November 10, 2011

Lisa Brines, Ph.D.
National List Manager
USDA/AMS/NOP, Standards Division
1400 Independence Ave. SW
Room 2646-So., Ag Stop 0268
Washington, DC 20250-0268

> RE: Petition for inclusion of L-carnitine on the National List at §205.605(b) as a non-agricultural (non-organic) substance allowed as an ingredient in or on processed products labeled as "organic" or "made with organic (specified ingredients)"
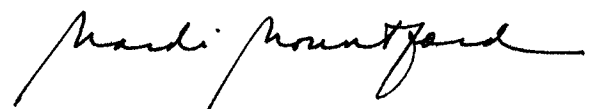
Dear Dr. Brines,

The International Formula Council (IFC) is an association of manufacturers and marketers of formulated nutrition products (e.g., infant formulas and adult nutritionals) whose members are based predominantly in North America. IFC members support the American Academy of Pediatrics' (AAP) position that breastfeeding is the preferred method of feeding infants. We also agree with the AAP that, for infants who do not receive breast milk, iron-fortified infant formula is the only safe and recommended alternative. IFC members are committed to providing infant formulas of the highest quality for those mothers who cannot or choose not to breastfeed, discontinue breastfeeding prior to one year or choose to supplement.

This petition seeks to add L-carnitine to the National List to permit its addition as a nonagricultural ingredient in infant formula. L-carnitine is currently used to fortify conventional infant formulas and infant formulas labeled as "organic" to the level of L-carnitine found in human milk, in accordance with the recommendations of independent professional associations, the European Directive for infant formula and follow on formula (Commission Directive 2006/141/EC), the Codex Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants (CODEX STAN 72-1981), and the 1998 recommendation of the Life Sciences Research Office (LSRO), American Societies for Nutritional Sciences, operating under a contract with the U.S. Food and Drug Administration (FDA).

The specific function of L-carnitine is as a "nutrient supplement" according to FDA 21 CFR 170.3(o)(20). L-carnitine has been added to soy-based infant formula products since 1986 and to cow's milk-based products since the mid-1990's. Infant formulas containing insufficient carnitine produced the symptoms of carnitine deficiency: failure to thrive; nonketotic hypoglycemia (low blood sugar); hypertonia (diminution of muscle tone); and cardiomyopathy.

Our petition and its appendices provide answers to all of the questions in the Guidelines on Procedures for Submitting National List Petitions, and in a manner that satisfies the criteria in the OFPA. We are available to provide any additional information that is required to complete your review process and recommendation.

Sincerely,

Mardi K. Mountford, MPH
Executive Vice President

INTRODUCTION

In October 1995, the National Organic Standards Board (NOSB) received a recommendation from its Processing, Handling and Labeling Committee ("the Committee") regarding the inclusion of synthetic vitamins, minerals, and accessory nutrients in organic foods.

The Committee had debated the issue of the inclusion of synthetic vitamins, minerals, and/or accessory nutrients in organic foods. Although it is generally considered that foods themselves are the best source of nutrients, in some cases, State regulations mandate the inclusion of vitamins and/or minerals to fortify foods.

The Committee also believed that recommendation by independent professional associations may also be taken into consideration. An example of this is infant cereals in which fortification of iron is highly recommended by the American Dietetic Association and various associations dealing with pediatric care and nutrition as a baby's stored iron supply from before birth runs out after the birth weight doubles.

The NOSB approved a Final Board Recommendation ("FBR") in October 1995. The Final Board Recommendation reads as follows:

 "Upon implementation of the National Organic Program, the use of synthetic vitamins, minerals, and/or accessory nutrients in products labeled as organic must be limited to that which is required by regulation or recommended for enrichment and fortification by independent professional associations."

The FBR includes a definition of the term "accessory nutrients," to mean nutrients not specifically classified as a vitamin or mineral but found to promote optimal health. Examples specifically cited in the FBR are omega-3 fatty acids, inositol, choline, carnitine, and taurine.

In creating the current regulation for organic foods, Code of Federal Regulations, Title 7, Part 205, the USDA implemented the FBR with respect to permitting the addition of nutrient vitamins and minerals at §205.605(b), albeit with an annotation ("in accordance with 21CFR 104.20") different than that approved by the NOSB. However, the current regulation is silent with respect to accessory nutrients.

On November 3, 2006, the USDA National Organic Program notified Accredited Certifiers that they could allow additional nutrients to be utilized in products certified as "organic" in accordance with 21 CFR 104.20(f).

On April 26, 2011, the Deputy Administrator of the National Organic Program announced that the National Organic Program planned to publish draft guidance that will clarify that the Food and Drug Administration's interpretation of 21 CFR 104.20 means that only nutrient vitamins and minerals listed in 21 CFR 104.20(d)(3) and those identified as essential nutrients in 21 CFR 101.9 are allowed under the National Organic Program standard at §205.605(b).

Infant formulas serve as the sole item of the diet of infants who are not fed human milk for the first four to six months of life. Several vitamins and accessory nutrients are included in infant formula either because their inclusion has been shown to enable infants fed these formulas to grow and develop similar to infants fed human milk or because their inclusion provides the infant with the same quantity of a vitamin or accessory nutrient as human milk provides and the accessory nutrient is essential for one or more other species of mammal, which is indirect evidence of its biological essentiality.

Each of the "accessory nutrients" cited in the FBR are currently added to infant formula. Two of these "accessory nutrients" – choline and inositol - are actually vitamins according to the infant formula regulations for infant formula established by the Food and Drug Administration in the Code of Federal Regulations, Title 21, at §107.10 and §107.100.

Two other nutrients cited in the FBR, carnitine and taurine, are less well-known amino acids that are essential to animal metabolism.

This petition specifically requests addition of the amino acid L-**carnitine** to the National List for use in infant formulas labeled as "organic."

ITEM A

This petition seeks inclusion of L-CARNITINE on the National List at §205.605 as a Non-agricultural (non-organic) substances allowed as an ingredient in or on processed products labeled as "organic" or "made with organic (specified ingredients or food group(s))."

ITEM B

1. The substance's chemical or common names.

The chemical name of carnitine, actually (-)-L-Carnitine, is (3-carboxy-2-hydroxypropyl)trimethyl-ammonium hydroxide, inner salt, L-. It is critical that the carnitine used in human nutrition be in the natural "L-" configuration common to nutritionally essential amino acids. The unnatural "D-" configuration is a competitive inhibitor of the natural form and must be avoided.

Alternate names are:
  1-Propanaminium, 3-carboxy-2-hydroxy-N,N,N-trimethyl-, hydroxide, inner salt, (R)- (9CI)
  3-Carboxy-2-hydroxy-N,N,N-trimethyl-1-propanaminium hydroxide, inner salt
  Ammonium, (3-carboxy-2-hydroxypropyl)trimethyl-, hydroxide, inner salt, L-
  gamma-trimethyl-ammonium-beta-hydroxybutyrate
  gamma-trimethyl-beta-hydroxybutyrobetaine
  levocarnitine

For over 50 years, L-carnitine has been referred to as "Vitamin B_T", in recognition that water-soluble L-carnitine was an essential nutrient for the confused flour beetle, *Tribolium confusum*. Infants fed a carnitine-free diet show biochemical changes. There are several anecdotal reports of abnormal clinical manifestations associated with diets low in carnitine.

The name "carnitine" originates in the prefix "carni-", Latin for "meat" (i.e., a "carnivore" is a meat eater). Carnitine was first isolated from a meat extract.

2a. The petitioner's name, address and telephone number and other contact information.

International Formula Council
1100 Johnson Ferry Road NE, Suite 300
Atlanta, GA 30342
Contact: Mardi Mountford, Executive Vice President
Phone: (678) 303-3027
Email: mmountford@kellencompany.com

2b. Sources of the petitioned substances

Food grade L-carnitine is a standard article of commerce available from many sources.

Recognized producers are Lonza Ltd., Basel, Switzerland and Sigma-Tau Industrie Farmaceutiche Riunite S.p.A., Rome, Italy.

Lonza Ltd.	Sigma-Tau is Sigma-Tau Pharmaceuticals, Inc.
Muenchensteinerstrasse 38, CH-4002	U.S. Office
Basel, Switzerland	9841 Washingtonian Blvd., Suite 500
Tel +41 61 316 81 11	Gaithersburg, MD 20878
Fax +41 61 316 91 11	Tel 1-800-447-0169 or 1-301-948-1041
	Fax 1-301-948-1862
	Email: sigmatauinfo@sigmatau.com

3. Current Use.

L-Carnitine is currently used to fortify conventional infant formulas and infant formulas labeled as "organic" with the nutrient carnitine to the level of L-carnitine found in human milk, in accordance with the recommendations of independent professional associations, the European Directive for infant formula, the Codex Alimentarius Commission International Infant Formula Standard CODEX STAN 72-1981, and the 1998 recommendation of the Life Sciences Research Office (LSRO), American Societies for Nutritional Sciences, operating under an FDA contract.

The specific function of L-carnitine is as a "nutrient supplement" [21 CFR 170.3(o)(20)].

4. Handling activities for which the substance is used.

L-carnitine has been added to soy-based infant formula products since 1986 and to milk-based products since the mid-1990's to provide a level of carnitine similar to that of human milk. Infant formulas containing insufficient carnitine produced the symptoms of carnitine deficiency: failure to thrive, nonketotic hypoglycemia (low blood sugar), hypertonia (diminution of muscle tone), and cardiomyopathy.

L-Carnitine is an integral component of the transport of long-chain fatty acids into the matrix of the mitochondria for oxidation. Carnitine plays an important role in the oxidation of medium-chain fatty acids in skeletal and cardiac muscle and in the transport of potentially toxic acylated metabolites outside the cell.

5. Source of the substances and a detailed description of the manufacturing process.

In mammals, L-carnitine is synthesized ultimately from the two essential amino acids L-lysine and L-methionine. Lysine provides the carbon backbone and methionine provides the three 4-N-methyl groups. Some proteins contain the carnitine precursor N^6-trimethyllysine (TML). The chemical structures of the metabolic precursors of L-carnitine are shown in Figure 1 on the next page.

In the body, it takes four enzymatic reactions to yield L-carnitine:

1. N^6-Trimethyllysine (TML) → 3-hydroxy- N^6-trimethyllysine (HTML)
2. HTML →4-N-trimethylaminobutyraldehyde (TMABA)
3. TMABA → 4-N-trimethylaminobutyrate (gamma-butyrobetaine)
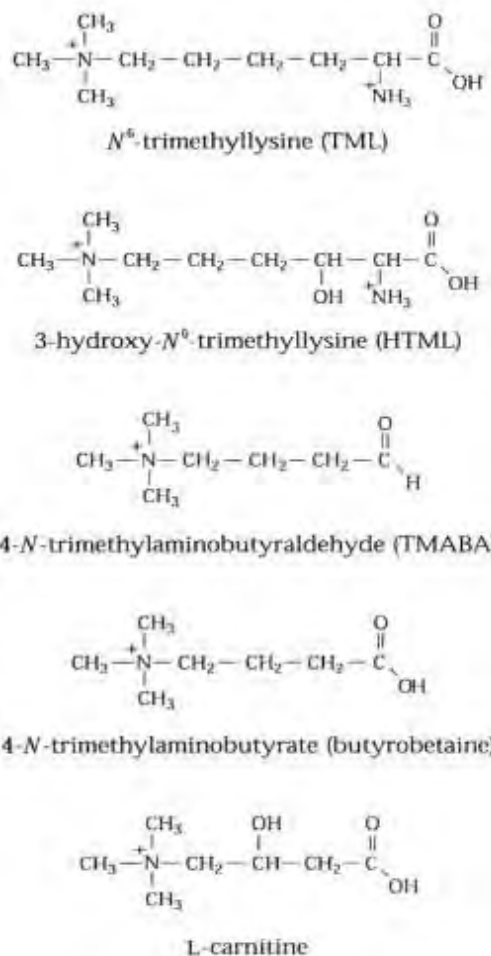4. Gamma-butyrobetaine → L-carnitine

Figure 1. The Mammalian Carnitine Biosynthetic Pathway

Carnitine Manufacturing Processes

L-Carnitine is produced commercially either by total chemical synthesis or by partial chemical synthesis with a final fermentation step. It is critical that the carnitine used in human nutrition be in the natural "L-" configuration common to nutritionally essential amino acids. The unnatural "D-" configuration is a competitive inhibitor of the natural form and must be avoided.

Fermentation of the penultimate chemical intermediate by an appropriate microorganism is an efficient and effective means of producing an L-carnitine free of the D-isomer. U.S. patents describing suitable microorganisms are included in Appendix A. The material that is fermented can be either gamma-butyrobetaine (the same precursor used in our body's metabolism) or crotonobetaine. Both of these materials are chemically synthesized. Crotonobetaine can be produced from the D-form of carnitine (Canadian Patent No. CA 2,023,744; Appendix A). A U.S. patent describing the chemical synthesis of butyrobetaine is included in Appendix A.

In a total chemical synthesis of L-carnitine, either the appropriate starting materials need to be chosen to ensure that the natural form is produced or the unnatural D-form must be totally removed at the end of the process.

A variety of processes involving total synthesis of L-carnitine have been patented (see Appendix A). For example, L-carnitine can be synthesized from optically pure (S)-beta-hydroxy-gamma-butyrolactone in a two-step process. (S)-3-Hydroxybutyrolactone is first converted to an ester and the ester is subsequently transformed to L-carnitine by treatment with trimethylamine in water. Optically pure (S)-beta-hydroxy-gamma-butyrolactone is produced by passing L-malic acid dissolved in methanol over a noble metal catalyst.

6. Summary of any available previous reviews of the petitioned substance.

a. Life Sciences Research Office (LSRO), American Societies for Nutritional Sciences (working under contract for the FDA) recommended the addition to any infant formula.[1] Page 2066S.

> *Carnitine*
>
> *Minimum*: **The Expert Panel recommended a minimum carnitine content of infant formulas of 1.2 mg/100 kcal, a level similar to that found in human milk.** Although the evidence that dietary carnitine is essential for the term infant is not convincing, biochemical changes are noted when infants are fed a carnitine-free diet and there are several anecdotal reports of abnormal clinical manifestations associated with diets low in carnitine. Infants nourished with soy protein-based formula with low carnitine content had lower plasma and urine carnitine levels and evidence of altered lipid metabolism, but no significant differences in rates of growth compared with supplemented infants. The functional significance of these metabolic differences in normal term infants is not known.
>
> *Maximum:* **The Expert Panel recommended a maximum carnitine content of infant formulas of 2.0 mg/100 kcal, a value similar to the upper limit reported for human milk.** The Expert Panel was unaware of any studies in which a NOAEL or LOAEL had been identified for carnitine exposure in infants. Consequently, in the absence of data the Expert Panel concluded that the maximum should be set at a level comparable to the upper ranges of carnitine concentrations reported for human milk.

b. Scientific Committee on Food. Report of the Scientific Committee on Food on the Revision of Essential Requirements of Infant Formulae and Follow-on Formulae. Brussels, European Commission 2003. SCF/CS/NUT/IF/65 Final 2003.

---

[1] LSRO. Assessment of Nutrient Requirements for Infant Formulas. J Nutr 1998;128(Supp):2059S–2298S.

Page 35:

> Taurine must be added to infant formula based on protein hydrolysates in amounts to achieve at least 5.25 mg/100 kcal (42 μmol/100 kcal) and **L-carnitine must be added to infant formulae based on protein hydrolysates and soy protein isolates** to achieve a content of at least 1.2 mg/100 kcal (7.5 μmol/100 kcal).

Page 45:

> Hydrolysed protein is permitted in the manufacturing of infant formula intended for healthy non-breast-fed infants at risk for atopic diseases. The method and extent of hydrolysis and processing must be documented but are not regulated. The minimum protein level is 2.25 g/100 kcal. The protein content is calculated with a conversion factor of 6.25 and both taurine (42 μmoles/100 kcal) and **L-carnitine must be added** (7.5 μmoles/100 kcal).

Pages 60 (corrected 17 September 2007):

> ### 4.7.2 Carnitine
>
> The addition of L-carnitine to infant formula based on soy protein isolate and hydrolyzed protein to give a content of at least 7.5 μmoles/100 kcal (1.2 mg/100 kcal) is required in the EU and the Committee does not propose a change. This value is similar or somewhat higher than in human milk (0.9 to 1.2 mg/100 kcal) because of a presumed reduced bioavailability from formula (Warshaw and Curry,1980). Cow's milk is rich in carnitine (around 5 mg/100 kcal) compared to human milk, therefore carnitine addition to cows' milk-based formula is not necessary. Carnitine is synthesized in the human body at a rate of approximately 0.3 mg/kg/day from lysine and using methionine as methyl donor (Rebouche and Seim, 1998). It is considered an indispensable nutrient for newborn infants (Rebouche, 1992) because of a temporarily compromised synthesizing capacity. Its function is the transport across membranes of carboxylic acids that have been activated to the co-enzyme A level, thereby delivering substrates for oxidation and removing toxic compounds.
>
> Infants receiving unsupplemented soy formula for 112 days showed lower serum levels of carnitine, higher levels of free fatty acids and an increased excretion of medium-chain dicarboxylic acids (Olson et al., 1989). The minimal dietary carnitine requirement of a newborn infant has been estimated to be 1.7 mg/kg/day due to a practically absent endogenous synthesis (Scholte and de Jonge, 1987).
>
> "The Committee considers the addition of carnitine to follow-on formula is not necessary. Supply from appropriate complementary food and from endogenous synthesis should be sufficient in older infants. Only the liver butyrobetaine hydroxylase, the last enzyme in carnitine biosynthesis, shows age-dependent low activity in young infants. The activity of the kidney enzyme and the other three biosynthetic enzymes in the liver and other tissues are not age-dependent (Vaz and Wanders, 2002).

c. Global Standard for the Composition of Infant Formula: Recommendations of an ESPGHAN Coordinated International Expert Group. ESPGHAN Committee on Nutrition. Journal of Pediatric Gastroenterology and Nutrition, 41:584–599 November 2005. Page 596.

> *L-carnitine*
>
> The recommendations of previous expert reviews (2,3) for a minimum L-carnitine content of 1.2 mg/100 kcal are supported. In contrast to the SCF, LSRO suggested a maximum level of 2 mg/100 kcal based on the upper end of the usual range found in human milk (2). In the absence of indications of any untoward effects of higher L-carnitine intakes in infants, the IEG concluded that no maximum level is needed to be set.

d. The European Commission Directive 2006/141/EC of 22 December 2006 on infant formulae and follow-on formulae and amending Directive 1999/21/EC, which is the current European regulation for infant formula composition, requires that the L-carnitine content shall be at least equal to 0.3 mg/100 kJ (1.2 mg/100 kcal) for infant formulas manufactured from protein hydrolysates and soy protein isolates. EC Directive 2006/1/EC of 22 December 2006 positively lists L-carnitine and its hydrochloride and L-carnitine-L-tartrate as permitted amino acid substances appropriate for use in infant formula.

e. The Pediatric Nutrition Handbook, 6th Edition, published by the Committee on Nutrition, American Academy of Pediatrics, in 2009, at page 327, states:

> Taurine and **carnitine** are amino acids that serve important and specific functions in the cell but are not incorporated into proteins. They can be synthesized by the body from cysteine and lysine, respectively, and are present in a mixed diet containing proteins of animal origin. The rates of synthesis in infants fed by total parenteral nutrition or receiving **synthetic formula devoid of** taurine and **carnitine** may be insufficient to meet all of their needs and **necessitate dietary supplementation**.

7. Information regarding the regulatory status of carnitine.

Carnitine has been added to soy isolate infant formulas since 1986, following reports of clinical manifestations of carnitine deficiency in infants fed soy-based formulas.[2] Since the mid-1990's, prior to the recommendation of the LSRO, commercial infant formulas in the United States have been supplemented with carnitine to compensate for the low amounts provided by bovine milk.

The Infant Formula Act passed into law in 1980 and amended in 1986 requires that manufacturers must provide FDA with assurances that the requirements specific to infant formula have been met for each "new" infant formula product prior to marketing. A "new infant

---

[2] MacLean WC Jr, Benson JD. Theory into practice: the incorporation of new knowledge into infant formula. Semin Perinatol. 1989 Apr;13(2):104-11.

formula" includes an infant formula manufactured by a person that has previously manufactured infant formula and in which there is a major change in processing or formulation from a current or any previous formulation produced by such manufacturer. [21 U.S.C. 350a(c)(2).] A "major change" is defined in an infant formula as ‚any new formulation, or any change in ingredients or processes where experience or theory would predict a possible significant adverse impact on levels of nutrients or availability of nutrients'. [21 C.F.R. 106.30 (c)(2)] Examples of "new" infant formulas include ‚any infant formula manufactured containing a new constituent not listed as a required nutrient under the act (in 21 CFR 107.100), such as taurine or **L-carnitine**'.[3] The FDA permitted L-carnitine supplementation of U.S. infant formulas in 1986.

The FAO/WHO Codex Alimentarius Commission adopted an international standard for infant formula in 1976 and adopted amendments in 1983, 1985, and 1987. It further revised the standard in 2007. CODEX STAN 72-1981 requires a minimum level of 1.2 mg/100 kcal of L-carnitine in all infant formulas but does not set a maximum level.

Carnitine is listed in the U.S. Pharmacopeia. A copy of the USP Reference Standard for carnitine is included at Appendix B.

8a. The Chemical Abstract Service (CAS) Number of L-Carnitine is 541-15-1.

8b. Labels of products that contain L-Carnitine.

See Appendix C (Labels).

9. The substance's physical properties and chemical mode of action.

(a) Physical Properties and Chemical Interactions

L-Carnitine forms white crystals or a white crystalline hygroscopic powder. It has a slight characteristic odor. L-Carnitine is extremely hygroscopic and even deliquescent and it can liquefy on exposure to air. L-Carnitine is highly soluble in water, in alcohol, in alkaline solutions, and in dilute mineral acids. It is practically insoluble in acetone or ethyl acetate.

(b) Toxicity and Environmental Persistence

Carnitine is irritating to the eyes, respiratory system and skin. Personnel should use personal protective equipment. Skin, eye, and respiratory protection should be used with any powdery ingredient. Carnitine is a biodegradable nutrient.

---

[3] http://www.fda.gov/ohrms/dockets/ac/02/briefing/3852b1_01.htm. Accessed May 21, 2011.

(c) Environmental Impacts from its Use or Manufacture

There are no negative environmental impacts known for L-carnitine. Production is in closed systems that maintain records of material input and output, as well as reuse of residual material in the production system. All non-usable waste material is handled according to a documented environmental management system.

The Lonza production sites for L-carnitine are certified ISO 14001:2004 by SQS. ISO 14001:2004 specifies the requirements for an environmental management system. To fulfill these requirements, objective evidence must be provided and audited to demonstrate that the environmental management system is operating effectively in conformity to the standard. In addition to verification that the production facility meets internal objectives, ISO 14001:2004 verifies compliance with environmental regulations.

The Policy on Safety, Health and Environment and an Environmental Statement from the L-carnitine supplier Lonza is included in Appendix D.

(d) Effects on Human Health

L-Carnitine is important in the transport of long-chain fatty acids into the cell organelles that convert fat into energy and in the oxidation of medium-chain fatty acids in skeletal and cardiac muscle. Thus it is vital to the energy efficiency of the body.

L-Carnitine is used as a dietary supplement by many individuals for many different reasons. L-Carnitine is used for conditions of the heart and blood vessels including heart-related chest pain, congestive heart failure (CHF), heart complications of a disease called diphtheria, heart attack, leg pain caused by circulation problems (intermittent claudication), and high cholesterol. Some people use L-carnitine for muscle disorders associated with certain AIDS medications, difficulty fathering a child (male infertility), a brain development disorder called Rett syndrome, anorexia, chronic fatigue syndrome, diabetes, overactive thyroid, attention deficit-hyperactivity disorder (ADHD), leg ulcers, Lyme disease, and to improve athletic performance and endurance.[4]

(e) Effects on Livestock

L-Carnitine has a positive effect on pregnancy in swine. Dietary supplementation with L-carnitine during pregnancy and lactation improves the reproductive performance of sows.[5] [6] Supplementation of sows' diets with L-carnitine increases body weights of their piglets at birth. Piglets from sows treated with L-carnitine did not differ in body weight gains, feed intake and

---

[4] http://www.webmd.com/vitamins-supplements/ingredientmono-1026-L-CARNITINE.aspx?activeIngredientId=1026&activeIngredientName=L-CARNITINE . Accessed May 22, 2011.

[5] Eder et al. Effect of L-carnitine supplementation on performance parameters in gilts and sows. J Anim Physiol Anim Nutr (Berl). 2001, Apr; 85(3-4):73-80.

[6] Musser et al., Effects of L-carnitine fed during gestation and lactation on sow and litter performance. J Anim Sci. 1999, Dec; 77(12):3289-95.

gain:feed ratio from those of control sows, but L-carnitine supplementation of their diets improves the growth performance in light piglets of primiparous sows.[7]

10a. Safety information about the substance including a Material Safety Data Sheet (MSDS).

A Material Safety Data Sheet for L-carnitine is attached in Appendix E.

10b. National Institute of Environmental Health Studies Substance Report.

A specific NIEHS report on L-carnitine does not exist, to our knowledge.

11. Research information about carnitine.

The full discussion of carnitine from the LSRO report of 1998, which discusses the pros and cons of carnitine addition to infant formula, is shown in Appendix F. This summary refers to several studies that have established that infants fed infant formulas devoid of carnitine show biochemical changes indicative of carnitine deficiency.

12. Petition Justification Statement.

The Scientific Committee on Food, in its Report of the Scientific Committee on Food on the Revision of Essential Requirements of Infant Formulae and Follow-on Formulae. Brussels, European Commission 2003. SCF/CS/NUT/IF/65 Final 2003, stated that "Cow's milk is rich in carnitine (around 5 mg/100 kcal) compared to human milk, therefore carnitine addition to cows' milk-based formula is not necessary." Although the level of carnitine in cow's milk is approximately twice that of human milk, cows' milk must be diluted by 50% or more to make its protein level appropriate for the infant. Cows' milk contains a high protein content: 20% of total energy, compared to about 7% of energy as protein in human milk. Consequently, the carnitine content of milk-based infant formula is approximately equal to that in human milk. However, as the Scientific Committee on Food pointed out, the carnitine in infant formulas has "presumed reduced bioavailability," based on the work of Warshaw and Curry, 1980[8]. Warshaw and Curry (1980) studied two groups of full-term newborn infants, 11 in each group, who were either

---

[7] Birkenfeld et al., Effect of dietary L-carnitine supplementation on growth performance of piglets from control sows or sows treated with L-carnitine during pregnancy and lactation. J Anim Physiol Anim Nutr (Berl). 2005, Aug; 89(7-8):277-83.

