, MK9 (Allison (1955) *Lancet* **I**, 669).
Natural Vitamin K, particularly Vitamin K_2-7, is not ordinarily ingested by humans. Large quantities of Menaquinone-7 (also known as MK-7 or Vitamin K_2-7 or K_2-7) are typically produced by fermentation of microorganisms. One of the well-studied methods of K2-7 production includes inoculation of soybean soup stock with *Bacillus subtilis natto*. Although the methods to prepare K_2-7 from *Bacillus subtilis natto* reportedly yield high quantities, their application may be limited due to higher fat-soluble contents. *Flavobacterium spps* for the production of K_2-7 has also been successful but the safety of the resulting product in the food industry is yet to be proven.

Fermentation process to obtain Vitamin K_2-7 known in the art typically involves culturing the microorganism in fermentation vessels containing a suitable substrate such as soya bean flour nutrient broth or other fermentation medium having a carbon source,

nitrogen source and salts as required. The bacteria are allowed to grow and produce K_2-7. The fermentation is terminated when the productivity reaches its optimum phase. US6677141 describes a method for culturing *Bacillus subtilis natto* inducing maximum storage of K_2-7 within the bacterial cells. The steps in the process include culturing *Bacillus subtilis natto* and recovering the cells of the microorganism before the vitamin K produced in the cells is released from the cells.

Vitamin K_2-7 being a fat-soluble vitamin, organic solvents is commonly used for its extraction from the fermentation broth. The extraction involves the commonly used solvents such as ethanol, isopropanol, diethyl ether, acetone or hexane that is used in a soxhlet extractor at a boiling temperature of the solvent to obtain a fat-soluble fraction. However, organic solvents are associated with the following drawbacks:

1. The effective cost of extraction increases as large quantities of solvents are normally used
2. Escaping solvent fumes are an environmental and work hazard.
3. Organic solvents like hexane, ether have low flash points and hence are a fire hazard.
4. Distillation of solvents is an energy intensive process.
5. Despite distillation, the product contains ppm levels of solvent thus increasing toxicity.

There is clearly a need to address some of the problems associated with the use of organic solvents for extraction. Experimentation with alternative extraction processes for extraction of K_2-7 resulted in the current invention, which is provided in detail in the instant application.

A fluid heated to above the critical temperature and compressed to above the critical temperature and pressure is known as a supercritical fluid and the use of these supercritical fluids for the extraction process is known as supercritical fluid extraction (SFE). Two supercritical fluids of particular interest are carbon dioxide (CO_2) and water. The properties of these fluids lie between that of gases and liquids. They have density

similar to that of liquids, while the viscosity and diffusion rates are closer to that of gases. Thus, a supercritical fluid can diffuse faster in a solid matrix than a liquid, yet possess a solvent strength to extract the solute from solid matrices. Major advantage of supercritical fluid is that a small reduction in temperature or a slightly larger reduction in pressure results in almost the entire solute precipitating out.

SFE has been used in the food industry with regard to different processes. US6761913 describes use of supercritical fluids in extraction of spices and pharmaceutically active herbal compounds from plants. Gast *et al* have described enrichment of fat soluble Vitamin E from natural resources namely crude palm oil and soy oil using supercritical fluid extraction method (*J* of Supercritical Fluids 2005, 34:17-25). Although the use of SFE has been known in the industry for extraction of fat soluble vitamins and oils, its use for extraction from fermentation broth has been not described previously. It is also important that any such extraction process at the industrial scale mainly focuses on reduction in the original volume which is otherwise cost prohibitive.

The present invention encompasses extraction of K_2-7 from fermentation broth by utilizing SFE. The described process effectively reduces the handling volume to about $1/10^{th}$ – $1/20^{th}$ of the original volume by adsorption on a solid prior to subjecting to SFE. The process described in the instant application has thus eliminated the use of organic solvents and provides an extraction procedure that is more economical and eco-friendly.

Summary of invention

The present invention is related to an economical and eco friendly process of supercritical fluid extraction, for the extraction and purification of Vitamin K_2-7 from the microbial fermentation broth.