[8] Warshaw, J. B. and E. Curry (1980). "Comparison of serum carnitine and ketone body concentrations in breast- and in formula-fed newborn infants." J Pediatr **97**(1): 122-125. See Appendix F.

breast-fed or fed unsupplemented milk-based infant formula over the first 42 hours after birth. The carnitine content of breast milk one to five days postpartum ranged from 70 to 95 nmoles/mL, with higher levels of up to 115 nmoles/mL in colostrum. The carnitine content of the commercial unsupplemented milk-based formula was between 40 and 80 nmoles/mL. Serum carnitine concentrations rose in the breast-fed infants but not in the formula-fed infants. At 42 hours, the concentration in the breast-fed infants was 60±8 nmoles/mL, compared to a statistically significantly lower concentration of 38±4 nmoles/mL in the formula-fed infants. The functional effect of the superior carnitine status of breast-fed infants compared to milk-based formula-fed infants was enhanced ketone body production by the breast-fed infants. Carnitine plays a central role in tissue oxidation of fatty acids. A copy of the Warshaw and Curry paper is attached as Appendix G.

Supplementing milk-based infant formula with L-carnitine increases the blood carnitine levels of infants.[9]

The FDA allowed carnitine supplementation of infant formulas in the United States in 1986 because of clinical reports of carnitine deficiency in infants fed carnitine-poor infant formulas. The goal of supplementation was to ensure that infants fed infant formula receive as much carnitine as infants who are breast-fed.

The evidence supporting the essentiality of carnitine in the diets of newborn infants includes clinical manifestations of deficiency in infants fed carnitine-poor diets. These manifestations include failure to thrive, nonketotic hypoglycemia, hypotonia, and cardiomyopathy. The LSRO report of 1998 (Appendix F) describes the adverse effects of low carnitine infant formula on fat metabolism in full-term infants. The conclusion of the Expert Panel was a recommendation that infant formula should contain not less than 1.2 mg/100 kcal, an amount comparable to the levels found in human milk (1.1 to 1.9 mg/100 kcal). The maximum recommended was 2.0 mg/100 kcal, a value similar to the upper ranges of carnitine concentrations reported for human milk.

The latest (6th) edition of the Pediatric Nutrition Handbook, published by the American Academy of Pediatrics, has the following statement. Page 327:

> Taurine and **carnitine** are amino acids that serve important and specific functions in the cell but are not incorporated into proteins. They can be synthesized by the body from cysteine and lysine, respectively, and are present in a mixed diet containing proteins of animal origin. The rates of synthesis in infants fed by total parenteral nutrition or receiving synthetic formulas devoid of taurine and **carnitine** may be insufficient to meet all of their needs and necessitate dietary supplementation.

Carnitine can be found in normal foods in the human diet. The highest concentrations of carnitine are found in red meat (especially beef) and dairy products. As noted earlier, the

---

[9] Campoy, C., R. Bayes, et al. (1998). "Evaluation of carnitine nutritional status in full-term newborn infants." Early Hum Dev **53 Suppl**: S149-164.

bioavailability of carnitine in bovine milk is too low for milk-based infant formulas to provide the infant with enough carnitine unless L-carnitine is added to the formula. According to the Reference Handbook for Nutrition and Health Counselors in the WIC and CSF Programs produced by the Food and Nutrition Service of USDA[10], "protein-rich foods are generally introduced to infants between 6 and 8 months old. Protein-rich foods include meat, poultry, eggs, cheese, yogurt, and legumes. . . . Introduction of protein-rich foods earlier than 6 months old may cause hypersensitivity (allergic) reactions."

Therefore, since infant formulas serve as the sole item of the diet of infants who are not fed human milk for the first four to six months of life, and carnitine found in normal foods in the human diet, red meat and dairy products, is not recommended or insufficient to provide the infant with the same quantity of carnitine as human milk provides, the addition of carnitine to organic infant formulas is essential to ensure healthy infant growth and development.

13. Confidential Business Information Statement.

This petition contains no Confidential Business Information.

---

[10] Infant Nutrition and Feeding. Publication FNS-288, 1993. Page94.

Appendices

Petition for addition to the National List of the substance L-CARNITINE, for use in infant formula products labeled as "organic."

Appendix A – L-Carnitine Process Patent Examples

- U.S. Patent No. 4,708,936
- U.S. Patent No. 5,028,538
- U.S. Patent No. 6,653,112
- Canadian Patent No. 2,023,744
- U.S. Patent No. 5,087,745
- U.S. Patent No. 6,429,319
- U.S. Patent No. 5,473,104
- U.S. Patent No. 7,718,414

Appendix B – USP Reference Standard for L-Carnitine

Appendix C – Product Labels

Appendix D – Lonza Environmental Statement
Lonza Policy on Safety, Health and Environment

Appendix E – Carnitine Material Safety Data Sheet (MSDS)

Appendix F – Carnitine discussion – 1998 LSRO report (Pp. 2120S-2121S and 2066S)

Appendix G – Warshaw, J. B. and E. Curry (1980). "Comparison of serum carnitine and ketone body concentrations in breast- and in formula-fed newborn infants." J Pediatr **97**(1): 122-125.

L-Carnitine Process Patent Examples

Fermentation production of L-carnitine

U.S. Patent No. 4,708,936 – Lonza – November 24, 1987
Process for the continuous production of L-carnitine
Abstract: Process for the continuous production of L-carnitine by the microbiological method. A microorganism of the strain DSM No. 3225 (HK 1331b) type is cultivated in a bioreactor with crotonobetaine and/or gamma-butyrobetaine in the presence of a growth substrate. The culture fluid passes outside of the bioreactor in a circulation in which a separation of the cell is carried out. A quantity of cell-free solution, which is as large as the amount fed to the bioreactor as a substrate, is withdrawn from the bioreactor. The L-carnitine is separated from the cell-free solution.

U.S. Patent No. 5,028,538 – Sigma Tau – July 2, 1991
Process for the production of L-carnitine and its derivatives
Abstract: The invention relates to a process for the L(-)-carnitine biochemical production, in an economically favorable way. This new process is technically achievable with easily available raw materials in simple reaction conditions and with not complicated substance separation techniques. This process is carried out with bacteria that stereo-specifically hydrates crotonobetaine to L(-)-carnitine.

U.S. Patent No. 6,653,112 – Sigma Tau – November 25, 2003
Method for producing L-carnitine from crotonobetaine using a two stage continuous cell-recycle reactor
Abstract: L(-)-carnitine is synthesized from crotonobetaine, crotonobetaine salts or derivatives in an ecologically advantageous manner by immobilizing cells of Escherichia coli 044 K74 on ceramics, glass beads or polyurethane disks in a two stage continuously operating cell recycle reactor containing a reaction medium. The medium preferably contains between 25 mM and 1 M crotonobetaine and at least 50 mM fumarate. Growing or resting cells of E. coli are retained in the reactor by micro or ultrafiltration membranes which are arranged as a flat membrane module or hollow fiber module. A first stage contains a reactor tank and a second stage contains an external recirculation loop connected to the tank for feeding the reaction medium through a filter unit. L-carnitine is synthesized under anaerobic conditions to produce a reaction medium containing L-carnitine and unreacted crotonobetaine. The reaction medium is transferred through the recirculation loop to the filter unit to produce a filtrate containing L-carnitine and a residue containing unreacted crotonobetaine and cells. The residue is recirculated to the reactor tank.

Production of Crotonobetaine

Canadian Patent No. 2,023,744
Process for the Production of Crotonobetaine Hydrochloride
Abstract: A process is disclosed for the production of crotonobetaine hydrochloride, which is a starting product in the microbiological production of L-carnitine. In the process, carnitine hydrochloride in its racemic form or in the form of its enantiomers is reacted at elevated temperature with acetic anhydride in the presence of an acid catalyst.

Production of Butyrobetaine

U.S. Patent No. 5,087,745 – Lanza - February 11, 1992
Process for the production of gamma-butyrobetaine
Abstract: Process, which is feasible on a commercial scale, for the production of gamma-butyrobetaine. For this purpose, butyrolactone with hydrogen chloride and an alcohol is converted to the chlorobutyric acid ester, the trimethylammonium butyric acid salt is formed with trimethylamine and then saponified to the end product.

Totally Synthetic Processes for Carnitine

U.S. Patent No. 6,429,319 – August 6, 2002
Continuous process for the production of optically pure (S)-beta-hydroxy-gamma-butyrolactone
Abstract: Disclosed is a process for the production of optically pure (S)-beta-hydroxy-gamma-butyrolactone through the hydrogenation of substituted carboxylic acid derivatives. A solution containing 1 to 50% by weight of a substituted carboxylic acid derivative is fed at a WHSV of 0.1 to 10 h.sup.-1, to a fixed bed reactor which is filled with a catalyst and maintained at a reaction temperature of 50 to 550.degree. C. under a halogen partial pressure of 15 to 5,500 psig. The catalyst is composed of a noble metal as a catalytically effective ingredient which is impregnated in an inorganic oxide as a support. The molar ratio of the hydrogen to the substituted carboxylic acid derivative is maintained at a molar ratio of 1:1 to 10:1. The process can produce optically pure (S)-beta-hydroxy-gamma-butyrolactone with higher purities at higher yields than can conventional techniques. In addition to being relatively simple and environmentally friendly, the process is so economically favorable as to apply to industrial production.

U.S. Patent No. 5,473,104 – December 5, 1995
Process for the preparation of L-carnitine
Abstract: There is disclosed a process for preparation of L-carnitine from (S)-3-hydroxybutyrolactone. The process is a two-step preparation in which (S)-3-hydroxybutyrolactone is first converted to a hydroxy-activated form and subsequently transformed to L-carnitine by treatment of the hydroxy-activated (S)-3-hydroxybutyrolactone with trimethylamine in water.

U.S. Patent No. 7,718,414 – CJ Cheiljedang Corp. – May 18, 2010
Microorganism of Enterobacteriacae genus harboring genes associated with L-carnitine biosynthesis and method of producing L-carnitine using the microorganism
Abstract: Provided is a microorganism that belongs to Enterobacteriacae and a method of producing L-carnitine using the same. The microorganism includes polynucleotide encoding activity of S-adenosylmethionine-6-N-lysine methyltransferase from Neurospora crassa, polynucleotide encoding activity of 6-N-trimethyllysine hydroxylase, polynucleotide encoding activity of 3-hydroxy-6-N-trimethyllysine aldolase, and polynucleotide encoding activity of .gamma.-trimethylaminoaldehyde dehydrogenase and y-butyrobetaine hydroxylase.

United States Patent [19]

Kulla et al.

[11] Patent Number: 4,708,936

[45] Date of Patent: Nov. 24, 1987

[54] **PROCESS FOR THE CONTINUOUS PRODUCTION OF L-CARNITINE**

[75] Inventors: **Hans Kulla**, Visp; **Pavel Lehky**, Naters; **Armand Squaratti**, Brig, all of Switzerland

[73] Assignee: **Lonza Ltd.**, Gampel, Switzerland

[21] Appl. No.: **717,546**

[22] Filed: **Mar. 29, 1985**

[30]   **Foreign Application Priority Data**

Feb. 27, 1985 [CH] Switzerland 890/85

[51] Int. Cl.^{4} **C12P 13/00; C12P 13/01**
[52] U.S. Cl. **435/128;** 435/106; 435/287; 435/824; 435/825
[58] Field of Search 435/128, 106, 287, 824, 435/825

[56]   **References Cited**

U.S. PATENT DOCUMENTS

4,371,618 2/1983 Cavazza 435/128

FOREIGN PATENT DOCUMENTS

0122794 10/1984 European Pat. Off. .

OTHER PUBLICATIONS

Ornston et al., *Biochemical and Biophysical Research Communications,* vol. 36, No. 1, (1969), pp. 179–184.

Perry's Chemical Engineers Handbook, 6th Edition, (1984), pp. 27–7.
J. H. Miller, "Experiments in Molecular Genetics", Cold Spring Laboratory, (1972), pp. 121 to 143, Experiments 13 through 17.
P. Gerhardt et al., (eds.), Manual of Methods for General Bacteriology, Am. Soc. for Microbiology, (1981), pp. 222 to 242.
Kulla et al., Arch. Microbiol. 135, 1 (1983), pp. 1 to 7.
J. P. Vandecasteele, Appl. Environ, Microbiol. 39, pp. 327 to 334, (1980).
Lindstedt et al., Biochemistry 6, pp. 1262–1270 (1967).
Lindstedt et al., Biochemistry 16, pp. 2181–2188 (1977).

Primary Examiner—Charles F. Warren
Assistant Examiner—Elizabeth A. Hanley
Attorney, Agent, or Firm—Fisher, Christen & Sabol

[57]   **ABSTRACT**

Process for the continuous production of L-carnitine by the microbiological method. A microorganism of the strain DSM No. 3225 (HK 1331b) type is cultivated in a bioreactor with crotonobetaine and/or γ-butyrobetaine in the presence of a growth substrate. The culture fluid passes outside of the bioreactor in a circulation in which a separation of the cell is carried out. A quantity of cell-free solution, which is as large as the amount fed to the bioreactor as a substrate, is withdrawn from the bioreactor. The L-carnitine is separated from the cell-free solution.

22 Claims, 1 Drawing Figure

U.S. Patent   Nov. 24, 1987   **4,708,936**

FIGURE
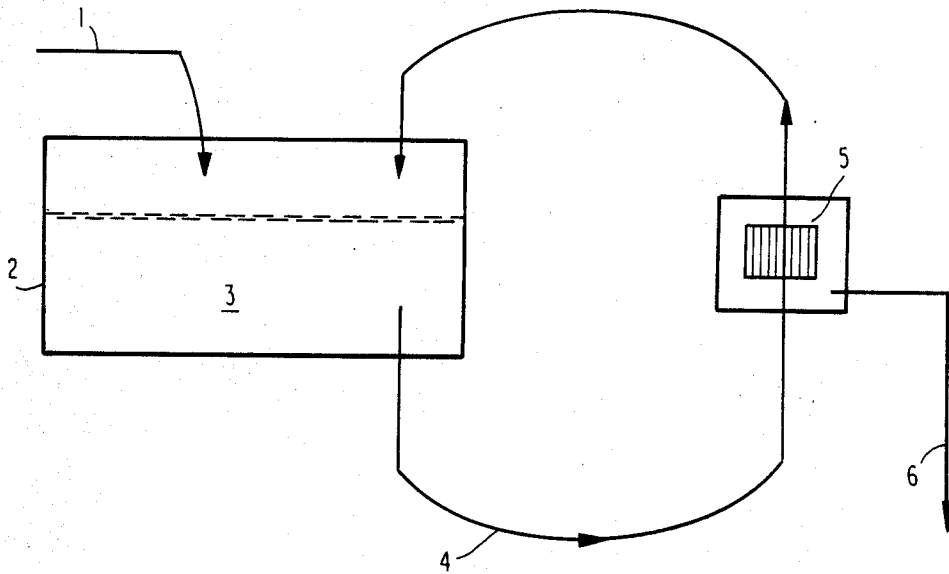
4,708,936

1

PROCESS FOR THE CONTINUOUS PRODUCTION OF L-CARNITINE

BACKGROUND OF THE INVENTION

1. Field Of The Invention

The invention relates to a continuous process for the production of L-carnitine by the microbiological or biotechnical method.

2. Prior Art Or Related Art

The production of L-carnitine from γ-butyrobetaine is known. The γ-butyrobetaine is brought into contact with a hydroxylase-enzyme, liberated from spores of Neurospora crassa (U.S. Pat. No. 4,371,618), in the presence of sodium-2-oxoglutarate, a reducing agent, an iron ion source and a hydroxyl group donor solvent. Such process has the disadvantage of needing a multiplicity of co-factors, which must be externally fed in. Thus, stoichiometric quantities of 2-oxoglutarate are decarboxylized oxidatively in the reaction to succinate. Fe^{2+} is needed as the O_2-activator, ascorbate is used in order to keep the iron in the reduced form, and catalase is needed to destroy the harmful H_2O_2 which develops in the traces.

Lindstedt et al., "The Formation and Degradation of Carnitine in Pseudomonas", (Biochemistry 6, 1262–1270 (1967)), isolated a microorganism of the species Pseudomonas which grows with γ-butyrobetaine as a C- and N-source. The first reaction of the composition path was the hydroxylation of the γ-butyrobetaine to L-carnitine, whereupon the intermediately developing L-carnitine was further completely catabolized into CO_2, H_2O and NH_3.

If such microorganism was used for the production of L-carnitine, such hydroxylase obtained from bacteria would also have the disadvantageous co-factor-requirements described by Lindstedt et al., "Purification and Properties of γ-Butyrobetaine Hydroxylase from Pseudomonas sp. AK 1", Biochemistry 16, 2181–2188, (1977).

BROAD DESCRIPTION OF THE INVENTION

An object of the invention is to provide a process which overcomes and/or avoids the above-stated disadvantages of the prior art and mentioned related art. Another object of the invention is to provide a process, which allows in a continuous system or manner, the production of L-carnitine enantio-selectively. A further object of the invention is to provide a process for the production of L-carnitine from crotonobetaine and/or γ-butyrobetaine by means of the microbiological process. Other objects and advantages are set out herein or are obvious herefrom to one skilled in the art.

The advantages and objects of the invention are achieved by the process of the invention.

As compared to the systems known from the above-description of the prior art, microorganisms used in the invention use H_2O, and not O_2, as the hydroxyl group donors, as was determined by investigations using $H_2^{18}O$ and $^{18}O_2$.

According to the invention, a microorganism of the strain DSM No. 3225 (HK 1331b) type is cultivated in a bioreactor with crotonobetaine and/or γ-butyrobetaine in the presence of a growth substrate. The culture fluid is guided outside of the bioreactor in a circulation in which a cell separation is carried out, whereby a quantity of cell-free solution, which is as large as the amount fed to the bioreactor as a substrate, is withdrawn from

2

the bioreactor. Finally the L-carnitine is separated from the cell-free solution.

By use of the advantageous invention method of operation, and as a result thereof of the retention of the biomass, a higher productivity as well as a greatly improved longtime stability of the continuous process is achieved.

Preferably crotonobetaine, γ-butyrobetaine or a mixture thereof is used in a quantity of 0.1 to 10 percent by weight, based on the weight of the culture medium. Preferably dimethylglycine, choline, glutamate, acetate and/or betaine is used as a growth substrate. The growth substrates are preferably used in a quantity of 0.1 to 10 percent by weight, based on the weight of the culture medium. The separation of the cells is accomplished preferably by centrifugation or preferably by ultrafiltration. Preferably a through-flow rate of 0.05 to 0.5 h^{-1} is used.

Preferably γ-butyrobetaine and/or crotonobetaine, which has been desalted and purified, is used. The γ-butyrobetaine and/or crotonobetaine prefereably is desalted and purified by electrodialysis or by ion exchange.

The isolation of L-carnitine from the cell-free solution is preferably accomplished by cation exchange chromotography. Or, preferably, the isolation of L-carnitine is accomplished from the cell-free solution by electrodialysis with subsequent recrystallization.

BRIEF DESCRIPTION OF THE DRAWING

In the drawing:

The Figure is a schematic view of a preferred embodiment of the continuous process of the invention.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, all parts, ratios, percentages and proportions are on a weight basis unless otherwise stated herein or otherwise obvious herefrom to one ordinarily skilled in the art.

The preferred microorganism DSM No. 3225 (HK 1331b) is particularly suitable for producing L-carnitine in the continuous process of the invention from crotonobetaine and/or γ-butyrobetaine and without catabolizing the produced L-carnitine. The new strain DSM 3225 (HK 1331b) was deposited on Feb. 8, 1985, in the German collection of microorganisms (DSM), Gesellschaft fuer Biotechnologische Forschung mbH., Griesebachstrasse 8, 4300 Goettingen, Federal Republic of Germany, under the designation or number DSM 3225. Such deposit of a culture of such new strain of microorganisms in such depository affords permanence of the deposit and ready accessibility thereto by the public if a patent is granted, under conditions which assure (a) that access to the culture will be available during the pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 C.F.R. 1.14 and 35 U.S.C. 122, and (b) that all restrictions on the availability to the public of the culture so deposited will be irrevocably removed upon the granting of the patent. The applicants or their assigns have provided assurance of permanent availability of the culture to the public through such depository.

Microorganism strain DSM 3225 is obtainable by the following selection methods of the invention:

(a) Microorganisms, which grow with betaine, γ-butyrobetaine, crotonobetaine and L-carnitine as the

4,708,936

3

C-and N-source, are mutated in the conventional or customary manner.

(b) From the culture obtained by cultivation of the mutated microorganisms, the mutated microorganisms are selected which are stable, do not catabolize L-carnitine and do not grow on L-carnitine, crotonobetaine or γ-butyrobetaine, but do grow with betaine.

(c) From the latter culture, such a strain is selected which is stable, does not catabolize L-carnitine, does not grow on L-carnitine, crotonobetaine or γ-butyrobetaine and shows good growth in a medium which contains L-glutamate as well as γ-butyrobetaine or crotonobetaine.

Preferably, following selection step (b), the microorganisms are selected which excrete L-carnitine and do not grow on L-carnitine, crotonobetaine or γ-butyrobetaine, but do grow with betaine.

Effectively, the mutated microorganisms are cultivated further in a betaine medium and these further-cultivated microorganisms are cultivated even further preferably in an L-carnitine medium in order to carry out selection step (b). The cultivation of strains growing with betaine, γ-butyrobetaine, crotonobetaine and L-carnitine as a C- and N-source is carried out effectively in such a manner that one produces mixed cultures from mixtures of bacteria by inoculation of crotonobetaine nutritional solutions and that, from these cultures, one establishes pure cultures of microorganism decomposing crotonobetaine with the help of traditional microbiological techniques. The mutation of such a culture, which grows with betaine, γ-butyrobetaine, crotonobetaine and L-carnitine as a C-and N-source can be carried out according to known methods. [J. H. Miller, "Experiments in Molecular Genetics", Cold Spring Harbor Laboratory, (1972), pages 121 to 143].

Methods usable effectively for the production of stable mutants are the frame-shift method, the deletion method and the transposon-insertion method. The microorganisms mutated in this way are then subjected to selection step (b), after further cultivation in a betaine medium and transfer into an L-carnitine medium, whereby means of known "counter-selecting agents", [P. Gerhardt et al., (eds.), Manual of methods for General Bacteriology, Am. Soc. for Microbiology, (1981), pages 222 to 242], those microorganisms are selected which are stable, do not catabolize L-carnitine and do not grow on L-carnitine, crotonobetaine or γ-butyrobetaine, but do not grow with betaine.

Starting out from these strains which produce L-carnitine corresponding to step (c), spontaneous well-growing colonies are isolated from the surface of a nutrient medium strengthened with agar, which contains L-glutamate and butyrobetaine (selectively also crotonobetaine or L-carnitine). These strains grow poorly with betaine. Thus, these strains are ideally suited in the continuous process according to the invention with biomass retention, since in a betaine plus butyrobetaine (selectively also crotonobetaine) medium, a productive equilibrium occurs without any additional growth of biomass. The quick growth desirable in the starting phase can be achieved by the addition of L-glutamate.

The preferred strain is the strain DSM No. 3225, (HK 1331b), as well as its descendants and mutants.

The scientific (taxonomic) description of the strain DSM No. 3225 (HK 1331b) is as follows:

4

Form (shape) of the cell	rods partly pleomorphic
length μm	1–2
width μm	0.5–0.8
mobility	+
flagellata	peritric
gram-reaction	−
spores	−
formation of poly-β-hydroxybutyrate	−
oxidase	+
catalase	+
growth:	
anaerobic	−
37° C.	+
41° C.	−
pH 5.6	−
Mac-Conkey-agar	+
SS-agar	−
cetrimide-agar	−
formation of pigment:	
not diffusing	−
diffusing	−
fluorescing	−
formation of acid (OF-Test) from:	
glucose aerobic	−
anaerobic	−
fructose aerobic	−
ASS glucose	+
xylose	+
trehalose	+
ethanol	−
gas formation from glucose	−
ONPG	+
arginine dihydrolase	−
lysine decarboxylase	−
phenylalanine deaminase	−
ornithine decarboxylase	−
H_2S	−
Voges-Proskauer	−
indol	−
nitrite from nitrate	+
denitrification	+
formation of levan	−
lecithinase	−
urease	+
decomposition of:	
starch	−
gelatin	−
casein	−
tyrosin	−
Tween 80	−
DNA	+
aesculin	+
utilization of substrate	
acetate	−
citrate	−
malonate	−
glycine	−
norleucin	−
xylose	+
fructose	+
glucose	+
autotrophic growth with H_2	−
3-ketolactose	−
growth:	
betaine	+
L-carnitine	−
γ-butyrobetaine	−
crotonobetaine	−
L-glutamate and crotono-betaine	+
L-glutamate and butyrobetaine	+
L-glutamate and L-carnitine	+

The continuous process of the invention for the production of L-carnitine can be carried out effectively in such a way that a preculture of a microorganism, preferably a microorganism strain DMS 3225, is cultivated in a sterilized, preferably vitamin containing mineral me-

4,708,936

5

dium [Kulla et al., Arch. Microbiol, 135, 1 (1983), pages 1 to 7], at 20° to 40° C., preferably at 30° C., at an effective pH value of 6 to 8, preferably 7, for 20 to 50 hours, preferably for 30 to 40 hours. This preculture effectively contains 0.1 to 10 percent by weight, preferably 0.1 to 5 percent by weight, of choline, glutamate, acetate, dimethylglycine or betaine as a growth substrate. Particularly preferred is betaine in a quantity of 0.2 to 5 percent by weight.

Furthermore, it is customary with microbiology techniques to add to the preculture also the starting compounds that are to be converted-in this case, γ-butyrobetaine, crotonobetaine or mixtures thereof in a quantity of 0.1 to 10 percent by weight, preferably 0.1 to 5 percent by weight, related to the reaction medium. The γ-butyrobetaine or crotonobetaine can be present as a hydrochloride salt or as a free inner salt as well as in the form of one of its derivatives.

Using the preculture produced according to the process mentioned above, further cultures can be inoculated. These further cultures have effectively the same composition as the precultures.

After that, one can change over to the continuous method of operation of the invention (according to the principle show in FIG. 1).

Effectively one proceeds in such a way that, with a through-flow rate D of 0.05 to 0.5h^{-1}, preferably 0.07 to 0.12h^{-1}, one simultaneously pumps γ-butyrobetaine and/or crotonobetaine in a concentration of effectively 1 to 100 g/1, preferably 10 to 50 g/1, and betaine in a concentration of effectively 1 to 100 g/1, preferably 2 to 20 g/1, by medium line **1** into bioreactor **2** (the concentrations are related to 1 1 of culture liquid). Culture fluid **3**, which is contained in reactor **2**, which corresponding to the vitamin-containing mineral medium according to Kulla et al., Arch. Microbiol. 135, 1 (1983), and which contains the L-carnitine-producing strain DSM 3225 (HK 1331b), at the same time is transported outside of bioreactor **2** in circulation line **4**, which contains cell separating arrangement **5** (it effectively is equipment for ultrafiltration and centrifugation).

As a result of separation of the cells, one achieves on the one hand, that the active biomass will not be withdrawn from bioreactor **2** but instead is brought back and, on the other hand that the L-carnitine containing cell-free solution **6** can be withdrawn from the circulation. At the same time, one proceeds in such a way that an equally large quantity of cell-free solution **6** is withdrawn from the cell separation as is fed to bioreactor **2** in medium inlet **1**. Usually, the concentration of L-carnitine in the cell-free solution **6** is equivalent to the reacted quantity of γ-butyrobetaine or crotonobetaine. Also usually, the cell-free solution **6** contains an additional 5 to 10 percent of unreacted γ-butyrobetaine or crotonobetaine.

The high stability of the system is surprising and unexpected. Thus, for weeks, no loss of activity is observed.

Relief of the culture can be using γ-butyrobetaine and/or the crotonobetaine which previously has been desalinated and purified by means of ion exchanges or by means of electrodialysis.

In order to achieve a 100 percent turnover, one can likewise proceed in such a way that a secondary reaction step is arranged in the form of a cascade.

Obtaining of L-carnitine from cell-free solution 6 can be carried out in such a way that the solution is freed of

6

the charged particles (cations and anions) by means of a laboratory electrodialysis installation. The terminal point of the desalination can be determined conductometrically. At the same time, the salts migrate into the concentrate circulation while the L-carnitine remains as an inner salt (betaine) in the diluent circulation. Thus, yields of L-carnitine in the diluent of more than 95 percent can be achieved after the desalination.

Alternatively to the electrodialysis, it is possible to desalinate the L-carnitine also by means of a strongly acidic cation exchange in the H$^+$-form [see J. P. Vandecasteele, Appl. Environ, Microbiol. 39, 327 (1980), pages 327 to 334]. At the same time, solution is allowed to flow over an ion exchange column for a length of time until the ion exchange is exhausted and L-carnitine breaks through. The anions pass as free acids into the passage. The cations remain on the ion exchange. After neutral washing of ion exchange with water, the L-carnitine can be eluted with aqueous ammonia solution. Thus, yields of L-carnitine of more than 95 percent can be achieved in the ammoniacal eluant.

The diluted L-carnitine solution obtained in the case of electrodialysis as well as by means of ion exchange can be upgraded by a reversing osmosis and can subsequently be azeotropically dehydrated.

The L-carnitine obtained thusly can then be converted into a pure, white L-carnitine by subsequent recrystallization from effectively isobutanol, acetone, methanol, ethanol, or n-butanol. Or, the recrystallization can be conducted using one of such materials in combination with a solvent which dissolves only a small amount of L-carnitine, such as, acetone, ethylacetate, butylacetate, isobutylmethyl ketone and acetonitrile, preferably isobutanol and activated charcoal treatment. According to such process, one can obtain L-carnitine with specific turns of $[\alpha]_D^{25}$ —30.5 to 31.0; c is 1 in H$_2$O (literature value —30.9°; Strack et al., Hoppe-Seyler's Z.f. physiolog. Chem., 318 (1960), 129) and a content of more than 99 percent (HPLC).

By way of summary, the invention involves the continuous production of L-carnitine from crotonobetaine and/or γ-butyrobetaine using the microbiology way or method.

The pertinent parts of commonly-owned concurrently-filed herewith application Ser. No. 717,698, filed on Mar. 29, 1985, (L.P. 1329), entitled "Process For The Production Of L-Carnitine By Way Of Microbiology", by Hans Kulla and Pavel Lehky are incorporated herein by reference.

The practical use of the invention is illustrated by the following examples:

EXAMPLE 1

A 0.1 1 preculture of the strain DSM 3225 (HK 1331b) was cultivated in the following nutrient medium at 30° C. and pH 7.0 for 24 hours:

Composition of the nutrient medium	
L-glutamate	2 g
betaine	2 g
crotonobetaine	2 g
buffer solution	100 ml
Mg—Ca—Fe solution	25 ml
trace elements solution	1 ml
vitamin solution	1 ml
with water on	1 1
Buffer solution	

4,708,936

-continued

Composition of the nutrient medium		
Na_2SO_4	1	g
$Na_2HPO_4.2H_2O$	25.08	g
KH_2PO_4	10	g
NaCl	30	g
with water on	1	l
Mg—Ca—Fe solution		
$MgCl_2.6H_2O$	16	g
$CaCl_2.2H_2O$	0.58	g
$FeCl_3.6H_2O$	0.032	g
with water on	1	l
Trace elements solution		
$ZnSO_4.7H_2O$	100	mg
$MnCl_2.4H_2O$	30	mg
H_3BO_3	300	mg
$CoCl_2.6H_2O$	200	mg
$CuCl_2.2H_2O$	10	mg
$NiCl_2.6H_2O$	22	mg
$NaMoO_4.2H_2O$	30	mg
with water on	1	l
Vitamin solution		
pyridoxal.HCl	10	mg
riboflavine	5	mg
nicotinamide	5	mg
thiamine.HCl	5	mg
biotine	2	mg
sodium pantothenate	5	mg
p-aminobenzoic acid	5	mg
folic acid	2	mg
vitamin B 12	5	mg
with water on	1	l

With the preculture, 2 l of nutritional medium of equal composition was inoculated in the fermenter and was cultivated for 24 hours at 30° C. and pH 7. The pH was kept constant at 7.0 by the addition of 8 percent phosphoric acid.

Subsequently, the continuous operation was started. With a flow rate D of 0.09 per hour, the above-described medium containing 15 g/l of betaine and 24.3 g/l of crotonobetaine (desalinated with the help of electrodialysis) without L-glutamate, was pumped through medium inlet 1 into the bioreactor 2. Culture fluid 3, which contained the L-carnitine and the HK 1331b biomass, was continuously pumped in circulation line 4 at the speed of 2 l per min. through a normal ultrafiltration arrangement. The active biomass was brought back into the bioreactor. The clear L-carnitine containing filtrate 6 was pumped off at a flow rate of likewise 0.09 per hour.

According to analysis (HPLC), the filtrate contained 25 g/l of L-carnitine and 2.0 g/l unreacted crotonobetaine. This corresponds to a 92 percent turnover of crotonobetaine and an L-carnitine yield of 99.6 percent, related to the reacted crotonobetaine. The betaine was completely catabolized. After reaching a maximum cell density of about 35 g dry weight per liter, the biomass concentration and the L-carnitine productivity remained constant for at least 1 month.

Isolation of L-carnitine

L-carnitine was isolated from the solution which contained 25 g of L-carnitine, 2 g of crotonobetaine and about 10 g of inorganic salts per liter of solution, as follows:

(1) Desalination

2 Liters of L-carnitine solution was desalinated by means of a commercial strongly acidic cation exchange in the H+-form and subsequently by means of elution with aqueous ammonia. The process was as follows:

400 g of DOWEX ® HCR-W2 was placed in an exchange glass column. Then, 2 l of L-carnitine solution was allowed to flow over the ion exchange for about 3 hours. At the same time, the L-carnitine did not yet come through the DOWEX column. After that, the column material was washed out with about 1.5 l of ice water. Subsequently the L-carnitine and crotonobetaine were eluted with 1 l of 5 percent ammonia and 0.5 l of ice water in about 3 hours—1550 ml of ammoniacally desalinated L-carnitine solution was obtained. 30.9 of L-carnitine and 2.47 g of crotonobetaine were found in each liter of the solution. This corresponds to a yield of 96 percent, in the case of desalination by means of ion exchange.

(2) Upgrading

The solution from the desalination using ion exchange (1550 ml) was concentrated at 50° C. and 25 mbar by means of a labor-rotation evaporator. For the removal of the water, the latter was taken out azeotropically with isobutanol on the rotation evaporator under vacuum (50° C., 25 mbar). The residue was dried at 50° C. and 25 mbar (34.05 g). containing 90.7 percent of L-carnitine and 7.2 percent of crotonobetaine. This corresponds to a practically quantitative yield of L-carnitine for the upgrading.

(3) Purification

The purificaton of the crude L-carnitine was accomplished in the same manner as described in Example 2 below. At the same time, a comparable yield and quality was achieved.

EXAMPLE 2

100 ml of the nutritional medium described in Example 1, which contained 2 g/l butyrobetaine instead of the crotonobetaine, was inoculated with strain HK 1331b and was cultivated at 30° C. and pH 7 for 24 hours. In this case, 2 l of the same nutritional medium were inoculated in the bioreactor and were cultivated like the preculture (at 30° C. and pH 7) for 24 hours. After that, the continuous operation was started. The nutrient, which had the following composition:

desalinated butyrobetaine	25	g
betaine	0.7	g
$MgCl_2.6H_2O$	200	mg
$CaCl_2.2H_2O$	14.5	mg
$FeCl_3.6H_2O$	0.8	mg
Na_2SO_4	100	mg
KCl	100	mg
solution of trace elements	1	ml
vitamin solution	1	ml
with water on	1	l

was pumped into the bioreactor at a flow rate of 0.09/h. The pH was kept constant at 7 by an addition of 8 percent H_3PO_4. As described in Example 1, the culture fluid was continuously filtered and the clear filtrate was collected. After reaching a maximum cell density of about 30 g dry weight per liter, the L-carnitine productivity of the culture remained constant for 4 weeks. 4.5 Liters of filtrate were obtained daily. According to HPLC analysis, the filtrate contained 25 g/l of L-carnitine and 1.9 g/l of butyrobetaine. This corresponds to a 92.4 percent turnover of the educt and to an L-carnitine yield of 97.5 percent, related to the reacted butyrobetaine. The betaine was completely catabolized.

4,708,936

| 9 | 10 |

Isolation of L-carnitine

Pure L-carnitine was isolated from the solution which contained about 25 g of L-carnitine, 2 g of butyrobetaine and about 4 g of inorganic salts per liter of solution, as follows:

(1) Desalination

2 Liters of L-carnitine solution was desalinated by means of a customary labor-electrodialysis installation (Berghof BEL-2).

At the same time, the experiment was conducted as follows:

1 Liter of 5 percent sodium sulfate solution was inserted into the electrode circuit, 1.9 1 of 0.1 percent cooking salt solution was placed in the concentrate circulation and 2.0 1 of 1-carnitine was inserted into the diluate circulation. Now, this was electrodialyzed for about 4 hours at a voltage which was limited at 24 volts and a current which was limited at 2 amps. The turnover velocity of the three circulations was about 1.8 1/min. At the beginning of the dialysis a conductive capacity of about 5 mS/cm was measured in the dialysate circulation (containing the L-carnitine and butyrobetaine). At the end of the electrodialysis, conductivity amounted to about 0.1 mS/cm. The L-carnitine solution in the diluate circulation was pumped off and was washed secondarily with about 200 ml of E-water (ice water). In this diluate L-carnitine solution (2150 ml), 22.1 g of L-carnitine and 1.77 g of butyrobetaine per liter were found analytically, which corresponds to a yield of 95 percent in the case of the desalination by means of electrodialysis. This solution was used for upgrading as explained below.

(2) Upgrading

The solution from the desalination by means of electrodialysis (10 1) was reconcentrated by means of a conventional reversing osmosis-module of 22 g of L-carnitine to about 160 g of L-carnitine/1 (1 molar).

At the same time, the experiment was conducted as follows:

The solution to be concentrated was placed in the operating container, and after building up the pressure with nitrogen to about 42 bar, it was turned over by means of a turnover pump for a sufficient length of time until about 8.61 1 of permeate had passed through (time needed: 2 to 4 hours). The part retained constituted an approximately 7-times reconcentrated solution, containing 152 g of L-carnitine and 12.1 g of butyrobetaine. Approximately 5 percent of the L-carnitine and of the butyrobetaine passed into the permeate, so that the yield for the upgrading in the case of the reversing osmosis amounted to about 95 percent. Further upgrading of the L-carnitine solution enriched by means of reversing osmosis into crude L-carnitine took place analogously to the method mentioned in Example 1 by evaporation on the rotary evaporator and azeotropic drying with isobutanol, which took place practically quantitatively.

(3) Purification

60 g of crude L-carnitine (approximately 93 percent of L-carnitine and 6 percent of butyrobetaine) and 6 g of activated charcoal were heated in 900 ml of isobutanol to reflux. The activated charcoal was filtered off hot and 580 ml of isobutanol was distilled off the filtrate, whereby the L-carnitine partially crystallized out. 300 ml of acetone was added and the material was cooled to ambient temperature. L-carnitine was subjected to suction and was washed twice with 60 ml of acetone. The recrystallization was carried out for a second time (without treatment with charcoal) in order to completely remove the butyrobetaine. After that, the material was dried at 70° C. and 25 mbar until the weight was constant. Then, 49.5 g of white, not-discolored L-carnitine [HPLC more than 99 percent; specific rotation $[\alpha]_D^{25} - 30.9°$, (c=1, H_2O)] was isolated at a yield of 87 percent above both recrystallizations, which corresponds to an average yield of 93 percent per recrystallization. The evaporation residues, 4.76 g from the first recrystallization and 3.46 g from the 2nd recrystallization (mainly L-carnitine and butyrobetaine), could be returned to the fermentation.

What is claimed is:

1. A process for the continuous production of L-carnitine comprising cultivating a microorganism of the strain *Achromobacter xylosoxydans* DSM 3225 (HK 1331b) type in a bioreactor with crotonobetaine and/or γ-butyrobetaine in the presence of a growth substrate, passing the culture fluid outside of the bioreactor in a circulation in which a separation of the cell is carried out, whereby a quantity of cell-free solution, which is as large as the amount fed to the bioreactor as a substrate, is withdrawn from the bioreactor, and separating the L-carnitine from the cell-free solution.

2. The process as claimed in claim 1 wherein crotonobetaine γ-butyrobetaine or a mixture thereof is used in a quantity of 0.1 to 10 percent by weight, in relation to the culture medium.

3. The process as claimed in claim 2 wherein dimethylglycine, choline, glutamate, acetate and/or betaine is used as a growth substrate.

4. The process as claimed in claim 3 wherein the growth substrate is used in a quantity of 0.1 to 10 percent by weight, in relation to the culture medium.

5. The process as claimed in claim 4 wherein the separation of the cells is accomplished by centrifugation.

6. The process as claimed in claim 4 wherein the separation of the cells is accomplished by ultrafiltration.

7. The process as claimed in claim 6 wherein a through-flow rate of 0.05 h^{-1} is used.

8. The process as claimed in claim 7 wherein a γ-butyrobetaine and/or crotonobetaine is used which has been desalted and purified.

9. The process as claimed in claim 8 wherein the γ-butyrobetaine and/or crotonobetaine is desalted and purified by electrodialysis.

10. The process as claimed in claim 8 wherein the γ-butyrobetaine and/or crotonobetaine is desalted and purified by ion exchange.

11. The process as claimed in claim 8 wherein the isolation of L-carnitine from the cell-free solution is accomplished by cation exchange chromotography.

12. The process as claimed in claim 8 wherein the isolation of L-carnitine is accomplished from the cell-free solution by electrodialysis with subsequent recrystallization.

13. The process as claimed in claim 1 wherein dimethylglycine, choline, glutamate, acetate and/or betaine is used as a growth substrate.

14. The process as claimed in claim 1 wherein the growth substrate is used in a quantity of 0.1 to 10 percent by weight, based on the culture medium.

4,708,936

11

15. The process as claimed in claim **1** wherein the separation of the cells is accomplished by centrifugation.

16. The process as claimed in claim **1** wherein the separation of the cells is accomplished by ultrafiltration.

17. The process as claimed in claim **1** wherein a through-flow rate of 0.05 to 0.5 h^{-1} is used.

18. The process as claimed in claim **1** wherein γ-butyrobetaine and/or crotonobetaine is used which has been desalted and purified.

12

19. The process as claimed in claim **18** wherein the γ-butyrobetaine and/or crotonobetaine is desalted and purfied by electrodialysis.

20. The process as claimed in claim **18** wherein the γ-butyrobetaine and/or crotonobetaine is desalted and purified by ion exchange.

21. The process as claimed in claim **1** wherein the isolation of L-carnitine from the cell-free solution is accomplished by cation exchange chromotography.

22. The process as claimed in claim **1** wherein the isolation of L-carnitine is accomplished from the cell-free solution by electrodialysis with subsequent recrystallization.

     *   *   *   *   *

United States Patent [19]

Seim et al.

[11] **Patent Number:**   **5,028,538**

[45] **Date of Patent:**   **Jul. 2, 1991**

[54] **PROCESS FOR THE PRODUCTION OF L-CARNITINE AND ITS DEIVATIVES**

[75] Inventors: **Hermann Seim; Heniz Loester; Rainer Claus; Hans-Peter Kleber; Erich Strack,** all of Leipzig, German Democratic Rep.

[73] Assignee: **Sigma Tau Industries Farmaceutiche Riunite S.p.A.,** Rome, Italy

[21] Appl. No.: **349,929**

[22] Filed: **May 9, 1989**

Related U.S. Application Data

[63] Continuation of Ser. No. 665,765, Oct. 29, 1984, abandoned.

[30]   **Foreign Application Priority Data**

Nov. 3, 1983 [DD] German Democratic Rep. 2562812

[51] Int. Cl.^{5} **C12R 1/19; C12P 13/00**
[52] U.S. Cl. **435/128; 435/252.8; 435/280**
[58] Field of Search 435/128, 252.8, 280

[56]   **References Cited**

U.S. PATENT DOCUMENTS

4,650,759 3/1987 Yokozeki et al. 435/128
4,708,936 11/1987 Kulla et al. 435/128

OTHER PUBLICATIONS

Chemical Abstracts, vol. 98, No. 15, abstract 122551z, Apr. 11, 1983; Seim et al., "Reductive Metabolism of L-carnitine . . . by Escherichia coli".

Chemical Abstracts, vol. 96, No. 21, abstract 177676h; May 24, 1982; Seim et al., "Formation of γ–butyrobetain . . . by Proteus vulgaris".

Seim et al., "Stimulation of Anaerobic Growth of *Salmonella typhanurium* by Reduction of L–Carnitine, Carnitine Derivatives and Structure–Related Trimethylammonium Compounds"; *Arch. Microbiol.* 132: 91–95 (1982).

Seim et al., "Reduktiver Stoffwechsel des L–Carnitins und strakturverwandter Trimethylammonium verbindungen in *Escherichia coli*"; *Acta Biol. Med. Germ.,* vol. 41, 11: 1009–1018 (1982).

Different Carnitine Acyltransferases in Calf Liver BBA 280, pp. 422–433 by Helge Erik Solberg.

Primary Examiner—Herbert J. Lilling
Attorney, Agent, or Firm—Ladas & Parry

[57]   **ABSTRACT**

The invention relates to a process for the L(−)-carnitine biochemical production, in an economically favorable way.

This new process is technically achievable with easily available raw, materials in simple reaction conditions and with not complicated substance separation techniques.

This process is carried out with bacteria that stereospecifically hydrates crotonobetaine to L(−)-carnitine.

8 Claims, No Drawings

5,028,538

1

PROCESS FOR THE PRODUCTION OF L-CARNITINE AND ITS DEIVATIVES

This is a continuation of co-pending application Ser. No. 06/665,765, filed on Oct. 29, 1984, now abandoned.

The present invention relates to a process for the production of optically active L(−)-carnitine(R-configuration) from an optically inactive precursor.

L-carnitine (3-hydroxy -4-trimethylaminobutyrate) is normally present in the organism where it exerts the role of activated long-chain free fatty acids' carrier, through the mitochondrial membrane. Since the mitochondrial membrane is impermeable to the acyl CoA's derivatives, the long-chain free fatty acids can cross it only when the esterification with L-carnitine has taken place. The importance of using L(−)-carnitine for therapeutical use has recently been underlined. ("Carnitine biosynthesis. metabolism, and functions" Editors: R. A. Frenkel and J. D. Mc Garry Academic Press 1980).

Since the needed quantity of L-carnitine was not available, it was often substituted with the DL-carnitine racemate, obtainable by total chemical synthesis. But the DL-carnitine, which contains the D(+) isomer, caused certain side effects which had not taken place using L(−)-carnitine (Curr. Ther. Res. 28 (1980), 195–198).

It is also known that the transferases necessary for the L(−)-carnitine formation, the carnitine acetyl-transferase (EC 2.3.1.7) and the carnitine palmitoyl-transferase (EC 2.3.1.21), are specific for the L(−) form, whereas the D(+) isomer is a competitive inhibitor of these two transferases.

Administration of D-carnitine can, moreover, create a decrease of L(−)-carnitine in the cardiac muscle or in the skeletal muscle, as has been demonstrated on laboratory animals (guinea-pigs) (Life Sciences 28 (1981) 2931–2938). The symptoms of human hyperthyrosis which had improved after L-carnitine treatment, worsened drastically with D-carnitine (Endokrinologie, 38, (1959) 218–225), thus for patients' treatment only L(−)-carnitine can presently be used. This is also and especially valid in the case of patients having chronical kidney failure who have no possibility of actively eliminating the D(+) isomer.

Hemodialysis induces an L-carnitine secondary deficiency since it is eliminated in the dialysate, given its low molecular weight (161.2). The oral administration or the addition of L(−)-carnitine in the dialysis solution has avoided carnitine decrease in the patients.

In the case of hyperlipoproteinemia, widespread in industrialised countries, a significant decrease of the levels of plasma risk factors, triglycerides and cholesterol, has been reached with L(−)-carnitine (Lancet II (1978) 805–807). Similar effects could have been obtained using acylcarnitine (DE-OS 2903579).

DESCRIPTION OF THE PRIOR ART

L(−)-carnitine has been isolated from meat extracts with a complicated purification procedure.

In the '50's chemical synthesis were perfected, though only DL-carnitine could be obtained.

Up till now,the isomer L(−)-carnitine has been obtained by the racmate's resolution with fractional crystallisation of carnitine's salts with optically active acids. Various carnitine derivatives have been used, such as

2

carnitine-nitrile, carnitinamide or even carnitine inner salt itself.

As optically active acids, tartaric, camphoric and camphor-sulphonic acids were used (p.e. DD-PS 23217; DD-PS 93 347; DE-OS 2927672).

DL-carnitine has also been resolved with specific L(−)-transferases.

But, since for the production of one mole of L(−)-carnitine, at least one mole of acyl CoA was needed, this procedure turned out to be too expensive to apply industrially.

All the other chemical synthesis with subsequent racemate resolution have the ulterior disadvantage that from synthesised DL-carnitine not more than 50% of the wanted isomer can be isolated.

This problem has been overcome with enzymatic stereospecific synthesis with achiralic precursors.

The U.S. Pat. No. 4, 221,869 describes the production of L(−)-carnitine from dehydrocarnitine with carnitine dehydrogenase (EC 1.1.1.108) isolated from "*pseudomonas fluorescens*" using NAD as coenzyme,.though other enzymes are necessary, such as glucose dehydrogenase or alcohol dehydrogenase for the regeneration of NADH. Moreover, dehydrocarnitine is very instable and it spontaneously decomposes in acetonyltrimethylammonium and carbon dioxide.

The Patent application DE-OS 3123975 describes L(−)-carnitine production from γ-butyrobetaine with γ-butyrobetaine hydroxylase (EC 1.14.11.1) isolated from "*Neurospora crassa*" mould. During this hydroxylase reaction α-ketoglutaric acid and a reducing agent (i.e. ascorbate) must be added to the incubation medium.

To have the maximum yield of L(−)-carnitine,catalase is also needed. γ-butyrobetaine hydroxylase is obtained after mould growth, isolation and purification of its spores with detergents and mechanical or ultrasonic treatment. The precursors of the L(−)-carnitine biosynthesis are L(−)-methionine and L-lysine. Through the ε-N,N,N trimethyl lysine, ε-N,N,N trimethyl-β-hydroxy-lysine and N,N,N trimethylamino butyraldehyde intermediates, γ-butyrobetaine is formed. It is hydroxylated to L-carnitine by γ-butyrobetaine hydroxylase in presence of molecolar oxigen, α-ketoglutaric acid, ferrous ions and a reducing agent. Crotonobetaine is not an intermediate in the biosynthesis.

The invention's purpose is that of overcoming the disadvanages of the known methods of L(−)-carnitine production and to indicate a procedure that allows, in a favorably economical way, the biochemical production of the compound.

DESCRIPTION OF THE INVENTION

The invention describes the technically realisable procedures to produce L(−)-carnitine from easily available raw materials under simple reaction conditions and with not complicated intermediate separation techniques.

Until now the only notions were:

(1) L(−)-carnitine, D(+)-carnitine and crotonobetaine are metabolized to γ-butyrobetaine.

(2) the enterobacter growth stimulation depends on the γ-butyrobetaine formation, obtained by the crotonobetaine reduction. (Arch. Microbiol. 132 (1982), 91–95).

5,028,538

3

(3) The "clostridia-bacteria" are capable of reducing crotonic acid to butyric acid in anaerobic conditions. (FEBS Lett. 109, (1980) 244–246).

Surprisingly it was found that even some bacteria, in certain particular conditions, had been capable of stereospecifically hydrating crotonobetaine to L(−)-carnitine; a process which had not been observed in presence of γ-butyrobetaine in the incubation medium.

These bacterial strains were:

Escherichia coli (*E. coil* 044 K74; 055 K 59; 0 111 K58; 0 114 K90) Salmonella (*S. typhimurium* LT₂; *cottbus; anatum; newington*)

Proteus (*P. vulgaris; mirabilis*)

Shigella (*S. flexneri* 1a) .

Hafnia (*H. alvei Biotyp* A and B)

Clostridium (*C. kluyveri; sporogenes*)

Citrobacter (*C. freundii*).

According to the invention, to the bacteria growing on complex or minimal mediums, or to resting cells, crotonobetaine (4-N,N,N-triethylamino crotonic acid) or one of its salts, such as for example chloride, iodide, perchlorate, nitrate phosphate, or crotonobetaine amide, nitrile and aryl or alkyl ester is added.

After a certain period of incubation the L(−)-carnitine formed is isolated from the reaction medium.

The crotonobetaine concentration in the incubation medium was included between 10 μ moles and 5 moles/1. As described in tables V, VI, VII the quantity of L(−)-carnitine formed increased with the increase of crotonobetaine concentration, but the percentual yield refered to the raw material decreased.

After the separation of the L(−)-carnitine from crotono betaine the latter may be used again.

The resting cells were incubated in a minimal culture medium with salt solutions, organic and inorganic buffer mixture which did not contain any C and/or N sources or of which the C and contents could not be used by the bacteria.

The incubation times were comprised between 3 hrs and 5 days. A preferred interval of time is 12 hrs–48 hrs.

The bacteria capable of hydrating crotonobetaine can grow on the more diverse complex culture mediums, solid or liquid, thus commercial nutritive mediums containing meat, yeast, malt and starch water extracts can be used, adding, in partially anaerobic conditions, C and/or N sources such as ammonium hydroxide,urea, alcohol, carbohydrates, organic acids including fatty acids. The cultures are grown in calibrated tubes, in anaerobic condition and without stirring.