In one aspect, the invention provides a process for production and extraction of Vitamin K_2-7 involving the sequential steps of

(a) culturing and harvesting *B. subtilis natto;*

(b) concentrating the broth containing K_2-7 cells by cross flow procedure;

(c) mixing the concentrate with an adsorbent;

(d) subjecting mixture to supercritical fluid extraction (SFE) and
(e) separation by expansion of gas/solute mixture.

In another aspect, the invention provides a process to reduce the volume of the starting material of the extraction process for use in industrial scale. The reduction in volume is achieved in the instant invention by concentrating the culture broth by using 0.2 micron hollow fiber by cross flow procedure. The volume reduction is about $1/10^{th}$ – $1/20^{th}$ of the original volume.

The invention provides supercritical fluid extraction of Vitamin K_2-7 from the fermentation broth by subjecting the mixture containing the reduced broth and adsorbent to supercritical fluid extraction. CO_2 is used as the preferred supercritical fluid for the extraction.

Description of the invention

Supercritical fluids can extract a product with no solvent residues thus overcoming many of the problems associated with use of organic solvents. CO_2 is the most common supercritical fluid widely used in the food industry. Due to the non-toxicity and low critical temperature it can be used to extract thermally labile food components without contaminating the product with residual solvent.

In one embodiment, the process of production and extraction of K_2-7 is carried out by
(a) culturing and harvesting *Bacillus subtilis natto*;
(b) concentrating the broth containing K_2-7 cells by cross flow procedure;
(c) mixing the concentrate with an adsorbent
(c) subjecting mixture to supercritical fluid extraction (SFE)
(d) separation by expansion of gas/solute mixture

The invention provides a process in which *B. subtilis natto* is cultured in a fermentor. The cells loaded with K_2-7 are concentrated using a filter operated in cross flow mode. For supercritical fluid extraction, CO_2 is circulated under pressure through the extraction chamber consisting of the solid matrix. The extraction of the soluble K_2-7 from the solid matrix is by simple dissolution of the solute from the matrix. Separation is brought about

by expansion of the gas/solute mixture resulting in the liquid CO_2 turning gaseous. The solute is collected in the collection chamber. The procedure is repeated till recovery of solute is negligible.

In one embodiment, there is provided a method for the production and harvest of K_2-7. The producer culture used is Bacillus subtilis natto which is cultured in a 5.0 liter fermentor using a medium comprising 10% soya flour, 5% glycerol, 0.5% yeast extract and 0.5% K_2HPO_4. Temperature is maintained at 40^0 - 45^0 C and aeration at 1 vvm. The culture conditions are maintained for 96 hrs during which period maximum production of K2-7 is reported in US7018630. K_2-7 levels are monitored by HPLC and the broth is harvested.

Alternatively the cells are allowed to sporulate at which point K_2-7 is present both intracellularly and extracellularly. The broth is then harvested and mixed with 10-30% of soluble filler such as maltodextrin, common salt, KCl or Na_2SO_4. This material is then spray dried using a commercial spray drier with a nozzle inlet temperature of about 110^0C-140^0C, more preferably at 125^0C-130^0C. The chamber temperature is maintained at 65^0C. The dried powder can then be directly used for super critical fluid extraction

In an advantageous aspect of the invention, there is provided a method for the reduction in the volume of the broth for economical application of the extraction process. The reduction in the volume is achieved using a 0.2-micron hollow fiber operating in a cross flow mode. The broth volume is reduced to about $1/10^{th}$ to $1/20^{th}$ of the starting volume. Preferably the reduction in the original volume is about 80-90%. More preferably the reduction achieved is 80% of the starting volume. The reduced broth is then mixed with a wet cake of adsorbent and is allowed to dry before introducing it in the extraction chamber. The adsorbents used for this procedure is precipitated silica (INSILICA, Degussa), dicalcium phosphate (DCP), deoiled rice bran, diatomaceous earth, bentonite, china clay or other materials known in the art that have high water retention capacity and absorbents that are oil free. Any of the above mentioned adsorbents can be used alone, but the preferred combination of adsorbents used in the invention is DCP: Insilica in the

proportion of 95:5. This combination is preferred due to economic reasons and the anticaking properties of insilica.