The reduction of crotonobetaine to γ-butyrobetaine leads to a loss of substance for L(−)-carnitine synthesis, thus this reduction reaction is inhibited by adding electron acceptors of the anaerobic respiration and other substances. Non limiting examples of the invention are oxygen, nitrates, trimethylamine N-oxide and other N-oxides, dimethyl sulphoxide, glucose, fructose, saccarose, maltose and electron acceptors such as fumarate, crotonate and acrylate. (Tables II, III, IV).

The bacteria grown on commercial nutritive mediums, supplemented with meat broth or pancreatic peptone were able to hydrate crotonobetaine to L(−)-carnitine (table IX). The rate of L(−)-carnitine synthesis is increased, though, by inducing crotonobetaine hydroxylase, adding to the culture medium of growing bacteria,crotonobetaine,DL-carnitine, fractions of DL-carnitine enriched with D(+) (obtained by the chemical resolution of the racemate) or its derivatives (i.e. car-

4

boxylic esters, O-acylcarnitine or O-acylcarnitine esters).

Crotonobetaine, its salts or its derivatives. were prepared according to the following known proceedings:

1) Dehydration with sulphuric acid or acetic anhydride of DL-carnitine, D(+)-carnitine or D(+) enriched DL-carnitine, or alternatively elimination of the acid from the corresponding O-acyl DL-carnitine or O-acyl D carnitine.

2) Exhaustive methylation of the 4-ammino crotonate with methyl halogenides.

3) Reaction of trimethylamine with 4-halogen substituted crotonate.

A good separation of the L(−)-carnitine from non reacted crotonobetaine or from the formed γ-butyrobetaine. by ion-exchange cromatography ("Recent research on carnitine" Ed. G. Wolf, pg 11–21 (1965), US-PS 4,221,869; DE-OS 3123975) can only be carried out for small quantities.

L(−)-carnitine in larger quantities has been separated after reaction of the hydroxy group with the acyl chlorides of medium and long chain fatty acids.

The O-acyl carnitines formed can be extracted from the water phase with n-butanol or isobutanol (Biochim. Biophys. Acta 280, (1972), 422–433).

The O-acyl carnitines are obtained by evaporation of the organic phase. They can be used directly in the biochemical research, or hydrolyzed with ammonium hydroxide to L(−)-carnitine which is then utilized for therapeutical application.

The crotonobetaine which remains in the water phase is added back to the incubation medium.

The L(−)-carnitine formed was measured using the carnitine acetyltransferase after the addition of acetyl CoA with the DTNB method.

The excess presence of crotonobetaine from 10 to 100 does not disturb the L(−)-carnitine determination.

Some of the advantages of this procedure are:

1) By dehydration of the D(+)-carnitine or of the D(+) enriched DL-carnitine, obtained by the chemical resolution of the racemate, crotonobetaine, which is a cheap raw material for the L(−)-carnitine synthesis, is formed.

2) In this process no biochemical substances or other enzymes are to be employed.

3) The cost of incubation and of the raw materials are very low.

The following non-limiting examples illustrate the present invention.

EXAMPLE 1

A 500 ml reaction vessel containing a complex culture medium with crotonobetaine:

pancreatic peptone	20 g/1	bidistilled H₂O
sodium chloride	5 g/1	bidistilled H₂O
crotonobetaine	50 m moles/1	bidistilled H₂O
pH7 (ΔE₅₄₀ = 0.050)		

was inoculated with a suspension of *E. coli* 044 K74, previously grown in 5% blood agar, in physiological solution.

The reaction flask was subsequently covered with paraffin oil and incubated at 37° C.

Table 1 shows the L(−)-carnitine formation in relation with the incubation time and the bacteria growth.

5,028,538

5

TABLE 1

relation between L(−)-carnitine synthesis, incubation time and bacteria growth.			
		L(−)-carnitine synthesis	
Incubation time [h]	Growth [Δ E$_{540}$]	L(−)-carnitine[moles]	$\dfrac{\text{L(−)-carnitine[moles]}}{\text{crotonobetaine[moles]}} \times 10^2$
0	0.0	—	—
3	0.100	—	—
6	0.120	0.5	0.9
12	0.190	2.0	3.9
24	0.200	6.7	13.4
48	0.175	6.6	13.3

EXAMPLE 2

The complex culture medium containing crotonobetaine, inoculated with *E. coli* (example 1), after 24 hrs of incubation was centrifugated at 6000 rounds per minute to separate the grown bacteria. The obtained centrifugate was washed with phosphate buffer according to Sorensen 0.01 M at pH 7.5 (0.20 g/l KH$_2$PO$_4$ and 3.05 g/l Na$_2$HPO$_4$ ·12 H$_2$O), suspended in a 1 l Sorensen phosphate buffer containinq 5 g/l crotonobetaine (34.9 m moles/1) (ΔE$_{540}$=0.080) and maintained for 24 hrs at 37° C. Subsequently it was again centrifugated and 9.08 m moles/1 of L(−)-carnitine were found in the solution. A yield of 26% in relation with crotonobetaine.

EXAMPLE 3

To a complex culture medium containing L(−)-carnitine (31 m moles/1 of L-carnitine corresponding to 5 g/1) and inoculated, as in example 1, with several strains of enterobacteria, electron acceptors of the anaerobic respiration and other substances (sodium succinate, sodium fumarate and glucose 10 g/1, potassium nitrate, trimethylamine N-oxide (TMO) 5 g/1) were added.

Table II expresses the yield in moles of γ-butyrobetaine in relation with the L(−)-carnitine utilized.

TABLE II

Influence of the electron acceptors and of other substances on the L(−)-carnitine reduction to γ-butyrobetaine						
	γ-butyrobetaine formation [% moles]					
Bacteria strains	−	succinate	fumarate	KNO$_3$	TMO	glucose
Escherichia coli 044 K74	71	77	13	0	0	0
Escherichia coli K12 Hfr H	45	43	5	0	0	0
Salmonella typhimurium LT$_2$	87	89	68	0	8	71
Proteus vulgaris	79	81	17	0	17	0

EXAMPLE 4

The complex culture medium (KM) described in example 1, or the minimal culture medium (MM) containing:

Na$_2$HPO$_4$.12H$_2$O	15.0 g
KH$_2$PO$_4$	3.0 g
NH$_4$Cl	1.0 g
MgSO$_4$.7 H$_2$O	0.15 g
CaCl$_2$	0.014 g
FeCl$_3$	0.0002 g
D-ribose	7.5 g
distilled H$_2$O sufficient for 1 l	

were additioned with 50 m moles of crotonobetaine and with sodium fumarate (10 g/l), potassium nitrate (5 g/l), and trimethylammine N-oxide (5 g/l). They were then

6

incubated with a layer of paraffin (anaerobic conditions) or without paraffin (aerobic conditions), at 37° C. with *E. coil* 044 K74 for 48 hrs.

TABLE III

Influence of electron acceptors on the L-carnitine formation in the growing bacteria					
Culture medium	L(−)-carnitine synthesis [m moles/l]				
	O$_2$ absence	O$_2$ presence	fumarate	KNO$_3$	TMO
KM	4.5	2.6	10.7	0.9	5.1
MM	2.7	1.5	10.8	0	5.9

EXAMPLE 5

A complex culture medium (KM) or a minimal culture medium (MM) containing 50 m moles/1 of crotonobetaine were inoculated with *E. coil* 044 K74 and incubated for 48 hrs. The cells, collected and washed twice (example 2), were incubated for 48 hrs in Sorensen buffer containing 50 m moles/1 of crotonobetaine to which the electron acceptors were added as in example 4

TABLE IV

Influence of the electron acceptors on the L-carnitine formation with resting cells					
Culture medium	L(−)-carnitine synthesis [m moles/l]				
	O$_2$ absence	O$_2$ presence	fumarate	KNO$_3$	TMO
KM	7.5	8.3	0	7.8	8.4
MM	9.1	12.1	0	9.8	10.4

EXAMPLE 6

Minimal culture mediums (MM), with various crotonobetaine concentrations, supplemented with D-ribose as C source (example 4) were inoculated with *E. coli* 044 K74 (example 1) and incubated at 37° C. for 48 hrs.

TABLE V

L(−)-cartinine synthesis with bacteria grown on minimal culture medium with various crotonobetaine concentrations				
		L-cartinine synthesis		
crotono-betaine [m moles/l]	[m moles/l]	$\dfrac{\text{L-carnitine moles}}{\text{crotonobetaine moles}} \times 10^2$		Growth [E$_{540}$]
500	5.3	1.1		0.120
50	4.3	8.6		0.450
5	0.3	6.0		0.385
0.5	0.09	18.0		0.220

EXAMPLE 7

A complex culture medium as described in example 1 (A) and a minimal culture medium supplemented with D-ribose, as described in example 4 (B), each containing 8 g/1 of L(−)-carnitine, were inoculated with *E. coli* 044 K74. After 48 hrs the collected bacteria, washed

5,028,538

7

twice, were suspended in Sorensen buffer, containing various crotonobetaine concentrations and incubated for 48 hrs a 37° C.

TABLE VI

L(−)-carnitine synthesis with induced resting cells by L(−)-carnitine at various crotonobetaine concentration

	crotono-betaine [m moles/l]	L-carnitine synthesis	
		[m moles/l]	$\dfrac{\text{L-carnitine moles}}{\text{crotonobetaine moles}} \times 10^2$
(A)	500	9.0	1.8
	50	7.5	15.1
	5	2.9	58.4
(B)	500	16.2	3.2
	50	8.3	16.5
	5	3.7	74.0

EXAMPLE 8

A complex culture medium containing crotonobetaine as in example 1 inoculated with *E. coli* 044 K74 was centrifugated after 48 hrs. The centrifugate, washed twice, was dispersed in a minimal culture medium, without C and N sources, containing various concentrations of crotonobetaine and incubated for 48 hrs.

TABLE VII

L-carnitine synthesis with resting cells induced by crotonobetaine at various crotonobetaine concentrations

crotonobetaine [m moles]	L-carnitine synthesis	
	[m moles/l]	$\dfrac{\text{L-carnitine moles}}{\text{crotonobetaine moles}} \times 10^2$
500	24.3	4.9
50	12.2	24.5
5	2.4	48.8
0.5	0.47	94.0

EXAMPLE 9

A minimal culture medium as described in example 4 which contains, instead of crotonobetaine, 50 m moles/1 of L(−)-carnitine was inoculated with *E. coli* 044 K74 and incubated for hrs. Subsequently the collected and twice washed bacteria were incubated at 37° C. in a minimal culture medium without C and N sources but with 50 m moles/1 of crotonobetaine. The optical density at the beginning of the incubation was $\Delta E_{540} = 0.210$.

TABLE VIII

L(−)-carnitine formation with resting cells in function of time

Incubation time [h]	L-carnitine synthesis	
	[moles/l]	$\dfrac{\text{L(−)-carnitine moles}}{\text{crotonobetaine moles}} \times 10^2$
3	2.5	5.1
6	3.7	7.5
12	6.6	13.2
24	9.0	18.0
48	10.1	20.3

EXAMPLE 10

A complex culture medium (KM) or a minimal culture medium (MM) without C sources, additioned with the substances indicated in table IX, at the concentrations of 50 m moles/1, was inoculated with *E. coil* 044 K74 and incubated for 48 hrs. Subsequently, the collected bacteria, washed twice with Sorensen buffer,

8

were incubated for 48 hrs in Sorensen buffer with 50 m moles/1 crotonobetaine.

TABLE IX

L-carnitine formation with resting cells in function of the various substances' addition in the growth medium

		Growing cells	Resting cells	
Medium	added substances	ΔE_{540} after 48 hrs	ΔE_{540} at the beginning	L-carnitine systhesis m moles/1
KM	—	0.315	0.225	1.4
MM	D-Ribose	0.196	0.136	0.3
MM	D-Glucose	0.325	0.260	0.6
MM	D-Ribose Fumarate	0.165	0.100	0
MM	D-Ribose, GABOB+	0.395	0.220	0.3
MM	D-Ribose L-carnitine	0.255	0.190	6.6

⁻DL- γ amino β-hydroxybutyric acid.

What is claimed is:

1. A process for producing L(−)-carnitine comprising:

   (a) preparing a bacterial culture medium comprising a crotonobetaine selected from the group consisting of crotonobetaine, a crotonobetaine salt, a crotonobetaine derivative or mixtures thereof;

   (b) inoculating the culture medium with *Escherichia .Coli* 044 K 74;

   (c) incubating the bacteria wherein the incubation takes place in anaerobic, partially anaerobic or aerobic conditions wherein the incubation occurs for a time period of 12 hours–120 hours.

   (d) recovering at least 3.9% yield of L(−)-carnitine from the medium.

2. The process according to claim **1**, wherein the crotonobetaine salts are selected from the group consisting of chloride, iodide, perchlorate, nitrate, and phosphate salts of crotonobetaine.

3. The process according to claim **1**, wherein the crotonobetaine concentration in culture medium is between 10 μ moles and 5 moles/1.

4. The process according to claim **1**, wherein the bacterial incubation takes place in a commercial complex or minimal nutritive medium, to which are added, to the growing bacteria and/or resting cells, electron acceptors or respiration, hydrogen acceptors and substrates that inhibit the reduction of crotonobetaine to γ-butyrobetaine.

5. The process according to claim **4** wherein the substrates that inhibit the reduction of crotonobetaine to γ-butyrobetaine are selected from the group consisting of oxygen, nitrates, trimetylamine N-oxides, glucose, fructose, saccarose, maltose, dimethylsulphoxide, fumarate, crotonate and acrylate.

6. The process as in claim **1**, wherein the incubation occurs for a time period of 12 hours–48 hours.

7. The process according to claim **1** wherein the crotonobetaine derivative is selected from the group consisting of crotonobetaine nitrile, crotonobetaine amide, aryl and alkyl crotonobetaine esters or mixtures thereof.

8. The process according to claim **1**, wherein the culture medium is inoculated with induced resting cells and the bacterial culture medium comprises one or more of the group selected from crotonbetaine, and carboxylic esters of crotonobetaine, O-acyl carnitine esters, derivatives of L(−), D(+), and DL-carnitine and salts thereof.

* * * * *

US006653112B2

(12) **United States Patent**   (10) **Patent No.:**   **US 6,653,112 B2**

Kleber et al.   (45) **Date of Patent:**   *Nov. 25, 2003

(54) **METHOD FOR PRODUCING L-CARNITINE FROM CROTONOBETAINE USING A TWO STAGE CONTINUOUS CELL-RECYCLE REACTOR**

(75) Inventors: **Hans-Peter Kleber**, Grossdeuben (DE); **Manuel Canovas-Diaz**, Santo Angel-Murcia (ES); **Jose Maria Obon**, Murcia (ES); **Jose Maria Iborra**, Murcia (ES)

(73) Assignee: **Sigma-Tau Industrie Farmaceutiche Riunite S.p.A.**, Rome (IT)

(*) Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/530,783**

(22) PCT Filed: **Nov. 9, 1998**

(86) PCT No.: **PCT/EP98/07124**

§ 371 (c)(1),
(2), (4) Date: **May 12, 2000**

(87) PCT Pub. No.: **WO99/24597**

PCT Pub. Date: **May 20, 1999**

(65) **Prior Publication Data**

US 2003/0073203 A1 Apr. 17, 2003

(30) **Foreign Application Priority Data**

Aug. 11, 1997   (DE) 197 49 480

(51) **Int. Cl.^{7}** **C12P 13/00**; C12P 7/40; C12N 11/14; C12N 11/08; C12N 1/20
(52) **U.S. Cl.** **435/128**; 435/136; 435/176; 435/180; 435/252.8; 435/280; 435/849
(58) **Field of Search** 435/128, 174, 435/176, 180, 136, 280, 252.8, 849

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,906,568 A * 3/1990 Jung et al. 435/128
4,978,616 A * 12/1990 Dean, Jr. et al. 435/70.3
5,028,538 A * 7/1991 Seim et al. 435/128

FOREIGN PATENT DOCUMENTS

EP   0 320 460   6/1989

OTHER PUBLICATIONS

Jung, et. al., Chemical Abstracts 117: 187989 d, 1992.*

Jung, et. al., Chemical Abstracts 115: 6956 f, 1991.*

Trevan, et. al., Immobilized Enzymes, John Wiley & Sons 1980, pp. 66–71.*

* cited by examiner

Primary Examiner—David M. Naff
(74) *Attorney, Agent, or Firm*—Nixon & Vanderhye P.C.

(57) **ABSTRACT**

L(−)-carnitine is synthesized from crotonobetaine, crotonobetaine salts or derivatives in an ecologically advantageous manner by immobilizing cells of *Escherichia coli* 044 K74 on ceramics, glass beads or polyurethane disks in a two stage continuously operating cell recycle reactor containing a reaction medium. The medium preferably contains between 25 mM and 1 M crotonobetaine and at least 50 mM fumarate. Growing or resting cells of *E. coli* are retained in the reactor by micro or ultrafiltration membranes which are arranged as a flat membrane module or hollow fiber module. A first stage contains a reactor tank and a second stage contains an external recirculation loop connected to the tank for feeding the reaction medium through a filter unit. L-carnitine is synthesized under anaerobic conditions to produce a reaction medium containing L-carnitine and unreacted crotonobetaine. The reaction medium is transferred through the recirculation loop to the filter unit to produce a filtrate containing L-carnitine and a residue containing unreacted crotonobetaine and cells. The residue is recirculated to the reactor tank.

9 Claims, No Drawings

US 6,653,112 B2

1

METHOD FOR PRODUCING L-CARNITINE FROM CROTONOBETAINE USING A TWO STAGE CONTINUOUS CELL-RECYCLE REACTOR

FIELD OF THE INVENTION

The invention relates to a process for producing L-carnitine from crotonobetaine, from salts of crotonobetaine, other derivatives of crotonobetaine or the like.

BACKGROUND OF THE INVENTION

It is known that L-carnitine, a ubiquitously occurring compound, plays an important role in metabolism, especially in transporting long-chain fatty acids through the inner mitochondrial membrane. Numerous clinical applications derive from the function of carnitine in the metabolism of eukaryotes, e.g., in the treatment of patients with carnitine deficiency syndromes, in the prevention and therapy of various heart diseases and in the treatment of hemodialysis patients. Further, L-carnitine is significant as a supplemental nutrient and also promotes, as an additive to fermentation media, the growth of yeasts and bacteria. The growing need for this biologically active L-carnitine enantiomer for these and other applications has led to a worldwide search for means of synthesizing this betaine in an optically pure form, since the chemically synthesized racemate cannot be used because it inhibits carnitine acetyl transferase and the carnitine carrier protein.

To isolate the L-isomer, up to now processes have been used that are based on splitting racemates by fractionated crystallization using optically active acids (e.g., U.S. Pat. No. 4,254,053, 1981), where D(+)-carnitine occurs as a waste product.

This problem can be overcome by various biological processes, starting with inexpensive achiral precursors (Adv. Biochem. Eng. Biotechnol., 1993, 50, 21–44) Of particular interest is stereospecific hydration of trans-crotonobetaine into L-carnitine using strains of the genera Escherlchia (ED 0121444, 1984; DD 221 905, 1987; EP 0320460, 1989) or Proteus (Agric. Biol. Chem., 1988, 52, 2415–2421; U.S. Pat. No. 5,300,430, 1994) The advantage of this method lies in the fact that this achiral precursor can also be obtained by chemical dehydration of the waste product D-carnitine.

The numerous processes described in the literature with immobilized microorganisms in a continuously operating reactor system have the advantage that

   pure reaction media can be used, thus facilitating the extraction and purification process,

   by using higher concentrations of the biocatalyst in the reaction medium, higher productivities are achieved while the possibility of contamination is reduced,

   there is reduced sensitivity to inhibitors or a nutrient deficiency,

   a higher stability of the biocatalyst is achieved.

The advantages mentioned can also be applied to a commercially used process.

A continuously operating reactor in which microorganisms are retained by micro- or ultrafiltration membranes is an immobilization process which, besides the above-mentioned advantage, also entails lower costs for the immobilization while making it possible to have a very slight upscaling.

SUMMARY OF THE INVENTION

Consequently the object of the invention is a process for producing L-carnitine from crotonobetaine, crotonobetaine

2

salts or other crotonobetaine derivatives in a continuous reactor with free or immobilized cells, growing or resting Escherlchla coil 044K74 (DSM 8828) cells, that are retained by micro- or ultrafiltration membranes in a flat membrane or hollow fiber module.

DETAILED DESCRIPTION OF THE INVENTION

E. coil is kept in the reactor mentioned at temperatures between 20 and 40° C., pH values between pH 6.0 8.0 and under anaerobic conditions that are necessary for the induction of the enzyme that metabolizes carnitine.

A minimal or complex medium is used as the reaction medium. In both cases, crotonobetaine, crotonobetaine salts or other crotonobetaine derivatives are added in concentrations between 25 mmol and 1M. The minimal medium contains varying concentrations of caseine hydrolyzate and salts $(NH_4)_2SO_4$, KH_2PO_4, K_2HPO_4, $MgSO_4x7H_2O$, $MnSO_4x4H_2O$, $FeSO_4x7H_2O$, while the complex medium contains varying concentrations of pancreatic peptone and NaCl. To improve the growth of E. coil, glycerine, glucose, ribose, saccharose or lactose are added. Also added to the medium are inhibitors that prevent the transformation of crotonobetaine into y-butyrobetaine (fumarate, glucose or nitrate) and inductors of carnitine-metabolizing enzymes such as D-, L-, DL-carnitine, their salts and derivatives or crotonobetaine, its salts or derivatives.

The course of the reaction in the continuous cell-recycle reactor used here can be divided into two stages. The one stage consists of a reactor tank in which cells of E. coil, together with the reaction medium, convert most of the crotonobetaine into L-carnitine. This reactor tank has monitoring elements for pH value, temperature and stirring speed and for the monitoring and correction of oxygen concentration. The feed of the reaction medium into the reactor is performed with a dosing pump. When necessary, excess medium must be removed from the reactor tank. The second stage consists of an external recycling loop that is connected to the reactor tank and conveys the contents of the reactor through a filter unit by means of a pump. While the filtrate is being collected, to isolate L-carnitine from it as the reaction product, the residue from the filtration is fed again to the reactor. For filtering the cell suspension, commercial filter systems of varying provenance can be used as long as they have a pore size below the cell size of E. coil. The speed of the recycling pump remains unchanged to achieve the best possible filtration rates and to minimize the formation of a polarization membrane during the filtration process. Filtering may be performed using commercial cross current filtration or hollow-fiber modules consisting of ultra-or microfiltration membranes composed of cellulose, polysulphone or polysulphonated polysulphone with a retention limit of 300 kDa or 0.211 μ. The continuous cell-recycle reactor may be operated at different levels of dilution adjusted by dosing and filtration pumps, and at different agitation speeds and different biomass concentrations. Rate of discharge from the filtration pump is controlled by process control means.

The expression free E. coil cells indicates the state in which whole cells are suspended in the reaction medium without preventing a cell outflow through the exit solution. The expression immobilized cells describes the state in which whole cells are bonded to soluble polymers or insoluble carriers, or are enclosed in membrane systems (in Methods in Enzymol. 1987, vol. 135, 3–30)

The concept growth conditions is defined as the situation in which whole cells use substrates and form products

US 6,653,112 B2

3

during their life cycle. Resting cells are understood as intact cells that are not growing and that show, under certain conditions, special metabolic functions (in "Biotechnology" (Kieslich, K.; Eds. Rehm, N.J. and Reed, G.) Verlag Chemie, Weinheim, Germany. 1984, Vol. 6a, 5–30)

The process is described below with several embodiments:

EXAMPLE 1

Escherlchla coil 044 K74 is cultivated in an Erlenmeyer flask that is filled to the top and sealed air-tight at 37° C. under anaerobic conditions, on a rotating shaker (150 r.p.m.). The complex medium used has the following composition: 50 mM of crotonobetaine, 50 muM fumarate, 5 g/l of NaCi and varying concentrations (between 0.5 and 10 g/l) of pancreatic peptone. The pH is set using KOH to pH 7.5. Table 1 summarizes the specific growth rates at varying peptone concentrations.

TABLE 1

Maximum specific growth rates of *Escherlchla coil* 044 K74					
Peptone (g/l)	0.5	1.0	2.5	5.0	10.0
pmax (h~)	0.224	0.296	0.351	0.372	0.325

Under the conditions described, growing cells of *E. coil* are able to produce 20–30 mM of L-carnitine up to the end of the test.

Concentrations higher than 5 g/l of peptone yield similar growth and kinetic parameters as well as biomass content (OD 600 nm) In contrast, at lower peptone concentrations, lower growth parameters are obtained.

EXAMPLE 2

Escherlchla coil 044 K74 is cultivated in an Erlenmeyer flask that is filled to the top and sealed air-tight at 37° C. under anaerobic conditions, on a rotating shaker (150 r.p.m.) The complex medium used has the following composition: 50 muM of crotonobetaine, 5 g/l of pancreatic peptone, S g/l of NaCl and graduated concentrations (between 0 and 75 muM) of fumarate. The pH is set using KOR to pH 7.5.

Table 2 shows that adding fumarate causes higher growth rates of *E. coil* 044 K74 and an OD of 600 nm of almost 1.0 in the steady state. Further, fumarate causes L-carnitine formation of 20–30 muM up to the end of the test. In the absence of fumarate, a carnitine concentration of only 5 muM is obtained.

TABLE 2

Biomass ($0D_{600}$~) at varying fumarate concentrations after a 10 hour test.				
Fumarate (mM)	0	25	50	75
~max (h^1)	0.21	0.37	0.38	0.39
Biomass OD (600 nm)	0.980	0.910	1.00	0.950

EXAMPLE 3

The ability of *Escherlchla coil* 044 K74 to form L-carnitine from crotonobetaine is induced by crotonobetaine. The induction studies were performed with crotonobetaine between 5 and 75 muM using resting cells. At higher crotonobetaine concentrations, conversion rates of above 60% of L-carnitine are achieved (Table 3)

4

TABLE 3

Production of L-carnitine by resting cells of *Escherlchla coil* 044 K74 as a function of varying crotonobetaine concentrations.				
Crotonobetaine (mM)	5	25	50	75
L-carnitine production (%)	55	60	62	E5

EXAMPLE 4

Escherlchla coil 044 K74 is cultivated in an Erlenmeyer flask that is filled to the top and sealed air-tight at: 37° C. under anaerobic conditions, on a rotating shaker (150 r.p.m.). The complex medium used has the following composition: 50 mM of crotonobetaine, 5 g/l of pancreatic peptone, 5 g/l of NaCl and 50 mM of fumarate. The pH is set using KON to pH 7.5.

To raise the biocatalyst concentration in the reactor and to make it possible to have L-carnitine production at dilution rates higher than the maximum specific growth rate, a membrane reactor was used. The cells were retained with a polysulfone microfiltration membrane with an exclusion threshold of 0.1 pm and were used again. The membranes were arranged in a plate module. Table 4 summarizes the biomass content and Table 5 summarizes the carnitine production, the crotonobetaine conversion and the productivity in this system.

TABLE 4

Biomass content of *E. coil* 044 K74 in a continuously operating membrane reactor.				
Dilution rate (h^1)	0.0	0.2	1.0	2.0
Biomass (gdryweight/1)	0.5	2.1	9.4	27.0

TABLE 5

L-carnitine production, crotonobetaine conversion and productivity in a continuously operating cell reactor wlih					
Escherlchla coil 044 K74					
Dilution rate (tf^1)	0.0	0.5	1.0	1.5	2.0
L-carnitine production (%)	0.0	38	42	42	38
Crotonobetaine conversion (%)	0.0	24	26	26	30
Productivity ~	0.0	1.75	3.5	5.5	6.5

It can be seen from the tables that, with immobilized cells of *Escherlchla coil* 044 K74 in a cell recycle reactor, 6.5 1/h of L-carnitine was formed from crotonobetaine with a metabolization rate of almost 40%.

What is claimed is:

1. A method for producing L-carnitine from crotonobetaine, comprising the steps of:

(1) introducing between 25 mM and 1 M crotonobetaine or a salt or derivative thereof and at least 50 mM fumarate into a reaction a two stage continuously operating cell-recycle reactor wherein a first stage consists of a reactor tank containing the reaction medium and cells of a strain of the genera Escherichia that convert crotonobetaine to L-carnitine, said cells being immobilized on a carrier selected from ceramics,

US 6,653,112 B2

5

glass beads and polyurethane disks, and a second stage consists of an external recirculation loop connected to the reactor tank, by means of which the contents of the reactor are fed through a filter unit;

(2) synthesizing the L-carnitine under anaerobic condition in the reaction medium containing fumarate and crotonobetaine to produce a reaction medium containing L-carnitine and unreacted crotonobetaine; and

(3) transferring said reaction medium through the recirculation loop to said filter unit where the reaction medium is filtered to produce a filtrate containing the L-carnitine from the reaction medium and recirculating from the filter unit a residue containing unreacted cronobetaine into the reactor tank of step (1).

2. A method according to claim **1**, wherein the cells are *E. coli* 044 K74 (DSM 8828).

3. A method according to claim **2**, wherein the carrier does not impair viability of the cells.

4. A method according to claim **1**, wherein the cells are immobilized on ceramics.

5. A method according to claim **2**, wherein the reaction medium contains 50 mM crotonobetaine, 5 g/l pancreatic

6

peptone, 5 g/l NaCl and 50 mM fumarate, has a pH of 7.5, and the cell-recycle reactor comprises a continuously operating membrane reactor.

6. A method according to claim **1**, wherein in step (3) the residue contains the cells and the cells are continuously recycled to the reactor tank, and the reaction medium is filtered by cross-flow filtration or hollow fiber modules consisting of ultra- or microfiltration membranes.

7. A method according to claim **6**, wherein the membranes are composed of cellulose, polysulphone or polysulphonated polysulphone with a retention limit of 300 kDa or 0.211μ.

8. A method according to claim **1**, wherein the cell-recycle reactor is operated at different levels of dilution which are adjusted by a dosing pump and a filtration pump, and at different agitation speeds and different biomass concentrations.

9. A method according to claim **8**, wherein a rate of discharge of the filtration pump is controlled by process control means.

*   *   *   *   *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO.   : 6,653,112 B2   Page 1 of 1
DATED   : November 25, 2003
INVENTOR(S) : Kleber et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,
Item [30], **Foreign Application Priority Data**, should read
-- November 8, 1997   (DE) .... 197 49 480 --

Signed and Sealed this

Twenty-fifth Day of May, 2004

JON W. DUDAS
Acting Director of the United States Patent and Trademark Office

CA2023744

Publication Title:

PROCESS   FOR   THE   PRODUCTION   OF   CROTONOBETAINE
HYDROCHLORIDE

Abstract:

A process is disclosed for the production of crotonobetaine hydrochloride, which
is a starting product in the microbiological production of L-carnitine. In the
process, carnitine hydrochloride in its racemic form or in the form of its
enantiomers is reacted at elevated temperature with acetic anhydride in the
presence of an acid catalyst.

Courtesy of http://worldwide.espacenet.com

Consommation et Corporations Canada	Consumer and Corporate Affairs Canada	(21) (A1)	2,023,744
Bureau des brevets	Patent Office	(22)	1990/08/22
Ottawa, Canada K1A 0C9		(43)	1991/03/08
		(52)	260-520.1

5,050,8/01

(51) INTL.CL.⁵ C07C-227/16; C07C-229/30

(19) (CA) APPLICATION FOR CANADIAN PATENT (12)

(54) Process for the Production of Crotonobetaine
     Hydrochloride

(72) Worsch, Detlev - Switzerland ;

(73) Lonza Ltd. - Switzerland ;

(30) (CH) 3253/89 1989/09/07

(57) 5 Claims

Notice: The specification contained herein as filed

Canada

2023744

ABSTRACT OF THE DISCLOSURE

A process is disclosed for the production of crotonobetaine hydrochloride, which is a starting product in the microbiological production of L-carnitine. In the process, carnitine hydrochloride in its racemic form or in the form of its enantiomers is reacted at elevated temperature with acetic anhydride in the presence of an acid catalyst.

1

This invention relates to a new process for the production of crotonobetaine hydrochloride from carnitine hydrochloride, especially from racemic carnitine hydrochloride or from D-carnitine hydrochloride.

5   Crotonobetaine hydrochloride is used, inter alia, as a starting material in the microbiological synthesis of L-carnitine, as described, for example, in European Published Patent Applications Nos. 0158194 and 0122794.

It is known according to Binon et al., _Chemical_
10 _Abstracts_, Vol. 59, 6248, to obtain crotonobetaine hydrochloride in a 78 percent yield from D,L-carnitine chloride by reaction with concentrated sulfuric acid at 130°C, then precipitation with acetone and treatment with barium chloride. A significant drawback of such process
15 is, besides the problem of handling concentrated sulfuric acid, the resultant and nonrecyclable barium sulfate.

Furthermore, in the standard chemical production of L-carnitine by resolution of racemates, e.g., according to DD 23.217, the problem inevitably arises that D-
20 carnitine results, for which so far it has not been possible to find any further use.

An object of the invention is to provide a process that avoids the above drawbacks.

Accordingly, the invention provides a process for
25 the production of crotonobetaine hydrochloride, which comprises reacting carnitine hydrochloride as the racemate or in the form of its enantiomers with from 1.5 to 15 mol of acetic anhydride in the presence of an acid catalyst at a temperature between 90° and 130°C.

30   In the process of the invention, racemic carnitine hydrochloride, D-carnitine hydrochloride or L-carnitine hydrochloride is reacted with 1.5 mol to 15 mol of acetic anhydride in the presence of an acid catalyst at a temperature of 90° to 130°C. Preferably, D-carnitine
35 resulting from the standard L-carnitine synthesis or low-cost D,L-carnitine is used. Preferably the amount of

2023744

2

acetic anhydride is selected in the range of 1.8 to 2 mol. Preferably p-toluenesulfonic acid is used as the acid catalyst in an amount of 0.5 to 2 percent by weight based on the carnitine hydrochloride used.   The reaction

5 temperature is preferably between 110° and 125°C, especially between 115° and 120°C.   At this temperature, the reaction is generally complete after about 2 hours.

By the addition of a lower aliphatic alcohol, preferably ethanol, to the skill hot, preferably at 70° to

10 80°C, reaction mixture, and by subsequent cooling, crotonobetaine hydrochloride can advantageously be precipitated and isolated with an already high purity. Yields of over 70 percent are achieved by the process according to the invention.   The crotonobetaine

15 hydrochloride can be desalted in the usual way, e.g. by electrodialysis, and converted into crotonobetaine.

The product produced by the process according to the invention is especially suitable for use in the microbiological production of L-carnitine.

20 The following Example illustrates the invention.

<u>EXAMPLE</u>

25.0 g (0.125 mol.) of D,L-carnitine chloride, 0.25 g (1.5 mmol) of p-toluenesulfonic acid and 25.0 g (0.245 mol) of acetic anhydride were heated for 2 hours at

25 120°C.   The darkly colored solution was allowed to cool to about 80°C and 20 ml of ethanol was added.   The mixture was again allowed to cool slowly, and the product began to precipitate.   The suspension was cooled to about 6°C with an ice water bath, subjected to suction and rewashed with

30 a little cold ethanol.   The product was dried under vacuum and 17.4 g of a beige-colored powder with a content of 96 percent (HPLC) was obtained.   This corresponded to a yield of 73.5 percent.   Other data regarding the product are as follows:

35

3

^{1}H-NMR, DMSO d$_6$: 3.19 (s, -CH$_3$, 9H)

4.38 (d 8Hz, -CH$_2$, 2H)

6.33 (d 15 Hz, CH=, 1H)

6.90 (dt 15 Hz, CH=, 1H)

5   13.0 (s broad, -COOH) 1H)

2023744

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A process for the production of crotonobetaine hydrochloride, which comprises reacting carnitine hydrochloride as the racemate or in the form of its enantiomers with from 1.5 to 15 mol of acetic anhydride in the presence of an acid catalyst at a temperature between 90° and 130°C.

2. A process according to claim 1, wherein from 1.8 to 2 mol of acetic anhydride is used.

3. A process according to claim 1, wherein the acid catalyst is p-toluenesulfonic acid in an amount of from 0.5 to 2 percent by weight based on the carnitine hydrochloride.

4. A process according to claim 1, 2 or 3, wherein the reaction is performed at a temperature in the range of 110° to 125°C.

5. A process according to claim 1, 2 or 3, wherein, after the reaction is completed, a lower aliphatic alcohol is added to the reaction mixture and the crotonobetaine hydrochloride is precipitated by subsequent cooling and isolated.

SUBSTITUTE

REMPLACEMENT

SECTION is not Present

Cette Section est Absente

US005087745A

United States Patent [19]

Hardt et al.

[11] Patent Number: **5,087,745**

[45] Date of Patent: **Feb. 11, 1992**

[54] **PROCESS FOR THE PRODUCTION OF GAMMA-BUTYROBETAINE**

[75] Inventors: **Peter Hardt**, Visp; **Andrej Stravs**, Ried bei Brig; **Pius Abgottspon**, Stalden, all of Switzerland

[73] Assignee: **Lonza Ltd.**, Gampel/Valais, Switzerland

[21] Appl. No.: **667,367**

[22] Filed: **Mar. 12, 1991**

Related U.S. Application Data

[63] Continuation of Ser. No. 409,792, Sep. 20, 1989, abandoned.

[30] **Foreign Application Priority Data**

Sep. 21, 1988 [CH] Switzerland 3514/88

[51] **Int. Cl.^{5}** C07C 227/18; C07C 229/06
[52] **U.S. Cl.** 562/553; 570/172
[58] **Field of Search** 562/553; 560/172

[56] **References Cited**

U.S. PATENT DOCUMENTS

2,313,573	3/1943	Orthner et al.	560/172
2,367,878	1/1945	Lee	560/172
3,466,364	9/1969	Takahashi et al.	560/172
3,711,549	1/1973	Phillips et al.	260/563
3,796,632	3/1974	Fukumura	195/29
4,371,618	2/1983	Cavazza	435/128
4,567,140	1/1986	Voelskow et al.	435/42
4,650,759	3/1987	Yokozeki et al.	435/128
4,708,936	11/1987	Kulla et al.	435/128
4,806,282	2/1989	Tinti et al.	562/553

FOREIGN PATENT DOCUMENTS

0122794	10/1984	European Pat. Off. .
1903076	8/1969	Fed. Rep. of Germany .
1939759	3/1970	Fed. Rep. of Germany .
2751134	5/1979	Fed. Rep. of Germany .
1198414	7/1970	United Kingdom .

OTHER PUBLICATIONS

Aksnes et al., J. Chem. Soc., (1959), pp. 103ff.

Lindstedt et al., Biochemistry, vol. 16, No. 10, pp. 2181–2188, (1977).

Lindstedt et al., Biochemistry, vol. 6, No. 5, pp. 1262–1270, (1967).

J. H. Miller, Experiments in Molecular Genetics, Cold Spring Laboratory, pp. 121 to 143, Experiments 13–17.

P. Gerhardt et al., "Manual of Methods for General Bacteriology", Am. Soc. Microbio., pp. 222–242, (1981).

J. P. Vandecasteele, Appl. Environ. Microbio., vol. 39, No. 2, pp. 327–334, (1980).

Kulla et al., Arch. Microbiology, vol. 135, pp. 1–7, (1983).

Perry's Chemical Engineer's Handbook, 6th Ed., p. 27-7, (1989).

L. N. Ornston et al., Biochem. and Biophys. Res. Comm., vol. 36, No. 1, pp. 179–184, (1969).

Seim et al., European Congress of Biotechnology, 3rd Ed., vol. 1, pp. 481–486, (1984).

Primary Examiner—J. E. Evans
Attorney, Agent, or Firm—Fisher, Christen & Sabol

[57] **ABSTRACT**

Process, which is feasible on a commercial scale, for the production of gamma-butyrobetaine. For this purpose, butyrolactone with hydrogen chloride and an alcohol is converted to the chlorobutyric acid ester, the trimethylammonium butyric acid salt is formed with trimethylamine and then saponified to the end product.

4 Claims, No Drawings

5,087,745

1

PROCESS FOR THE PRODUCTION OF GAMMA-BUTYROBETAINE

This application is a continuation of prior U.S. application Ser. No. 409,792, filing date Sept. 20, 1989, now abandoned.

BACKGROUND OF THE INVENTION

1. Field Of The Invention

The invention relates to a process for the production of gamma-butyrobetaine.

2. Background Art

Gamma-butyrobetaine is finding increasing use as an initial product for the microbiological production of L-carnitine. Laboratory processes for the synthesis of the individual intermediate steps have been sufficiently described.

According to West German OS 2,751,134, gamma-butyrolactone can be reacted with thionyl chloride and methanol in a 91 percent yield into 1-chlorobutyric acid methyl ester. The necessary disposal of the resultant SO_2 is a drawback in this process.

It can be seen from West German OS 1,903,076 that gamma-butyrolactone can be converted into the gamma-chlorobutyric acid methyl ester with dry hydrochloric acid and methanol with 4-hour refluxing and after one-week standing of the reaction solution.

It can be gathered from West Germany OS 1,939,759 that gamma-butyrolactone can be converted into the gamma-chlorobutyric acid methyl ester in a two-step process (first step with zinc chloride and hydrochloric acid; second step with methanol under reflux conditions) with a yield of 90 to 95 percent. But a great disadvantage is the amount of zinc salt formed that cannot be recycled and heavily loads the waste water.

From Aksnes et al., J. Chem. Soc., (1959), p. 103 ff, it is further known that gamma-bromobutyric acid methyl ester can be converted into the 4-trimethylammonium butyric acid methyl ester by heating with alcoholic trimethylamine in a yield of only 20 percent.

The above-mentioned process steps, thus, produce either highly unsatisfactory yields or, because of the disposal problems of the resulting by-products, are not feasible on a commercial scale.

BROAD DESCRIPTION OF THE INVENTION

The object of the invention is to provide a simple process, feasible on a large scale, for the production of gamma-butyrobetaine from butyrolactone, which provides good yields and is quite safe from the ecological aspect.

The object of the invention is achieved by the process according to the invention. The invention process involves the production of gamma-butyrobetaine. The process includes converting gamma-butyrolactone with hydrogen chloride to gamma-chlorobutyric acid. The latter, without isolation, is converted with a lower aliphatic alcohol into the corresponding gamma-chlorobutyric acid lower alkyl ester. The lower alkyl ester is converted with trimethylamine to trimethylammonium butyric acid lower alkyl ester chloride. The latter, without isolation, is finally saponified with a base to the end product.

2

DETAILED DESCRIPTION OF THE INVENTION

In a first step, gamma-butyrolactone is converted with hydrogen chloride into the gamma-chlorobutyric acid. The operation is suitably performed at a pressure of 1 to 25 bars, preferably at 8 to 20 bars. In this case, the reaction temperature can vary between 40° and 150° C., preferably between 80° and 120° C.

The hydrogen chloride is usually used in an excess of 5 to 40 percent relative to the gamma-butyrolactone. The conversion to gamma-chlorobutyric acid lasts about 2 to 10 hours and generally takes place practically quantitatively. An advantage of the process according to the invention is that the gamma-chlorobutyric acid does not have to be isolated but the reaction solution of the first step can be added directly to the lower aliphatic alcohol necessary for the ester formation. The hydrogen chloride excess from the first step can, thus, function directly as an esterification catalyst. But it can prove necessary to supply additional hydrogen chloride for the esterification.

Methanol, ethanol, propanols and butanols, preferably methanol or ethanol, are suitably used as the lower aliphatic alcohols.

The esterification reaction advantageously takes place at a pressure of 1 to 15 bars, but advantageously at 1 to 10 bars, and at a temperature of suitably 40° to 150° C., preferably 70° to 120° C.

But it is also possible to perform the conversion of gamma-butyrolactone to gamma-chlorobutyric acid ester in one step. For this purpose, the reactants of gamma-butyrolactone, hydrogen chloride and the corresponding alcohol are added together and converted directly to the corresponding gamma-chlorobutyric acid ester at a pressure of 1 to 10 bars and a temperature of 40° to 150° C.

In the third step, the conversion of the gamma-chlorobutyric acid ester with trimethylamine to trimethylammonium butyric acid ester chloride takes place. Suitably this reaction is performed at a pressure of 1 to 10 bars and a temperature of 20° to 180° C., advantageously at 80° to 150° C.

The trimethylamine can be added in a slight excess or in a stoichiometric amount, but preferably in a small excess. It is advantageous to use the alcohol corresponding to the ester radical as the solvent. But trimethylamine-soluble solvents, such as, toluene, can also be used. The conversion of the gamma-trimethylammonium butyric acid ester chloride generally lasts 2 to 6 hours and takes place practically quantitatively.

The reaction solution can further be fed directly to the last step of the saponification.

In principle, all strong bases can be used as bases for the saponification. But preferably aqueous solutions of inorganic bases, such as, alkaline-earth or alkali hydroxides (e.g., NaOH or KOH) or alkaline-earth or alkali carbonates (e.g., sodium carbonate), are used. A reaction temperature of 20° to 100° C. has proved advantageous for the saponification.

Working up or purification of the gamma-butyrobetaine from the reaction solution——adapted to further uses——can take place, e.g., by desalination by ion exchangers, specific crystallization methods or by electrodialysis. The latter method is successfully used to obtain a completely desalted gamma-butyrobetaine solution which optionally, after dilution, can be fed directly to a microbiological carnitine synthesis.

5,087,745

3

The gamma-butyrobetaine produced according to the process of the invention generally exhibits a purity greater than 99.5 percent. Further, with the new process an overall yield of gamma-butyrobetaine greater than 65 percent, relative to gamma-butyrolactone, is 5 achieved.

U.S. Pat. No. 4,708,936 discloses a process for the continuous production of L-carnitine by the microbiological method. A microorganism of the strain DSM No. 3225 (HK 1331b) type is cultivated in a bioreactor 10 with γ-butyrobetaine in the presence of a growth substrate. The culture fluid passes outside of the bioreactor in a circulation in which a separation of the cell is carried out. A quantity of cell-free solution, which is as large as the amount fed to the bioreactor as a substrate, 15 is withdrawn from the bioreactor. The L-carnitine is separated from the cell-free solution.

EXAMPLE

(a) Production of gamma-chlorobutyric acid ethyl ester 20

51.7 kg (0.6 kmol) of gamma-butyrolactone (100 percent) was placed in an enamel pressure agitator. The closed system was heated to 100° C. with good agitation, and starting from 60° C. a total of about 26.5 kg (0.72 kmol) of HCl was pressed on. The temperature 25 and pressure quickly rose, caused by the exothermia. The addition of HCl and heat output were regulated so that the reaction could be performed isothermally at 100° C. and isobarically at 11 bars of pressure. Addition was continued until no more HCl was absorbed (about 30 5 to 6 hours). Then it was cooled to 20° C. and the residual HCl was discharged. Then 62.3 kg (1.35 kmol) of ethanol was added to the reaction solution. Then 2 kg (0.055 kmol) of HCl was again pressed on. It was heated to 100° C. and kept at this temperature for 2 hours (pressure was 6 bars) and then cooled to 20° C. The solution was then mixed with 92 kg of toluene and made basic with 23 kg of aqueous NaOH (30 percent) (pH 8 to 8.5). The phases were separated and the organic phase was washed with 26 kg of water. The combined water phases were again extracted with 46 kg of toluene. The organic phase was distilled. Thus, toluene, ethanol and water were separated. 87 kg of crude gamma-chlorobutyric acid ethyl ester (content 88 percent) remained as still residue, which could be used directly in 45 the following step. The yield was 85 percent, relative to the gamma-butyrolactone.

(b) Production of gamma-butyrobetaine

159 kg (0.92 kmol) of crude gamma-chlorobutyric 50 acid ethyl ester (content 88 percent) and 107 kg (2.3 kmol) of ethanol were placed in a pressure agitator. Then 57 kg (0.96 kmol) of trimethylamine was added in 15 to 30 minutes. The temperature in this case rose to 30° to 50° C. Then heating to 130° C. was performed. 55 The pressure in this case rose to 5 to 7 bars and then dropped back to about 4 bars. After a stable pressure was reached, it was cooled to 20° C. and the remaining triethylamine was removed. The reaction solution was adjusted to a pH greater than 11 with 138 kg of aqueous 60 NaOH (30 percent). Then it was kept at 60° C. for 1 hour and the pH was optionally readjusted. Then it was cooled to 20° C. and the precipitated NaCl was filtered off. After removal by distillation of the excess triethylamine and solvent, the residue was diluted with water. 65

4

This solution was adjusted to pH 8, filtered and desalted by electrodialysis. The resultant solution contained 32 percent of gamma-butyrobetaine corresponding to a yield of 80 percent, relative to the gamma-chlorobutyric acid ethyl ester. The content was 99.5 percent (HPLC determination of a dehydrated specimen).

What is claimed is:

1. Process of the production of gamma-butyrobetaine, comprising (a) conducting a step consisting of converting gamma-butyrolactone only with an excess of 5 to 40 weight percent, relative to the gamma-butyrolactone, of hydrogen chloride at a pressure of 1 to 25 bars and a temperature of 40° to 150° C. to obtain gamma-chlorobutyric acid, (b) a step consisting of converting the gamma-chlorobutyric acid from step (a), without isolation, only with a lower aliphatic alcohol selected from the group consisting of methanol, ethanol, a propanol and a butanol, in the presence of an amount of hydrogen chloride sufficient to act as an esterification catalyst at a pressure of 1 to 15 bars and a temperature of 40° to 150° C. to obtain the corresponding gamma-chlorobutyric acid lower alkyl ester, (c) converting the corresponding gamma-chlorobutyric acid lower alkyl ester with trimethylamine to trimethylammonium butyric acid lower alkyl ester chloride, the conversion being done at a pressure of 1 to 10 bars and a temperature of 20° to 180° C., and (d) saponifying the trimethylammonium butyric acid lower alkyl ester chloride, without isolation, with a base to the gamma-butyrobetaine, the saponficiation being done at a temperature of 20° to 100° C.

2. Process of the production of gamma-butyrobetaine, comprising (a) conducting a step consisting of converting gamma-butyrolactone only with an excess of 5 to 40 weight percent, relative to the gamma-butyrolactone, of hydrogen chloride at a pressure of 1 to 25 bars and a temperature of 40° to 150° C. to obtain gamma-chlorobutyric acid, (b), then without any intermediate step between this step and step (a), discharging the residual hydrogen chloride, (c), then without any intermediate step between this step and step (b), conducting a step consisting of converting the gamma-chlorobutyric acid from step (a), without isolation, only with a lower aliphatic alcohol selected from the group consisting of methanol, ethanol, a propanol and a butanol, in the presence of an amount of hydrogen chloride sufficient to act as an esterification catalyst at a pressure of 1 to 15 bars and a temperature of 40° to 150° C. to obtain the corresponding gamma-chlorobutyric acid lower alkyl ester, (d) converting the corresponding gamma-chlorobutyric acid lower alkyl ester with trimethylamine to trimethylammonium butyric acid lower alkyl ester chloride, the conversion being done at a pressure of 1 to 10 bars and a temperature of 20° to 180° C., and (e) saponifying the trimethylammonium butyric acid lower alkyl ester chloride, without isolation, with a base to the gamma-butyrobetaine, the saponfication being done at a temperature of 20° to 100° C.

3. Process according to claim 1 wherein methanol or ethanol is used as the lower aliphatic alcohol.

4. Process according to claim 2 wherein methanol or ethanol is used as the lower aliphatic alcohol.

* * * * *

US006429319B1

(12) **United States Patent**   (10) **Patent No.:**   **US 6,429,319 B1**

Kwak et al.   (45) **Date of Patent:**   **Aug. 6, 2002**

(54) **CONTINUOUS PROCESS FOR THE PRODUCTION OF OPTICALLY PURE (S)-BETA-HYDROXY-GAMMA-BUTYROLACTONE**

(75) Inventors: **Byong-Sung Kwak; Ki-Nam Chung; Tae-Yun Kim; Ki-Ho Koh; Jin-Woong Kim; Choon-Gil Kim**, all of Taejon (KR)

(73) Assignee: **SK Corporation**, Seoul (KR)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 84 days.

(21) Appl. No.: **09/767,668**

(22) Filed: **Jan. 22, 2001**

(30)   **Foreign Application Priority Data**

Jul. 27, 2000   (KR) 2000-43472

(51) **Int. Cl.^{7}** **C07D 307/56**
(52) **U.S. Cl.** **549/313**
(58) **Field of Search** 549/313

(56)   **References Cited**

U.S. PATENT DOCUMENTS

5,292,939 A   3/1994   Hollingsworth 562/515

5,319,110 A	6/1994	Hollingsworth	.... 549/313
5,374,773 A	12/1994	Hollingsworth	.... 562/515
5,473,104 A	12/1995	McCarthy	.... 562/567
5,808,107 A	9/1998	Hollingsworth	.... 549/323
5,998,633 A	12/1999	Jacks et al.	.... 549/313

Primary Examiner—Amelia Owens
(74) *Attorney, Agent, or Firm*—Abelman, Frayne & Schwab

(57)   **ABSTRACT**

Disclosed is a process for the production of optically pure (S)-beta-hydroxy-gamma-butyrolactone through the hydrogenation of substituted carboxylic acid derivatives. A solution containing 1 to 50% by weight of a substituted carboxylic acid derivative is fed at a WHSV of 0.1 to 10 h^{-1}, to a fixed bed reactor which is filled with a catalyst and maintained at a reaction temperature of 50 to 550° C. under a halogen partial pressure of 15 to 5,500 psig. The catalyst is composed of a noble metal as a catalytically effective ingredient which is impregnated in an inorganic oxide as a support. The molar ratio of the hydrogen to the substituted carboxylic acid derivative is maintained at a molar ratio of 1:1 to 10:1. The process can produce optically pure (S)-beta-hydroxy-gamma-butyrolactone with higher purities at higher yields than can conventional techniques. In addition to being relatively simple and environmentally friendly, the process is so economically favorable as to apply to industrial production.