In yet another aspect, the invention details a novel process of K_2-7 extraction from the fermentation broth using supercritical fluid extraction method. The supercritical fluid used is CO_2, which is circulated under a pressure ranging from 100-400 bars in the extraction chamber. The extraction of the soluble material from the solid matrix is by simple dissolution of the solute from the matrix. The separation procedure consists of expansion of the gas/solute mixture that results in liquid CO_2 turning gaseous and fall out of the solute is taken into a collection chamber. One such cycle of extraction usually takes 45 min after which the pressure is reduced and the extracted oil is separated from the gas. The residual cake is further subjected to extraction cycles till the oil is negligible. This K_2-7 oil fraction can be further subjected to fractionation/purification by chromatographic methods known to a person skilled in the art. The percentage recovery is about 90 – 100% as compared to 80%-90% obtained when a solvent system like hexane-propanol is used.

The invention is more fully understood by reference to the following examples. These examples should not, however, be construed as limiting the scope of the invention.

Example 1

Production of K_2-7

B. subtilis natto was cultured in a 5.0 liter fermentor using medium comprising 10% soya flour, 5% glycerol, 0.5% yeast extract and 0.5% K_2HPO_4. The culture was maintained at 40^0 - 45^0C and aeration at 1 vvm for 96 hrs at which point maximum K_2-7 was produced. K_2-7 levels were assayed by HPLC and the broth was harvested.

Example 2

Extraction of K_2-7 from broth

The broth containing K_2-7 was concentrated using a 0.2 micron hollow fibre operating in the cross flow mode. The permeate was checked and found to contain negligible or no activity. The retentate was reduces to a volume of 500 ml from 2.5 liter. 500 ml broth was than mixed into a DCP: Insilica mixture (95:5). A wet cake was obtained and allowed to dry at room temperature.

The cake was than scraped out and introduced in the extraction chamber wherein CO_2 gas was passed in the Supercritical fluid extraction unit to a pressure of 200 bars. This was circulated through the extraction chamber. After 45 min the pressure in the collection chamber was reduced and the extracted oil was separated from the gas. The oil was collected and residual cake further subjected to extraction till the oil content was negligible.

By this method it was possible to recover more than 95% of the K_2-7 present in the harvested broth. This K_2-7 oil fraction was further fractionated/purified by column chromatography.

Claims

We claim:

1. A process of production and extraction of Vitamin K_2-7 comprising the sequential steps of :

   (a) culturing and harvesting *B. subtilis natto*;

   (b) concentrating the broth containing K_2-7 cells by cross flow procedure;

   (c) mixing the concentrate with an adsorbent;

   (d) subjecting mixture to supercritical fluid extraction (SFE) and

   (e) separation by expansion of gas/solute mixture.

2. The process of claim 1, wherein the broth is concentrated using a 0.2 micron hollow fiber.

3. The process of claim 2, wherein the concentration procedure yields a volume reduction of about $1/10^{th}$ to $1/20^{th}$ of the original broth volume.

4. The process of claim 1, wherein the adsorbent is selected from the group consisting of precipitated silica (INSILICA, Degussa), Dicalcium Phosphate (DCP), deoiled rice bran, diatomaceous earth, bentonite, china clay and combinations thereof.

5. The process of claim 4, wherein the adsorbent is DCP:Insilica at a ratio of 95:5.

6. The process of claim 1, wherein the SFE is carried out in extraction chamber using CO_2 under pressure ranging from 100-400 bars.

7. The process of claim 6, wherein the pressure at which CO_2 is circulated in the extraction chamber is 200 bars.

8. A process of production and extraction of K_2-7 comprising the

   (a) culturing B. subtilis natto in a culture medium comprising 10% soya flour, 5% glycerol, 0.5% yeast extract and 0.5% K_2HPO_4 for 96hr at 37ºC and aeration at 1vvm;

   (b) harvesting and concentrating the broth with 0.2 micron hollow fibre operating in the cross flow mode;

(c) mixing the reduced broth with DCP:Insilica (95:5);

(d) extraction with CO_2 gas passed in supercritical fluid extraction unit at a pressure of 200 bars and

(e) separation of extracted oil from the gas.

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