13 Claims, 1 Drawing Sheet

FIGURE

FIG. 1
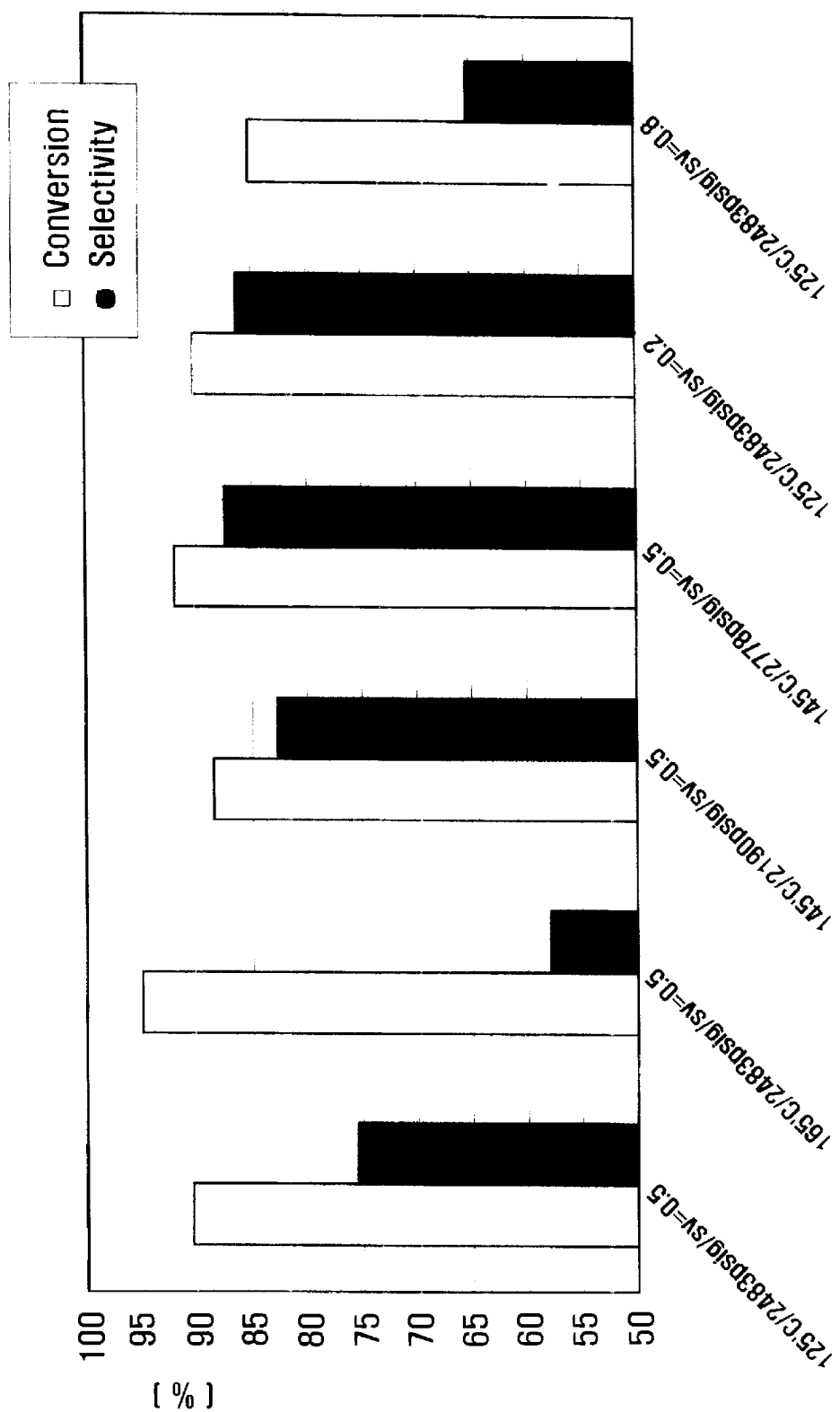
US 6,429,319 B1

1

CONTINUOUS PROCESS FOR THE PRODUCTION OF OPTICALLY PURE (S)-BETA-HYDROXY-GAMMA-BUTYROLACTONE

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a process for the production of optically pure (S)-beta-hydroxy-gamma-butyrolactone in a continuous manner. More particularly, the present invention relates to a continuous process for the production of optically pure (S)-beta-hydroxy-gamma-butyrolactone by hydrogenation of substituted carboxylic acid derivatives in a fixed-bed reactor which is filled with a catalyst comprising a noble metal and a support.

2. Description of the Prior Art

Optically pure, substituted gamma-butyrolactone is used as an intermediate for the synthesis of a variety of compounds, including medicines such as L-carnitine and ECHB (ethyl (S)-4-cyano-3-hydroxybutyrate), agricultural chemicals, chemical seasonings, and flavorings (U.S. Pat. No. 5,473,104).

Synthesis processes of (S)-β-hydroxy-γ-butyrolactone can be found in many patents. For example, U.S. Pat. Nos. 5,292,939, 5,319,110 and 5,374,773 disclose preparation method of substituted gamma-butyrolactone by the oxidation of water-soluble hydrocarbons. This process, however, is disadvantageous in that the heat of reaction is too great to conduct the oxidation in high concentrations of the reactant. No separation processes, except chromatography, are described in the above patents. Also, nowhere are mentioned yields. Thus, the processes disclosed in the above patents are not suitable for use in the industrial production.

There are reported multi-step processes for preparing gamma-butyrolactone using L-malic acid or L-aspartic acid as a starting material (J. Org. Chem. 1981, 46, 4319; Synth. Commun. 1986, 16, 183). They, however, have a drawback in that optical activity of intermediates is not maintained during the reaction, in addition to being unsuitable for large-scale production.

Starting from (S)-malic acid ester derivatives, a reducing process for preparing gamma-butyrolactone by use of borane-dimethylsulfide and sodium borohydride has been reported (Chem. Lett. 1984, 1389). However, this process is of a batch type with high production costs and being difficult to apply for industrial production. In addition, the reducing process produces wastes in large quantities, which are detrimental to the environment.

A process disclosed in U.S. Pat. No. 5,808,107 is to prepare optically active (S)-beta-hydroxy-gamma-butyrolactone by reducing L-malic acid dimethyl ester with lithium chloride and sodium borohydride to give (S)-3,4-dihydroxybutyric acid, and treating the intermediate with HCl in a methanol solution. However, this process suffers from the disadvantage of being performed in a complicated batch type manner and brings about pollution of the environment. In addition, the use of sodium borohydride, an expensive and explosive reducing agent, increases the production cost and thus is not suitable for large-scale production. When being used in large quantities, ether employed as a reaction solvent may create a noxious effect on the body owing to its narcotic nature and has the danger of exploding.

In U.S. Pat. No. 5,998,633 is described a process for the preparation of substituted gamma-butyrolactone, in which a hydrocarbon is oxidized to give acetonide, followed by the

2

treatment of the intermediate with an inorganic acid (aqueous HCl solution). This process is also industrially disadvantageous in that it is complicated and produces waste in large quantities.

As described above, the conventional processes are of batch types employing liquid or solid oxidizing or reducing reagents, so that they have the disadvantage of low productivity as well as the production of waste in large quantities. Furthermore, the conventional processes are limited in industrial applications since the low production yield is obtained owing to the complexity thereof.

SUMMARY OF THE INVENTION

Leading to the present invention, the intensive and thorough research on the production of optically active (S)-beta-hydroxy-gamma-butyrolactone, conducted by the present inventors, resulted in the finding that a catalyst, having as a catalytic ingredient a noble metal impregnated in an inorganic oxide support, for hydrogenating optically active, substituted carboxylic acid derivatives is very useful in synthesizing optically pure (S)-beta-hydroxy-gamma-butyrolactone and a fixed bed of the catalyst makes it possible to produce the compound of interest in a continuous manner.

Therefore, it is an object of the present invention to provide a process for the production of optically pure (S)-beta-hydroxy-gamma-butyrolactone from substituted carboxylic acid derivatives, which is improved in the production yield, friendly to the environment, and simple.

Based on the present invention, the above object could be accomplished by a provision of a method for producing optically pure (S)-beta-hydroxy-gamma-butyrolactone from a substituted carboxylic acid derivative by hydrogenation, in which a solution containing 1 to 50% by weight of the substituted carboxylic acid derivative is fed at a WHSV of 0.1 to 10 h^{-1}, to a fixed bed reactor which is filled with a catalyst and maintained at a reaction temperature of 50 to 550° C. under a hydrogen partial pressure of 15 to 5,500 psig, said catalyst having a noble metal as a catalytically effective ingredient and an inorganic oxide as a support, said hydrogen being maintained at a molar ratio of 1:1 to 10:1 relative to the substituted carboxylic acid derivative.

BRIEF DESCRIPTION OF THE DRAWING

FIG. **1** is a histogram showing the conversion and selectivity under various reaction conditions when substituted carboxylic acid derivatives are converted to optically pure (S)-beta-hydroxy-gamma-butyrolactone by hydrogenation in the presence of a catalyst in accordance with the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention pertains to continuous hydrogenation of esters of substituted carboxylic acids into optically pure (S)-beta-hydroxy-gamma-butyrolactone in the presence of a catalyst in a fixed bed reactor. By virtue of its superior production yield and productivity, this continuous process is far more economical than conventional processes. The process of the present invention also has an economical benefit in that the catalyst can be recovered and used repeatedly. Additionally, the above process requires no complicated post-processes, such as filtering off of the catalyst.

A catalyst suitable for use in the hydrogenation according to the present invention comprises a noble metal as a

US 6,429,319 B1

3

catalytically active ingredient. A useful noble metal is selected from the group consisting of nickel (Ni), palladium (Pd), platinum (Pt), rhodium (Rh), iridium (Ir), ruthenium (Ru), osmium (Os) and mixtures thereof. Such catalytically effective ingredient is impregnated on a support, which is preferably selected from the group consisting of alumina, silica, silica-alumina, zirconia, titania, zeolite and a molecular sieve.

The hydrogenation according to the present invention can be illustrated by the following reaction formula 1:

1
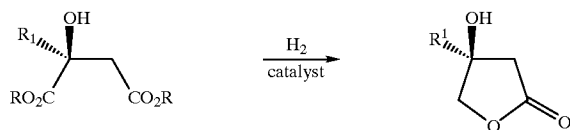
wherein R is a linear alkyl containing 1 to 10 carbon atoms, a cycloalkyl, or an aryl; and R' is a hydrogen atom or a methyl.

According to the present invention, to synthesize an ester compound of a substituted carboxylic acid from which optically pure (S)-beta-hydroxy-gamma-butyrolactone is produced, there is employed an alcohol selected from a linear alcohol containing 1 to 10 carbon atoms such as methanol, ethanol and n-propanol, a cycloalcohol or an aromatic alcohol. The alcohol is used at an amount of 2.0 to 40 equivalents of the carboxylic acid used. As for the carboxylic acid, for example, optically pure malic acid or citramalic acid may be used. The esterification reaction is carried out at 50 to 150° C. under a pressure of 1 to 300 psig with a weight hourly space velocity (WHSV) ranging from 0.1 to 10 h^{-1} in the absence of or in the presence of a catalyst. Suitable as a catalyst is a solid acid with a preference to a sulfonate-substituted strong acid resin. If the reaction conditions are outside the above ranges, the production yield of the esters of carboxylic acid is lowered and the deactivation rate of the catalyst increases. Accordingly, the advantages that the continuous process of the present invention enjoys are lost.

In accordance with the present invention, the hydrogenation of esters of substituted carboxylic acid into optically active (S)-beta-hydroxy-gamma-butyrolactone is carried out at 50 to 550° C. under a hydrogen partial pressure of 15 to 5,500 psig with a WHSV ranging from 0.2 to 10 h^{-1}. This conversion through hydrogenation is preferably conducted at 100 to 250° C. under a hydrogen partial pressure of 1,000 to 4,000 psig with a WHSV ranging from 0.2 to 10 h^{-1}, and more preferably at 110 to 200° C. under a hydrogen partial pressure of 1,200 to 3,000 psig with a WHSV ranging from 0.3 to 5 h^{-1}. A reaction condition outside any of the ranges causes a decrease in the production yield and an increase in the deactivation rate of the catalyst, resulting in loss of the advantages that the continuous process of the present invention enjoys.

To completely convert an ester derivative of substituted carboxylic acid into a desired compound, the mole ratio of hydrogen to the ester derivative of substituted carboxylic acid is required to be at least 1.0. No limits are given to the amount of hydrogen if the mole ratio exceeds about 1. However, when an economical aspect is taken into consideration, the ratio of hydrogen to an ester of carboxylic acid is preferably maintained in the range from 2.0 to 10. The hydrogen which passes through the reactor while remaining unreacted is re-compressed and recycled into the

4

reactor. Depending on reaction conditions, the reaction may be directly separated into desirable products or may be recycled to further convert the unreacted reactant, followed by separation.

To convert esters of substituted carboxylic acid into (S)-beta-hydroxy-gamma-butyrolactone by hydrogenation, there is required a solvent suitable for dissolving highly viscous carboxylic derivatives so effectively as to smoothly feed them to the reactor. Further, the solvent requires to remove the heat of reaction which occurs during the esterification and the hydrogenation and not to react with any of the reactants, e.g., neither carboxylic acid derivatives nor hydrogen. For example, one selected from the group consisting of methyl alcohol, ethyl alcohol, n-propyl alcohol, isopropyl alcohol, dioxane, gamma-butyrolactone, tetrahydrofuran, water and mixtures thereof may be used as a solvent. Of these, isopropyl alcohol and water are preferred with greater preference for the latter. In the solvent, the ester of carboxylic acid is maintained at a concentration of 1 to 50% by weight and preferably at a concentration of 10 to 40% by weight.

As mentioned above, the hydrogenation according to the present invention is conducted with the aid of a catalyst. This has a noble metal as a catalytically effective ingredient. Examples of suitable noble metals include Ni, Pd, Pt, Rh, Ir, Ru, Os and mixtures thereof with preference to Ru. The catalytically active ingredient may be used in a bare form or in a combination with a support. In the latter case, the noble metal is impregnated on the support. Suitable as a support is an inorganic oxide selected from the group consisting of alumina, silica, silica-alumina, zirconia, titania, zeolite and molecular sieves. Of them, silica is most preferred.

The support may be in any form, such as a spherical form, a cylindrical form, a granular form, etc. For mechanical properties, the support preferably has a spherical or a cylindrical form.

In the catalyst, the noble metal is preferably contained at an amount of 0.1 to 15% by weight based on the catalyst, and more preferably at an amount of 0.5 to 10% by weight. For instance, a catalyst containing less than 0.1% by weight of the noble metal is poor in hydrogenation activity and selectivity. On the other hand, more than 15% by weight of the noble metal is economically unfavorable.

For impregnating the noble metal into the support, there may be used various techniques, including incipient wetness impregnation, excess water impregnation, spraying, and physically mixing. After completion of the impregnation, the composite is sintered for 2 hours or more in the air or an inert gas atmosphere. The sintering temperature is preferably maintained at 300 to 700° C. and more preferably at 300 to 550° C. For instance, when the sintering is carried out at less than 300° C., precursors of the metal impregnated are insufficiently decomposed. On the other hand, a sintering temperature higher than 700° C. lowers the dispersion degree of the metal impregnated, resulting in a catalyst with poor performance.

In a fixed bed reactor is filled the sintered catalyst. Before feeding the reactant to the reactor, the catalyst should be in a reduced state. To this end, the catalyst is maintained for at least 2 hours in a hydrogen atmosphere at 50 to 500° C. depending on the kind of the metal impregnated.

In the presence of the catalyst system which has a noble metal impregnated in a support, substituted carboxylic acid derivatives are hydrogenated to give optically pure (S)-beta-hydroxy-gamma-butyrolactone at a high yield. In accordance with the present invention, the employment of a fixed bed reactor in this hydrogenation allows the process to be

US 6,429,319 B1

5

conducted in a continuous manner, bringing about a great improvement in the production yield. In addition, the process is economically favorable in that the used catalyst may be recovered easily. Furthermore, the product recovery following the conversion is simple because there is no need to filter off the catalyst.

By adopting a fixed bed reaction system, the present invention shows a higher production yield per reactive space time than do conventional processes, has an economical benefit owing to the repeated use of the catalyst, and is simple without the need of filtering off the catalyst upon recovering the product. In the fixed bed reaction system, no limitations are imposed as to the form of the reactor or the reactant feeding and flowing direction. In order for reactants to come in smooth contact with each other, a trickle-bed type reactor is preferably used in which the reactants hydrocarbon and hydrogen are flowed downward together and dispersed uniformly throughout.

Effluents from the reactor are passed to a solvent-recovering unit in which the solvent is at least partially separated from the product. For this purpose, any recovering unit, such as a distillation column or a flash vaporizer, may be provided to the reactor system. Products or concentrates drained from the lower portion of the recovering unit are transferred to a vacuum distillation unit.

A better understanding of the present invention may be obtained in light of the following examples which are set forth to illustrate, but are not to be construed to limit the present invention.

EXAMPLE 1

Preparation of Catalyst

In a 100 cc flask containing secondary distilled water was added 17.9 g of ruthenium chloride ($RuCl_3$) to give a aqueous ruthenium solution. In a vessel for impregnating metal, equipped with a speed-controllable motor, 100 g of silica (⅛ cylindrical) was added, after which the ruthenium solution was poured while rotating the vessel. As a result, the ruthenium solution was uniformly dispersed over the silica. For 30 min, after completion of the pouring of the ruthenium solution, the vessel was further rotated at the same speed. Subsequently, the resulting ruthenium-supported catalyst was sintered at 550° C. for 6 hours under the air atmosphere in a muffle furnace. The catalyst was found to have a ruthenium content of 3.0% by weight as measured by fluorescent X-ray analysis.

EXAMPLE 2

Continuous Preparation of Dimethyl (S)-Malate

In an automatic high-pressure reactor made of stainless steel 316 was filled 25 g of a solid acid catalyst. After being purged with nitrogen, the inside of the reactor was heated from room temperature to 84° C. and maintained at a pressure of 100 psig. L-malic acid was dissolved in 8 equivalents of methanol and the resulting solution was fed at a WHSV of 4.0 h^{-1} into the reactor to produce the title compound at a yield of 90%: Conversion 99%. Selectivity 99%.

The effluent from the reactor was distilled in vacuum to separate at a separation yield of 90% dimethyl (S)-malate which was 99.8% in purity and 99.9% in optical purity. The preparation could be effected in a batch type. In this case, the reaction period of time was set to be 2 to 4 hours.

EXAMPLES 3 THROUGH 8

Continuous Preparation of (S)-Beta-Hydroxy-Gamma-Butyrolactone

In an automatic, stainless-steel 316, high pressure reactor (inner diameter 2.52 cm×length 60 cm) was filled 50 g of the

6

catalyst prepared in Example 1. The catalyst was converted to a reduced state by raising the temperature at a rate of 1° C. per min to 350° C. and maintained at this temperature for 6 hours in a hydrogen atmosphere. After being cooled, the inside of the reactor was purged with nitrogen gas. While the inside of the reactor was heated at a rate of 1° C. per min to 145° C. from room temperature, hydrogen was fed at a rate of 100 sccm. The hydrogen was added at an amount twice as much as necessary for the reaction. The dimethyl (S)-malate prepared in Example 2 was dissolved in water to give a 30 wt % solution. This dimethyl (S)-malate solution was fed under the conditions shown in Table 1, below. Reaction products were taken every 9 hours and analyzed by gas chromatography using a flame ionization detector (FID) (beta-DEX column 60 cm×0.25 mm×0.25 μm). The results are given in Table 1, below.

TABLE 1

Exam. No	Temp. (° C.)	Press. (psig)	WHSV (h^{-1})	Conversion (%)	Selectivity for S)-HGB* (%)
3	125	2,483	0.5	90.5	75.5
4	165	2,483	0.5	95.0	57.9
5	145	2,190	0.5	88.5	82.7
6	145	2,778	0.5	92.0	87.5
7	125	2,483	0.2	90.2	86.3
8	125	2,483	0.8	85.2	65.3

*(S)-beta-hydroxy-γ-butyrolactone

EXAMPLES 9 THROUGH 12

Continuous Preparation of (S)-beta-Hydroxy-gamma-Butyrolactone

While the solvent was being changed as shown in Table 2, below, the hydrogenation of dimethyl (S)-malate was conducted at 145° C. under a hydrogen partial pressure of 2,628 psig with the reactant being fed at a WHSV of 0.5 h^{-1} in the same manner as in Example 3. A measurement was made of the conversion and selectivity and the results are given in Table 2, below.

TABLE 2

Example No.	Solvent	Conversion. (%)	Selectivity for(S) - HGB* (%)
9	30% H_2O	92.0	85.5
10	20% H_2O	94.0	72.0
11	10% H_2O	95.0	78.0
12	10% i-PrOH	72.5	54.2

*(S)-beta-hydroxy-γ-butyrolactone

EXAMPLE 13

Low-Pressure Hydrogenation and Product Recycling

In the presence of the catalyst prepared in Example 1, the hydrogenation process was carried out in the same manner as in Example 6, except using a hydrogen partial pressure of as low as 1,460 psig. Product effluents from the reactor were recycled twice more. During the recycling, no deactivation of the catalyst was observed while an improvement was brought about in the conversion and selectivity. The results are given in Table 3, below.

US 6,429,319 B1

7

TABLE 3

Rxn Cycle No.	Conversion (%)	Selectivity (%)
1	75	65
2	90	75
3	95	85

EXAMPLE 14

Long-Term Continuous Reaction for (S)-beta-Hydroxy-gamma-Butyrolactone

Using the catalyst prepared in Example 1, a long-term continuous conversion reaction was performed in a reactor similar to that described in Example 2. Even after 500 hours of the reaction, no deactivation was observed in the catalyst. The results are given in Table 4, below.

TABLE 4

	Rxn Time (h)		
	100	250	500
Conversion (%)	92.5	92.4	91.4
Selectivity (%)	74.3	78.8	77.7

EXAMPLE 15

Separation of (S)-beta-Hydroxy-gamma-Butyrolactone

Using 50 g of the catalysts prepared in Example 1, a hydrogen reaction was conducted in a reactor similar to that described in Example 2. During hydrogenation, the hydrogen partial pressure was maintained at 2,438 psig while there were various changes to the reaction temperature and the WHSV. After 200 hours of the hydrogenation, 30 liters of a solution containing (S)-beta-hydroxy-gamma-butyrolactone with a selectivity of 75.2 wt % was obtained. This solution was neutralized with an aqueous 10% $NaHCO_3$ solution and deprived of the solvent, after which the residue was extracted three times with ethyl acetate to recover (S)-beta-hydroxy-gamma-butyrolactone. In 10 L glass reactor equipped with a vacuum distillater, the extract was distilled at 60° C. under 100 mbar to evaporate the solvent. In a thin film evaporator, the concentrate was further distilled at 100 to 120° C. under 0.6 to 1.7 torr to give (S)-beta-hydroxy-gamma-butyrolactone 99.0% in purity at a separation yield of 65%.

As described hereinbefore, the present invention can produce optically pure (S)-beta-hydroxy-gamma-butyrolactone with higher purities at higher yields than can conventional techniques. In addition to being relatively simple and environmentally friendly, the present invention is so economically favorable as to apply to industrial production.

The present invention has been described in an illustrative manner, and it is to be understood that the terminology used is intended to be in the nature of description rather than of limitation. Many modifications and variations of the present

8

invention are possible in light of the above teachings. Therefore, it is to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

What is claimed is:

1. A method for producing optically pure (S)-beta-hydroxy-gamma-butyrolactone from a substituted carboxylic acid derivative by hydrogenation, in which a solution containing 1 to 50% by weight of the substituted carboxylic acid derivative is fed at a WHSV of 0.1 to 10 h^{-1}, to a fixed bed reactor which is filled with a catalyst and maintained at a reaction temperature of 50 to 550° C. under a hydrogen partial pressure of 15 to 5,500 psig, said catalyst having a noble metal as a catalytically effective ingredient and an inorganic oxide as a support, said hydrogen being maintained at a molar ratio of 1:1 to 10:1 relative to the substituted carboxylic acid derivative.

2. The method as set forth in claim **1**, wherein said noble metal is selected from the group consisting of palladium (Pd), platinum (Pt), rhodium (Rh), iridium (Ir), ruthenium (Ru), osmium (Os), and mixtures thereof.

3. The method as set forth in claim **1**, wherein the noble metal is impregnated on the inorganic acid support at an amount of 0.1 to 15% by weight based on the catalyst.

4. The method as set forth in claim **1**, wherein said reaction temperature is maintained at 100 to 250° C.

5. The method as set forth in claim **1**, wherein said hydrogen partial pressure is maintained at 1,200 to 3,000 psig.

6. The method as set forth in claim **1**, wherein the reactants are fed at a WHSV of 0.2 to 5.0 h^{-1}.

7. The method as set forth in claim **1**, wherein said solution contains the carboxylic acid derivative at an amount of 10 to 40% by weight.

8. The method as set forth in claim **1**, wherein said solvent is selected from the group consisting of methyl alcohol, ethyl alcohol, n-propyl alcohol, isopropyl alcohol, dioxane, gamma butyrolactone, tetrahydrofuran, water and mixtures thereof.

9. The method as set forth in claim **1**, wherein said inorganic oxide support is selected from the group consisting of alumina, silica, silica-alumina, zirconia, titania, zeolite and molecular sieves.

10. The method as set forth in claim **1**, wherein said fixed bed reactor is a trickle-bed reactor.

11. The method as set forth in claim **1**, wherein said substituted carboxylic acid derivative is prepared by reacting a carboxylic acid with an alcohol in the presence of a solid acid in a reactor which is maintained at a reaction temperature of 50 to 150° C. under a reaction pressure of 1.0 to 300 psig with a WHSV being controlled within the range of 0.5 to 10 h^{-1}, said alcohol being selected from the group consisting of linear alcohols containing 1 to 10 carbon atoms, cyclic alcohols and aromatic alcohols and being used at an amount of 2.0 to 40 equivalents of said carboxylic acid.

12. The method as set forth in claim **11**, wherein said solid acid is a sulfonate-substituted strong acidic resin.

13. The method as set forth in claim **11**, wherein said carboxylic acid is optically pure malic acid or citramalic acid.

* * * * *

US005473104A

United States Patent [19]

McCarthy

[11] **Patent Number:**   **5,473,104**

[45] **Date of Patent:**   **Dec. 5, 1995**

[54] **PROCESS FOR THE PREPARATION OF L-CARNITINE**

[75] Inventor: **James R. McCarthy**, Solana Beach, Calif.

[73] Assignee: **Neurocrine Biosciences, Inc.**, San Diego, Calif.

[21] Appl. No.: **306,502**

[22] Filed: **Sep. 13, 1994**

[51] **Int. Cl.^{6}** **C07C 229/12**

[52] **U.S. Cl.** **562/567**

[58] **Field of Search** 562/567

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,135,788	6/1964	Noguchi et al.	260/531
3,151,149	9/1964	Strack et al.	260/465.5
3,830,931	8/1974	DeFelice	424/319
3,968,241	7/1976	DeFelice	424/319
4,070,394	1/1978	Wiegand	260/465.5 R
4,075,352	2/1978	DeFelice	424/319
4,898,977	2/1990	Herold et al.	564/191
5,292,939	3/1994	Hollingsworth	562/515

OTHER PUBLICATIONS

CA112:179887a (1989).

CA118:191741e (1992).

Masaji Tomita and Yuzo Sendju, "Synthesis of L–carnitine," *Z. Physiol. Chem* 169:263 (1927).

Voeffray et al., "193. L–Carnitine. Novel Synthesis and Determination of the Optical Purity," *Helv. Chim. Acta* 70:2058 (1978).

Strack et al., "Synthesis of DL–carnitine," *Ber.* 86:525 (1953).

Carter and Bhattacharyya, "Improvements in the Synthesis of DL–Carnitine," *J. Am. Chem. Soc.* 75:2503 (1953).

Strack and Lorenz, "Synthesis of DL,–D–, and L–carnitine from epichlorohydrin," *Z. Physiol. Chem* 318:129 (1960).

Jon Bremer, "Carnitine and its role in fatty acid metabolism," *TIBS* 2:207 (1977).

Jung et al., "Synthesis of L–Carnitine by Microorganisms and Isolated Enzymes," *Adv. Biochem. Eng.* 50:21 (1993).

Primary Examiner—José G. Dees
Assistant Examiner—Barbara S. Frazier
Attorney, Agent, or Firm—Seed and Berry

[57]   **ABSTRACT**

There is disclosed a process for preparation of L-carnitine from (S)-3-hydroxybutyrolactone. The process is a two-step preparation in which (S)-3-hydroxybutyrolactone is first converted to a hydroxy-activated form and subsequently transformed to L-carnitine by treatment of the hydroxy-activated (S)-3-hydroxybutyrolactone with trimethylamine in water.

19 Claims, No Drawings

5,473,104

1

PROCESS FOR THE PREPARATION OF L-CARNITINE

TECHNICAL FIELD

This invention relates generally to the preparation L-carnitine from (S)-3-hydroxybutyrolactone.

BACKGROUND OF THE INVENTION

L-carnitine plays a significant role in β-oxidation of fatty acids and, as a result, has created an increasing demand for this compound in medicine (see, e.g., Bremer, *Trends Biochem. Sci.* 2:207–9, 1977). Such demand has led to the development of numerous procedures lbr production of L-carnitine, including its isolation from natural sources, synthetic chemical procedures (such as resolution of racemic mixtures of DL-carnitine), and transformation by enzymes and microorganisms.

A number of chemical methods for the synthesis of DL-carnitine are known. For example, U.S. Pat. No. 3,135, 788 is directed to the preparation of DL-carnitine hydrochloride in which epichlorohydrin is first treated with trimethylamine to provide 1-chloro-2-hydroxy-4-(trimethylammonio)butane. Subsequent displacement of chloride by treatment with potassium cyanide produces the corresponding cyano compound which, upon acidic hydrolysis, yields DL-carnitine. A similar strategy was employed in U.S. Pat. No. 4.070,394 where the chloride of epichlorohydrin was initially displaced by trimethylamine and the product thus obtained treated with various metal cyanides to yield 1-cyano-2-hydroxy-4-(trimethylammonio)butane, which is then converted to DL-carnitine by hydrolysis.

The discovery of adverse effects of D-carnitine, and the questionable therapeutic effectiveness of the racemic mixture of DL-carnitine, has driven the search for a practical and economically efficient preparation of L-carnitine. To this end, optically pure L-carnitine has now been prepared by resolution from its racemic mixture. For example, U.S. Pat. No. 3,151,149 is directed to such a resolution by recrystallization using D-(+)-camphor-10-sulfonic acid. The synthesis of L-carnitine from optically pure precursors resolved from their respective racemic mixtures has also been reported. For example, racemic mixtures of 1-chloro-2-hydroxy-4-(trimethylammonio)butane have been resolved using L-(+)-tartaric acid, and the resulting enantiomerically pure chlorobutane chemically transformed to L-carnitine (Voeffray et. al., *Helv. Chim. Acta* 70:2058–64, 1987).

Optically pure L-carnitine has also been prepared from chiral compounds including (R)-4-chloro-3-hydroxybutyrate. For example, a chemomicrobiological synthesis of L-carnitine has been reported (Zhou et al., *J. Amer. Chem. Soc.* 105:5925–26, 1983) wherein optically pure (R)-4-chloro-3-hydroxybutyrate was prepared from ethylacetoacetate by reduction by baker's yeast, and then converted to L-carnitine by standard methods. Another enzymatic synthesis of (R)-4-chloro-3-hydroxybutyrate employing a coupled enzyme system of glucose dehydrogenase and alcohol dehydrogenase has also been reported (Wong et al., *J. Amer. Chem. Soc.* 107: 4028–31, 1985).

Despite the availability of L-carnitine, existing methods for its production are indirect, laborious, and economically prohibitive. Accordingly, there is a need in the art for a direct and efficient route to optically pure L-carnitine. The present invention fulfills this need, and provides further related advantages.

2

SUMMARY OF THE INVENTION

In brief, a two-step preparation of L-carnitine from commercially available (S)-3-hydroxybutyrolactone is disclosed. The process comprises reacting a hydroxy-activated (S)-3-hydroxybutyrolactone with an aqueous solution of trimethylamine. In the practice of this invention, L-carnitine is readily produced in enantiomeric excess from the optically pure starting material (i.e., (S)-3-hydroxybutyrolactone) at levels of at least 90%, and more typically in enantiomeric excess of at least 95%.

More specifically, in one embodiment of this invention, L-carnitine is produced from a hydroxy-activated (S)-3-hydroxybutyrolactone by treatment with a saturated solution of trimethylamine in water. In another embodiment, a co-solvent is optionally present in the aqueous trimethylamine solution. Such co-solvents include ethanol, methanol, tetrahydrofuran and acetonitrile, and may be present in the aqueous trimethylamine solution in amounts ranging from about 5% to about 50% by volume of the total solution.

Generally, the hydroxy-activated (S)-3-hydroxybutyrolactones of this invention include (S)-3-hydroxybutyrolactone alkyl and aryl sulfonates. More specifically, the hydroxy-activated (S)-3-hydroxybutyrolactones include alkyl and haloalkyl sulfonates as well as aryl, haloaryl, alkylaryl, alkoxyaryl, and nitroaryl sulfonates: alkyl sulfonates include methane, ethane, propane, isopropane, and butane sulfonates; haloalkyl sulfonates include halogenated derivatives of the above-mentioned alkyl sulfonates, and specifically include trichloromethane sulfonate, trifluoromethane sulfonate, chloroethane sulfonate, chloropropane sulfonate, and perfluorobutane sulfonate; aryl sulfonates include benzene and napthalene sulfonates, as well as various derivatives, including alkylaryl sulfonates such as alkylbenzene sulfonates (e.g., toluene, mesitylene, and triisopropylbenzene sulfonates), alkoxyaryl sulfonates including alkoxybenzene sulfonates such as methoxybenzene sulfonates, halobenzene sulfonates such as bromo-, chloro-, and fluorobenzene sulfonates, and nitroaryl sulfonates such as nitrobenzene sulfonates.

Other aspects of the invention will become evident upon reference to the following detailed description.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is generally directed to a process for the preparation of carnitine, 3-hydroxy-4-(trimethylammonio)butanoate, and, more specifically, to the preparation of the optically pure L-carnitine, (R)-3-hydroxy-4-(trimethylammonio)butanoate.

Carnitine is a chiral molecule by virtue of the fact that it possesses an asymmetric carbon atom. C-3, which bears as substituents a hydrogen, a hydroxy group, a trimethylamino methyl group, and a carboxymethyl group. As such, the configuration of substituents about C-3 results in to possible absolute configurations, and therefore, two optical isomers (i.e., enantiomers) of carnitine exist. One optical isomer causes leverorotation of a plane of polarized light and is designated as the L-isomer, while the other optical isomer causes dextrorotation and is designated as the D-isomer.

As mentioned above, the present process produces L-carnitine in enantiomeric excess corresponding to the optical purity, of the starting material, (S)-3-hydroxybutyrolactone. Using such an optically pure starting material, L-carnitine in enantiomeric excess of 90%, and more typically in enantio-

5,473,104

3

meric excess of 95% may readily be achieved. As used herein, the term "enantiomeric excess" refers to the optical purity of the chiral product (i.e., the percentage of one optical isomer in relation to the total amount of all of the optical isomers of the product produced). Accordingly, when the production of L-carnitine achieves an enantiomeric excess of 95% this means that 97.5% of the carnitine thus produced is the L-optical isomer.

The optical purity of L-carnitine achieved by this invention results from the stereoselective nature of the reaction of the present invention coupled with the use of an optically pure starting material. In other words, the process of the present invention is stereoselective (i.e., substantially produces a single optical isomer). Subjecting an appropriately reactive optically pure starting material (i.e., a single optical isomer of the starting material) to the process of the present invention provides an optically pure product. Thus, in the practice of the present invention, treatment of hydroxy-activated (S)-3-hydroxybutyrolactone produces L-carnitine, treatment of hydroxy-activated (R)- 3-hydroxybutyrolactone produces D-carnitine, and treatment of racemic hydroxy-activated 3-hydroxybutyrolactone produces racemic DL-carnitine.

As used herein, the term "hydroxy-activated" refers to a chemical moiety derived from a hydroxy group such that the derivative of the hydroxy group is reactive toward displacement (i.e., the derivative of the hydroxy group is a good leaving group). In the practice of the present invention, the hydroxy group of (S)-3-hydroxybutyrolactone has been activated toward displacement and subsequent L-carnitine formation by appropriate derivatization.

A representative process for the preparation of L-carnitine is presented schematically in below.
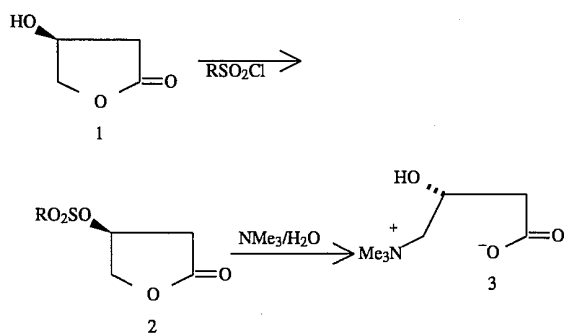
The above reaction involves a two-step process: first, an optically pure hydroxy-activated (S)-3-hydroxybutyrolactone (2) is produced from an optically active starting material, (S)-3-hydroxybutyrolactone (1); second, the hydroxy-activated (S)-3-hydroxybutyrolactone (2) is converted to L-carnitine (3) by reacting with trimethylamine in water.

The optical purity of hydroxy-activated (S)-3-hydroxybutyrolactone (2) is due to the optical purity of (S)-3-hydroxybutyrolactone (1), which is commercially available (Kaneka America Corp., New York, N.Y.) in optically pure form. The conversion of (1) to (2) is an esterification reaction and does not involve bond breakage or formation at C-3. Consequently, conversion of (1) to (2) occurs without racemization at C-3 and without loss of optical purity.

By a stereoselective reaction, the hydroxy-activated (S)-3-hydroxybutyrolactone (2) is then converted to L-carnitine (3). The transformation from (2) to (3) occurs upon treatment of (2) with an aqueous solution of trimethylamine. In

4

the particular reaction illustrated above, the hydroxy-activated (S)-3-hydroxybutyrolactone (2) is (S)-3-hydroxybutyrolactone sulfonate, and thus the activated hydroxy group is the —SO_3R moiety where R represents an alkyl or aryl group (or substituted derivatives thereof). In this embodiment, the hydroxy group of (S)-3-hydroxybutyrolactone (1) is activated toward displacement by treatment with a sulfonyl chloride to yield a sulfonic acid ester (i.e., a sulfonate). The activated hydroxy group, —SO_3R, is a good leaving group and thus facilitates the process of the present invention.

Without being limited to the following theory, for the formation of L-carnitine from the hydroxy-activated (S)-3-hydroxybutyrolactone, the mechanism of reaction is believed to involve the following steps. Nucleophilic attack by hydroxide ion (a species present in an aqueous solution of trimethylamine) on the lactone carbonyl of (2) is believed to occur, resulting in lactone ring opening and the generation of an alkoxide ion. The alkoxide ion then acts as a nucleophile and displaces the leaving group on its neighboring carbon atom, C-3. Such an intramolecular displacement results in an inversion of configuration of C-3. The transient product formed by the stereoselective displacement reaction is believed to be an epoxide which is immediately susceptible to nucleophilic attack by trimethylamine at C-4. The nucleophilic attack by trimethylamine results in epoxide ring opening to yield the optically pure product, L-carnitine (3), which possesses a trimethylammonium group at C-4 and a hydroxy substituted, asymmetric carbon at C-3.

In a preferred embodiment of the present invention, L-carnitine is produced from a hydroxy-activated (S)-3-hydroxybutyrolactone by treatment with an aqueous solution of trimethylamine which preferably contains a saturated amount of trimethylamine which may generally range from about 20% to about 25% by weight trimethylamine in water. In a preferred embodiment, the aqueous solution of trimethylamine is a saturated solution containing from about 23–25% by weight trimethylamine in water.

In another embodiment, a co-solvent is optionally present in the aqueous trimethylamine solution. The co-solvent serves to enhance the solubility of -the hydroxy-activated (S)-3-hydroxybutyrolactone in the predominantly aqueous reaction mixture, and thus promote L-carnitine formation. Suitable co-solvents include (but are not limited to) ethanol, methanol, tetrahydrofuran, and acetonitrile, and may be present in the aqueous trimethylamine solution in amounts ranging from about 5% to about 50% by volume of the total solution. In a preferred embodiment, the co-solvent is ethanol and is present in the aqueous trimethylamine solution in about 20% by volume of the total solution. Alternatively, the co-solvent may be added directly to the hydroxy-activated (S)-3-hydroxybutyrolactone immediately prior to the addition of the aqueous trimethylamine solution.

As mentioned above, in one embodiment of this invention the hydroxy-activated (S)-3-hydroxybutyrolactones are generally (S)-3-hydroxybutyrolactone alkyl and aryl sulfonates. Such hydroxy-activated (S)-3-hydroxybutyrolactone alkyl and aryl sulfonates include substituted alkyl and aryl sulfonates. Suitable substituted alkyl and aryl sulfonates include haloalkyl sulfonates, haloaryl, alkylaryl, alkoxyaryl, and nitroaryl sulfonates. Alkyl sulfonates include C_{1-4} alkyl (e.g., methane, ethane, propane, isopropane and butane) sulfonates; and haloalkyl sulfonates include halogenated derivatives of the above-mentioned alkyl sulfonates, such as trichloromethane sulfonate, trifluoromethane sulfonate, chloroethane sulfonate, chloropropane sulfonate, and perfluorobutane sulfonate. Similarly, aryl sulfonates include

5,473,104

5

benzene and napthalene sulfonates, as well as various derivatives thereof including alkylaryl sulfonates such as alkylbenzene sulfonates (e.g., toluene, mesitylene, and tri-isopropylbenzene sulfonates), alkoxyaryl sulfonates (e.g., alkoxybenzene sulfonates such as methoxybenzene sulfonates), haloaryl sulfonates (e.g., benzene sulfonates such as bromo-, chloro-, and fluorobenzene sulfonates), and nitroaryl sulfonates (e.g., nitrobenzene sulfonates). The aryl sulfonate derivatives of this invention may be substituted at one or more of the benzene or naphthalene ring positions. In a preferred embodiment, the hydroxy-activated (S)-3-hy-droxybutyrolactone is (S)-3-hydroxybutyrolactone methane sulfonate. (The preparation of (S)-3-hydroxybutyrolactone methane sulfonate from (S)-3-hydroxybutyrolactone and its conversion to L-carnitine is described in the Example.)

The following example is provided for purposes of illustration, not limitation.

EXAMPLE

The Preparation of L-Carnitine

This example presents a representative synthesis of L-carnitine. The synthesis is a two-step process: (1) formation of a hydroxy-activated (S)-3-hydroxybutyrolactone (i.e., a methane sulfonate); and (2) conversion of the hydroxy-activated (S)-3-hydroxybutyrolactone to L-carnitine by reaction with trimethylamine in water. All solvents and reagents used in this example are commercially available (e.g., Aldrich Chemical Co., Milwaukee, Wis.) and are used as received without further purification unless otherwise stated. Optically pure (S)-3-hydroxybutyrolactone is commercially available from Kaneka America Corp. (New York, N.Y.) and is used without further purification.

A. Synthesis of a Hydroxy-Activated (S)-3-Hydroxybutyro-lactone: (S)-3-Hydroxybutyrolactone Methane Sulfonate

To a solution of (S) 3-hydroxybutyrolactone (1) (102 g, 1 mol) in toluene (1 L) and triethylamine (172 mL, 1.25 mol) is added a catalytic amount of 4-N,N-dimethylaminopyridine (5 g). The resulting solution is cooled to 5° C. in an ice bath and methanesulfonyl chloride (148 g, 1.3 mol) is slowly added dropwise via an addition funnel. The reaction mixture is then allowed to warm to ambient temperature with stirring. After stirring at room temperature for 16 hours, the reaction mixture is poured into ice cold brine (500 mL). After separation of the aqueous and organic layers, the organic layer is washed with ice cold 1N hydrochloric acid (2×250 mL) and brine (250 mL). The organic solution is dried over anhydrous magnesium sulfate and evaporated to dryness in vacuo. The resulting oil, (S)-3-hydroxybutyro-lactone methane sulfonate ((2) where R=CH₃), is used directly in the next step.

B. Synthesis of L-Carnitine

To an aqueous solution of trimethylamine (250 mL), 25% by weight solution of trimethylamine in water, is added (S)-3-hydroxybutyrolactone methane sulfonate (98 g, 0.5 mol) prepared as described above. The resulting solution is stirred at room temperature 1 hr, and then heated at 100° C. in a sealed vessel for 16 hrs. Upon cooling the solution to room temperature, the reaction mixture is evaporated to dryness in vacuo using ethanol and toluene (5:1) to remove residual water by azeotropic distillation. The crude L-carnitine is triturated with anhydrous acetone (100 ml) and the purified L-carnitine thus produced is collected by filtration. $[\alpha]_D^{22}$ −31° (c 10.0, H₂O).

From the foregoing, it will be appreciated that, although specific embodiments of this invention have been described

6

herein for the purposes of illustration, various modifications may be made without departing from the spirit and scope of invention. Accordingly, the invention is not limited except by the appended claims.

I claim:

1. A process for preparing L-carnitine comprising reacting a hydroxy-activated (S)-3-hydroxybutyrolactone with an aqueous solution of trimethylamine.

2. The process of claim 1 wherein L-carnitine is produced in enantiomeric excess of at least 90%.

3. The process of claim 1 wherein L-carnitine is produced in enantiomeric excess of at least 95%.

4. The process of claim 1 wherein the aqueous solution of trimethylamine contains from 20–25% by weight trimethylamine.

5. The process of claim 1 wherein the aqueous solution of trimethylamine is a saturated solution of trimethylamine in water.

6. The process of claim 1 wherein the aqueous solution contains a co-solvent.

7. The process of claim 6 wherein the co-solvent is selected from the group consisting of ethanol, methanol, tetrahydrofuran and acetonitrile.

8. The process of claim 6 wherein the co-solvent is present in the aqueous solution in an amount form 5 to 50% by volume.

9. The process of claim 1 wherein the hydroxy-activated (S)-3-hydroxybutyrolactone is an (S)-3-hydroxybutyrolactone alkyl sulfonate.

10. The process of claim 9 wherein the (S)-3-hydroxybutyrolactone alkyl sulfonate is selected from the group consisting of methane sulfonate, ethane sulfonate, propane sulfonate, isopropane sulfonate and butane sulfonate.

11. The process of claim 9 wherein the (S)-3-hydroxybutyrolactone alkyl sulfonate is methane sulfonate.

12. The process of claim 1 wherein the hydroxy-activated (S)-3-hydroxybutyrolactone is an (S)-3-hydroxybutyrolactone haloalkyl sulfonate.

13. The process of claim 12 wherein the haloalkyl sulfonate is selected from the group consisting of trichloromethane sulfonate, trifluoromethane sulfonate, chloroethane sulfonate, chloropropane sulfonate and perfluorobutane sulfonate.

14. The process of claim 1 wherein the hydroxy-activated (S)-3-hydroxybutyrolactone is an (S)-3-hydroxybutyrolactone aryl sulfonate.

15. The process of claim 14 wherein the aryl sulfonate is selected from the group consisting of benzene sulfonate, alkylbenzene sulfonate, alkoxybenzene sulfonate, halobenzene sulfonate, nitrobenzene sulfonate and napthalene sulfonate.

16. The process of claim 15 wherein the alkylbenzene sulfonate is selected from the group consisting of toluene sulfonate, mesitylene sulfonate and triisopropylbenzenesulfonate.

17. The process of claim 16 wherein the hydroxy-activated (S)-3-hydroxybutyrolactone is toluene sulfonate.

18. The process of claim 15 wherein the alkoxybenzene sulfonate is methoxybenzene sulfonate.

19. The process of claim 15 wherein the halobenzene sulfonate is selected from the group consisting of bromobenzene sulfonate, chlorobenzene sulfonate and fluorobenzene sulfonate.

* * * * *

US007718414B2

(12) **United States Patent**
Kang et al.

(10) **Patent No.:** US 7,718,414 B2
(45) **Date of Patent:** May 18, 2010

(54) **MICROORGANISM OF *ENTEROBACTERIACAE* GENUS HABORING GENES ASSOCIATED WITH L-CARNITINE BIOSYNTHESIS AND METHOD OF PRODUCING L-CARNITINE USING THE MICROORGANISM**

(75) Inventors: **Whan-Koo Kang**, Daejeon (KR); **Bheong-Uk Lee**, Busan (KR); **Young-Hoon Park**, Seongnam (KR); **Eun-Sung Koh**, Suwon (KR); **Jae-Yeong Ju**, Seongnam (KR); **Jin-Ho Lee**, Yongin (KR); **Hye-Won Kim**, Seongnam (KR); **Hye-Jin Choi**, Seoul (KR)

(73) Assignee: **CJ Cheiljedang Corp.** (KR)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 114 days.

(21) Appl. No.: **11/994,969**

(22) PCT Filed: **Jul. 7, 2006**

(86) PCT No.: **PCT/KR2006/002661**

§ 371 (c)(1),
(2), (4) Date: **Jan. 7, 2008**

(87) PCT Pub. No.: **WO2007/007987**

PCT Pub. Date: **Jan. 18, 2007**

(65) **Prior Publication Data**

US 2008/0171368 A1   Jul. 17, 2008

(30) **Foreign Application Priority Data**

Jul. 7, 2005   (KR) 10-2005-0061142

(51) **Int. Cl.**
C12P 13/00 (2006.01)
C12N 1/20 (2006.01)

(52) **U.S. Cl.** **435/252.3**; 435/252.33; 435/128

(58) **Field of Classification Search** .... None
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,221,869 A	9/1980	Vandecasteele et al.
4,371,618 A	2/1983	Cavazza
4,650,759 A	3/1987	Yokozeki et al.
4,708,936 A	11/1987	Kulla et al.
5,028,538 A	7/1991	Seim et al.

FOREIGN PATENT DOCUMENTS

DE	3123975	6/1981
JP	62118899	5/1987
WO	2007007986 A1	1/2007
WO	WO 2007/011087	*	1/2007

OTHER PUBLICATIONS

Borum et al. JBC 1977, 252 pp. 5651-5655.*
Sambrook et al Molecular cloning A laboratory Manual 2nd ED 1989, pp. 8.46-8.52, 11.2-11.19.*
International Search Report for corresponding International Application No. PCT/KR2006/002661 dated Aug. 25, 2006.
Written Opinion of the International Searching Authority for corresponding International Application No. PCT/KR2006/002661 dated Aug. 25, 2006.
Borum, et al., "Purification of S-adenosylmethionine: epsilon-N-L-Lysine Methyltransferase," The Journal of Biological Chemistry, 252(16), 5651-5655 (1977).
European Search Report for 06769203.8—1212/1904620 dated Sep. 9, 2009.
Galagan, et al., "The genome sequence of filamentous fungus *Neurospora crassa*," Nature, 422(6934), 859-868 (2003).
McClung, et al., "Characterization of the Formate (for) Locus, Which Encodes the Cytosolic Serine Hydroxymethyltransferase of *Neurospora crassa*," Molecular and Cellular Biology, 12(4), 1412-1421 (1992).
Sweigers, et al., "Carnitine biosythesis in *Neurospora crassa*: identification of a cDNA coding for epsilon-N-trimethyllysine hydroxylase and its functional expression in *Saccharomyces cerevisiae*," FEMS Microbiology Letters, 210, 19-23 (2002).
XP-002541633 retrieved from EBI accession No. Q7S7R7 (Dec. 15, 2003).
XP-002541634 retrieved from EBI accession No. Q96UB1 (Jun. 20, 2002).
XP-002541635 retrieved from EBI accession No. P34898 (Feb. 1, 1994).
XP-002541636 retrieved from EBI accession No. Q7RYT1 (Dec. 15, 2003).
XP-002541637 retrieved from EBI accession No. Q7S4C5 (Dec. 15, 2003).

* cited by examiner

Primary Examiner—Delia M Ramirez
Assistant Examiner—Md. Younus Meah
(74) *Attorney, Agent, or Firm*—Cantor Colburn LLP

(57) **ABSTRACT**

Provided is a microorganism that belongs to Enterobacteriacae and a method of producing L-carnitine using the same. The microorganism includes polynucleotide encoding activity of S-adenosylmethionine-6-N-iysine methyltransferase from Neurospora crassa, polynucleotide encoding activity of 6-N-trimethyllysine hydroxylase, polynucleotide encoding activity of 3-hydroxy-6-N-trimethyllysine aldolase, and polynucleotide encoding activity of γ-trimethylaminoaldehyde dehydrogenase and y-butyrobetaine hydroxylase.

5 Claims, 13 Drawing Sheets

FIG. 1

LYSINE

↓ TRIMETHYL TRANSFERASE

ε-N-TRIMETHYLLYSINE

↓ N-TRIMETHYLLYSINE HYDROXYLASE

β-HYDROXY-e-N-TRIMETHYLLYSINE

↓ 3-HYDROXY-6-N-TRIMETHYLLYSINE ALDOLASE

γ-N-TRIMETHYLAMINOBUTYRALDEHYDE

↓ γ-N-TRIMETHYLAMINOBUTYRALDEHYDE
      DEHYDROGENASE

γ-BUTYROBETAINE

↓ γ-BUTYROBETAINE HYDROXYLASE

L-CARNITINE

Appendix A

Page A44

U.S. Patent

May 18, 2010

Sheet 2 of 13

US 7,718,414 B2

FIG. 2
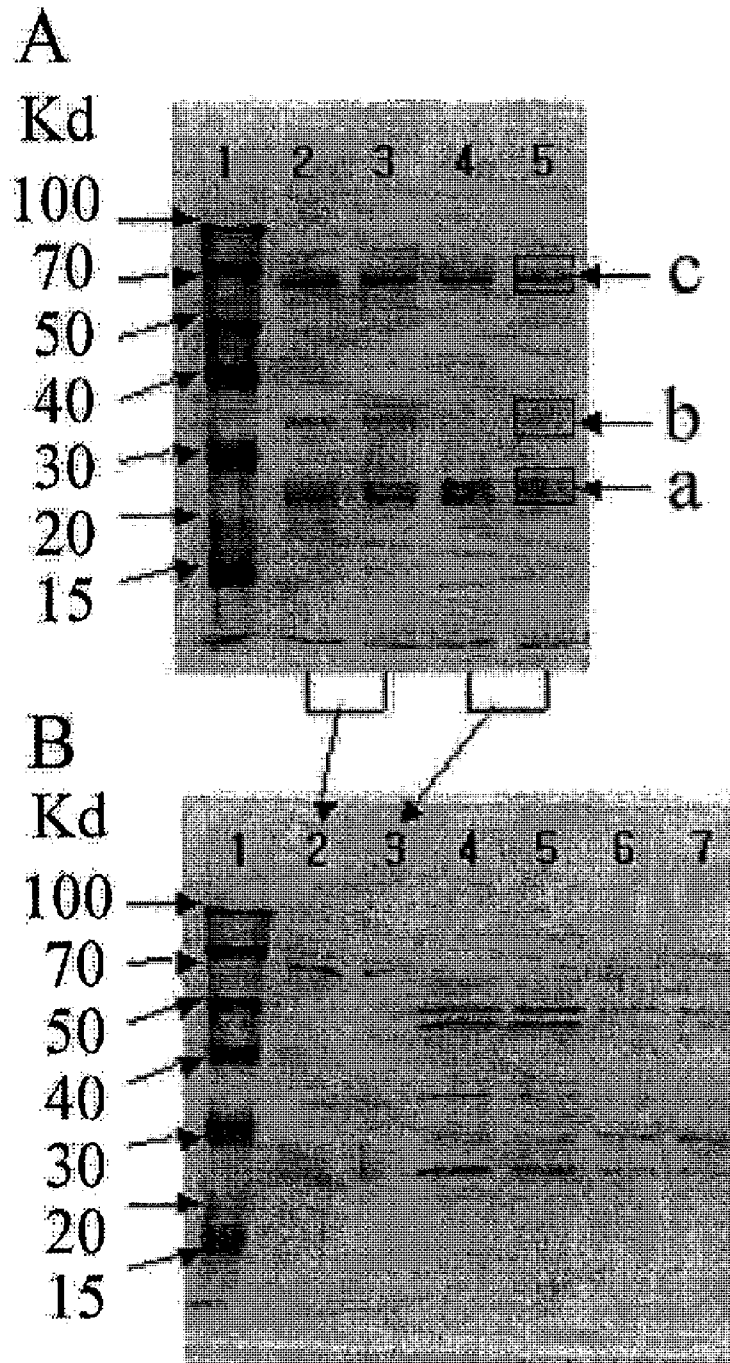
FIG. 3
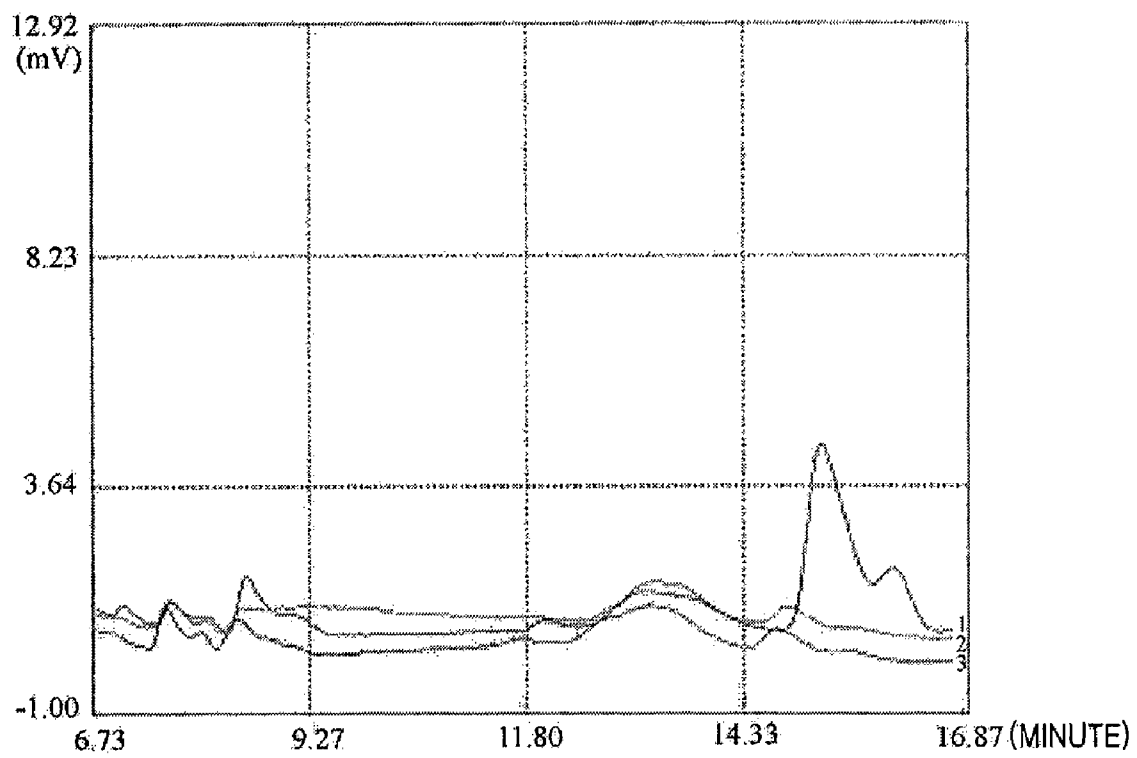
FIG. 4
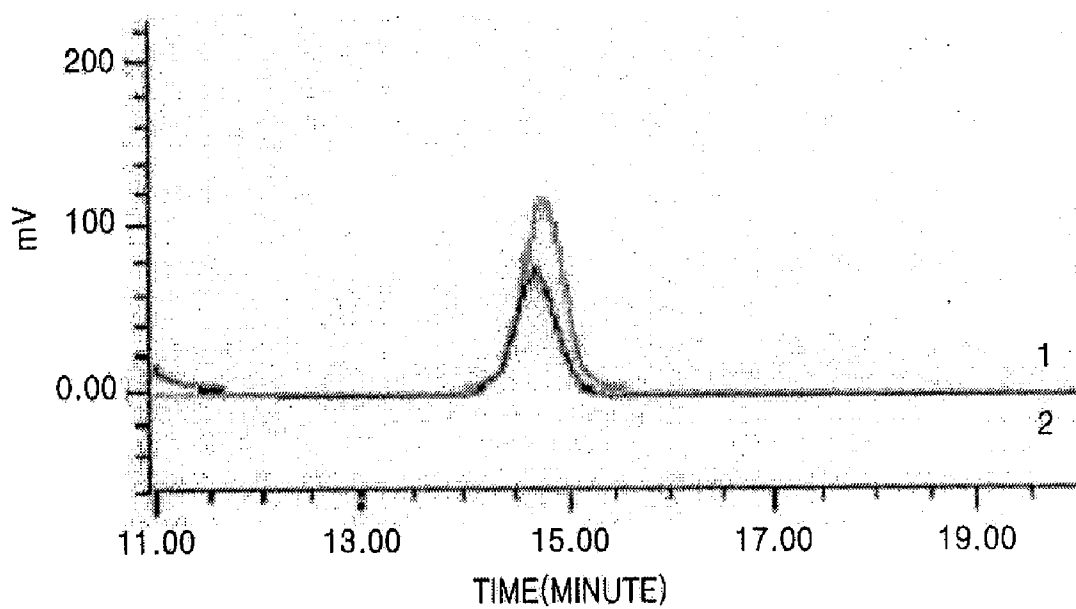
FIG. 5
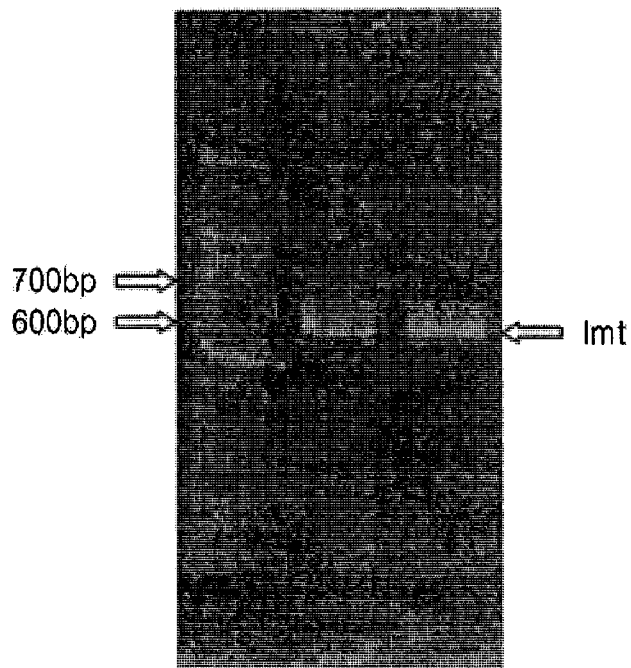
FIG. 6
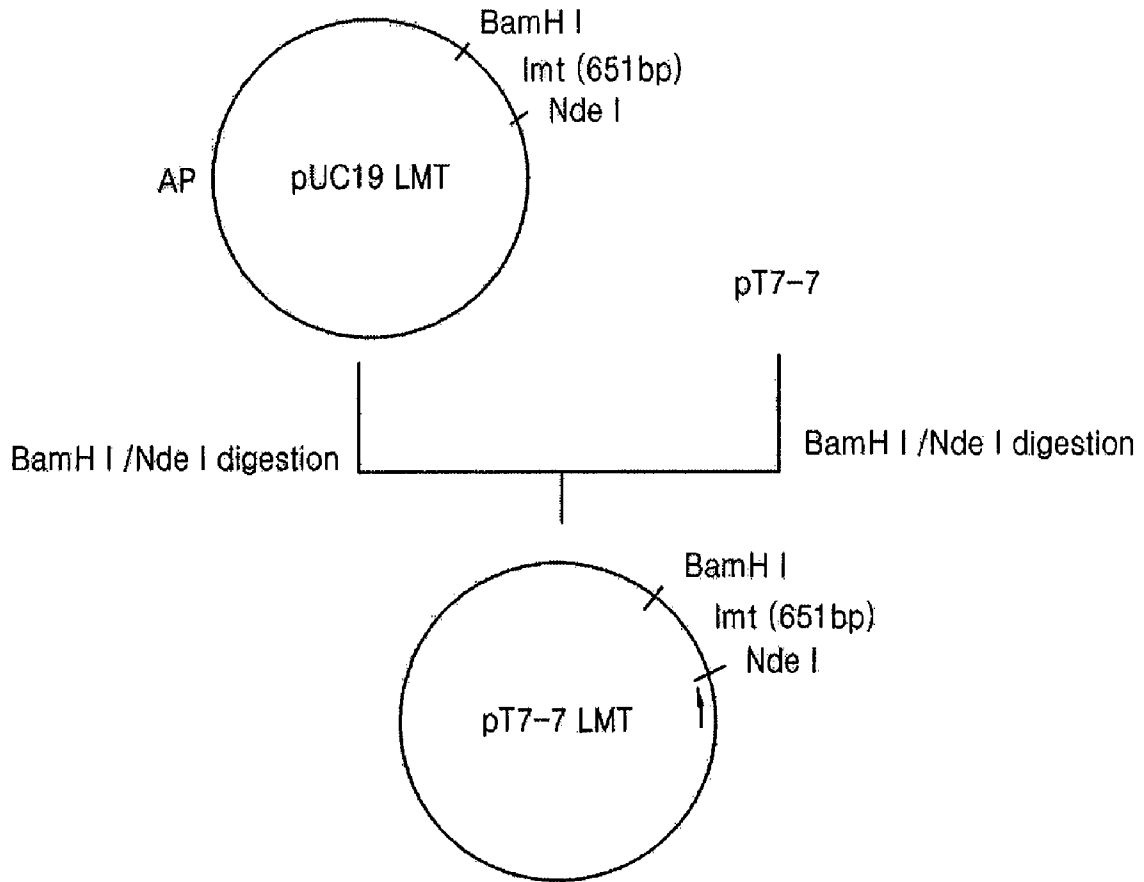
FIG. 7
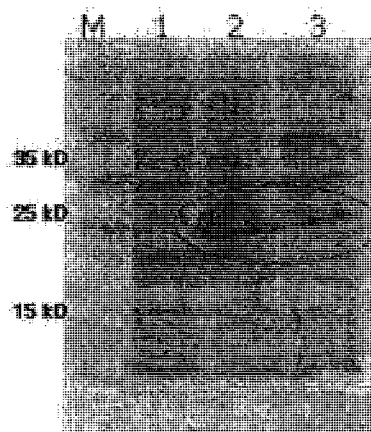
FIG. 8
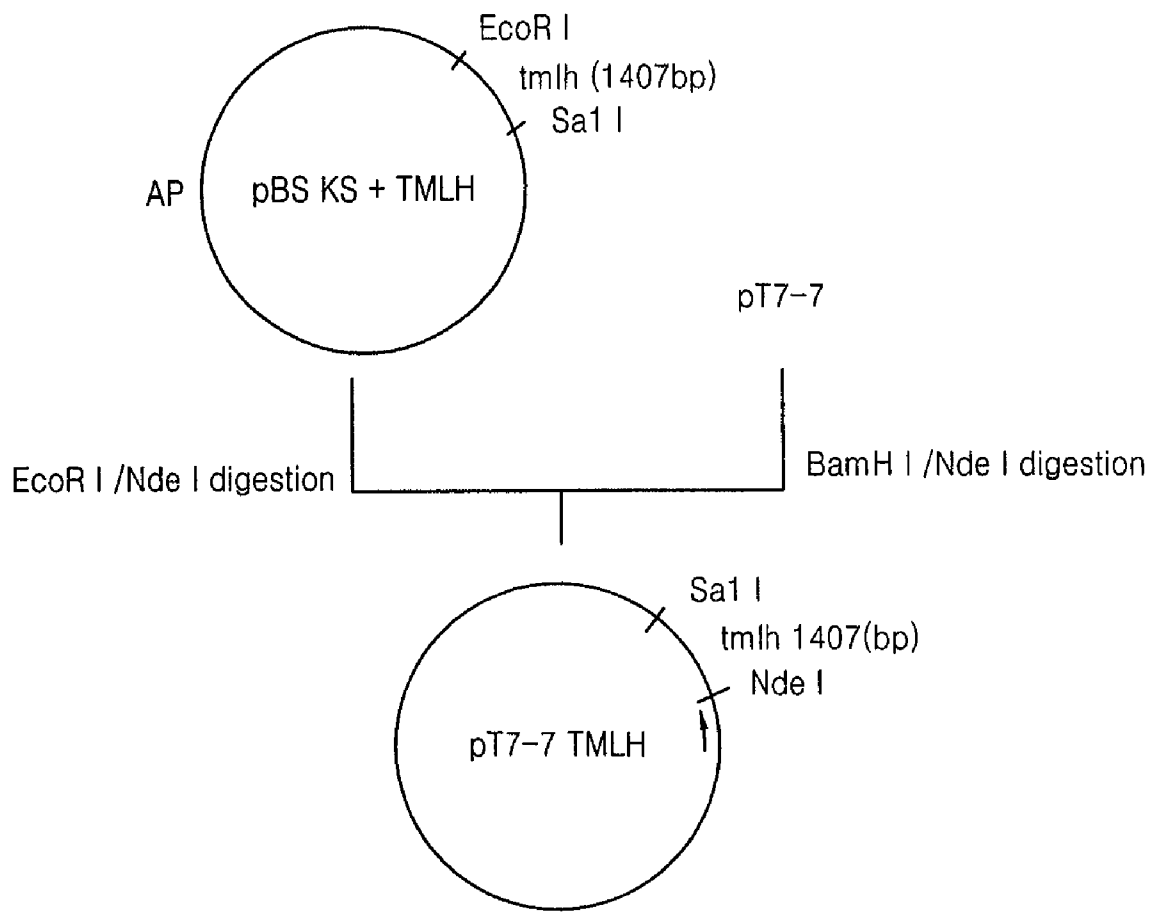
FIG. 9

FIG. 10

FIG. 11

FIG. 12

2,322 bp
2,027 bp

500 bp

1   2   3   4   5
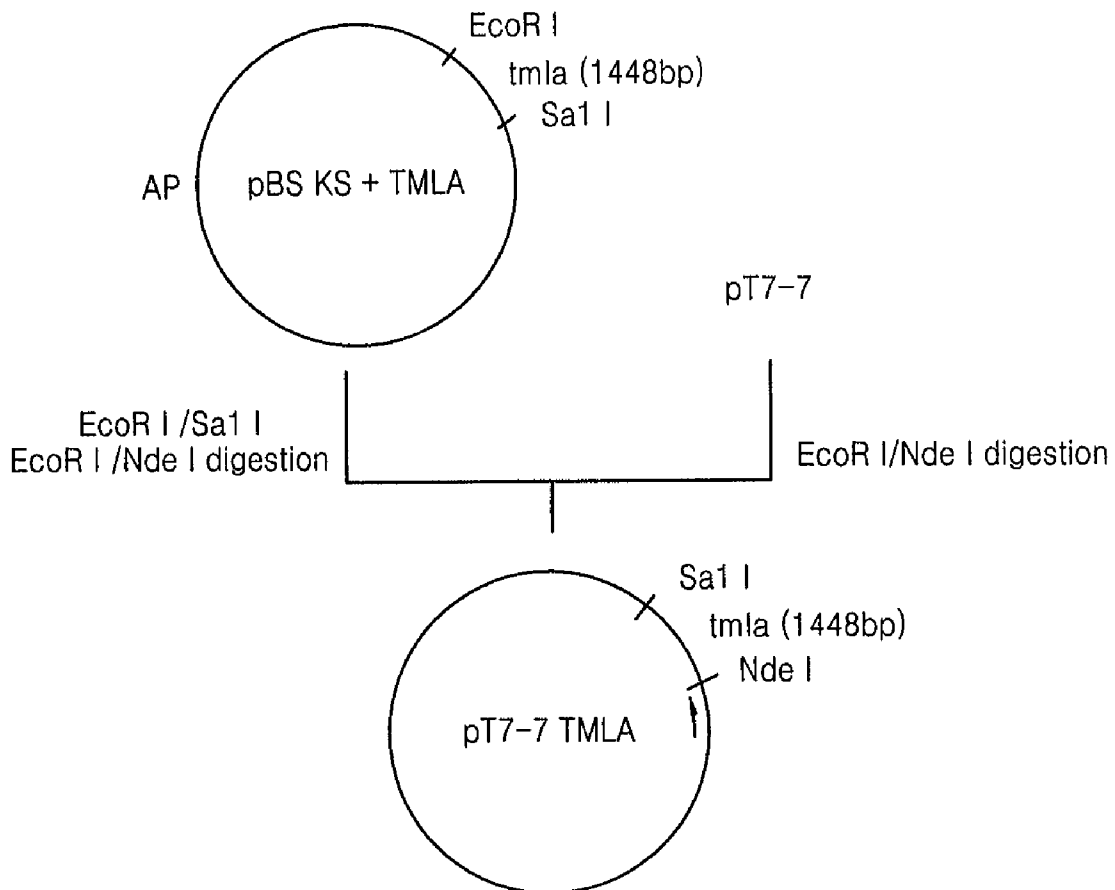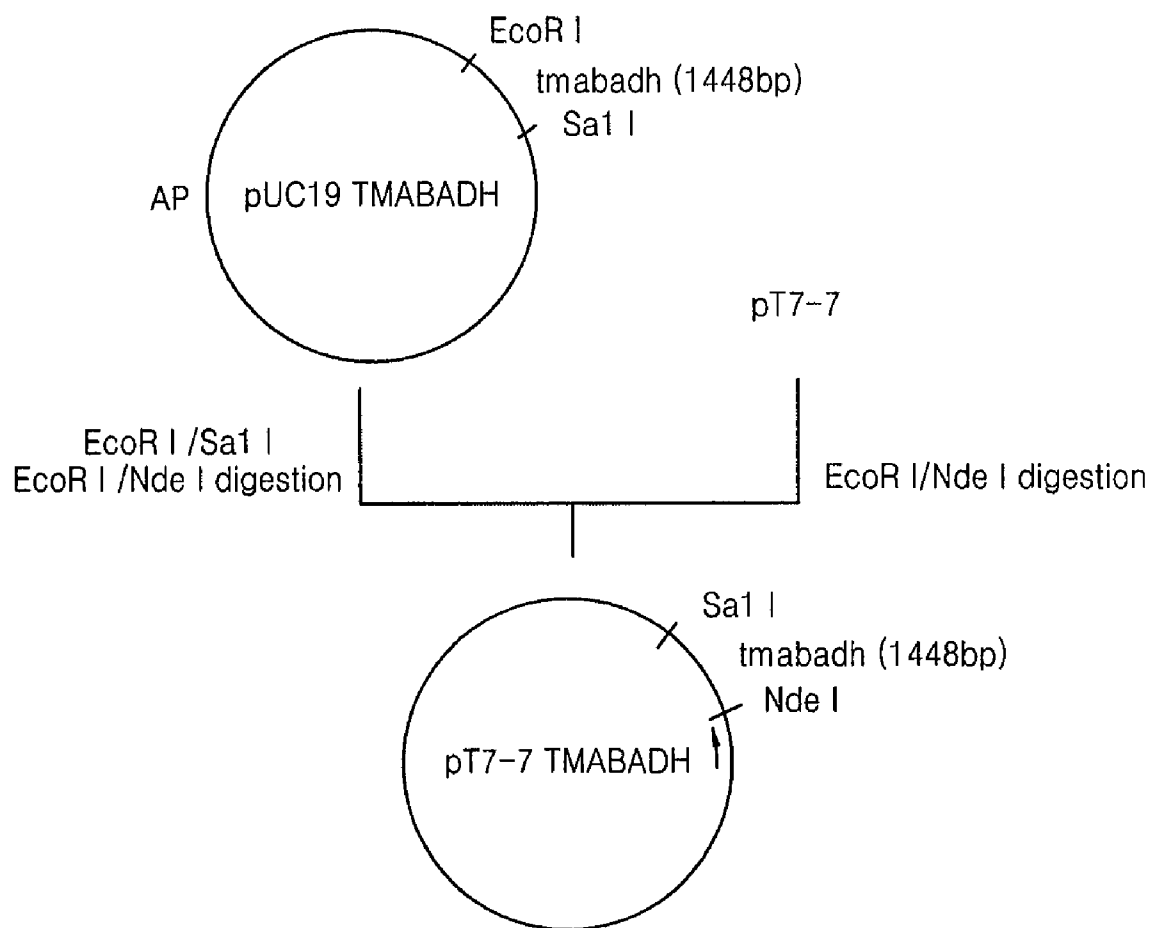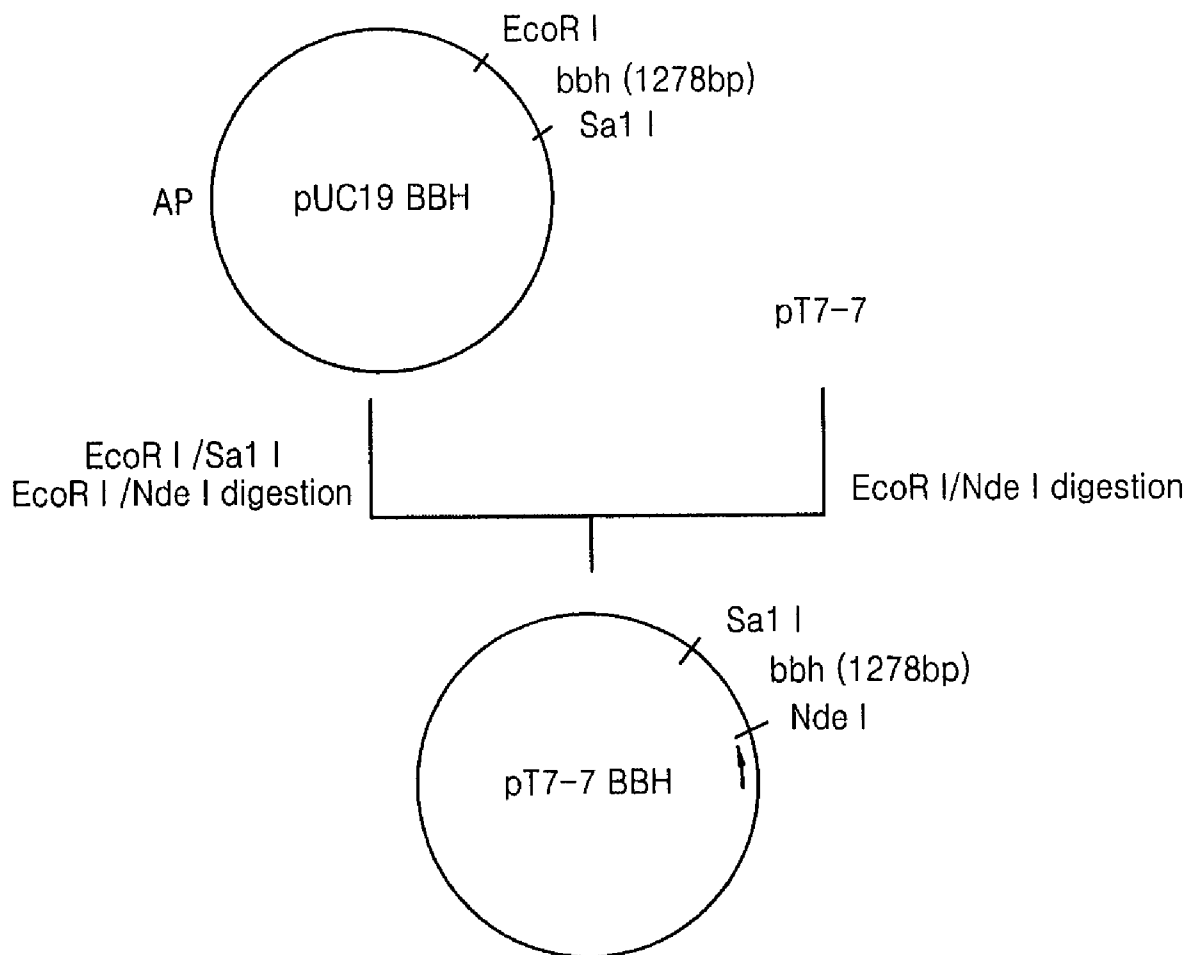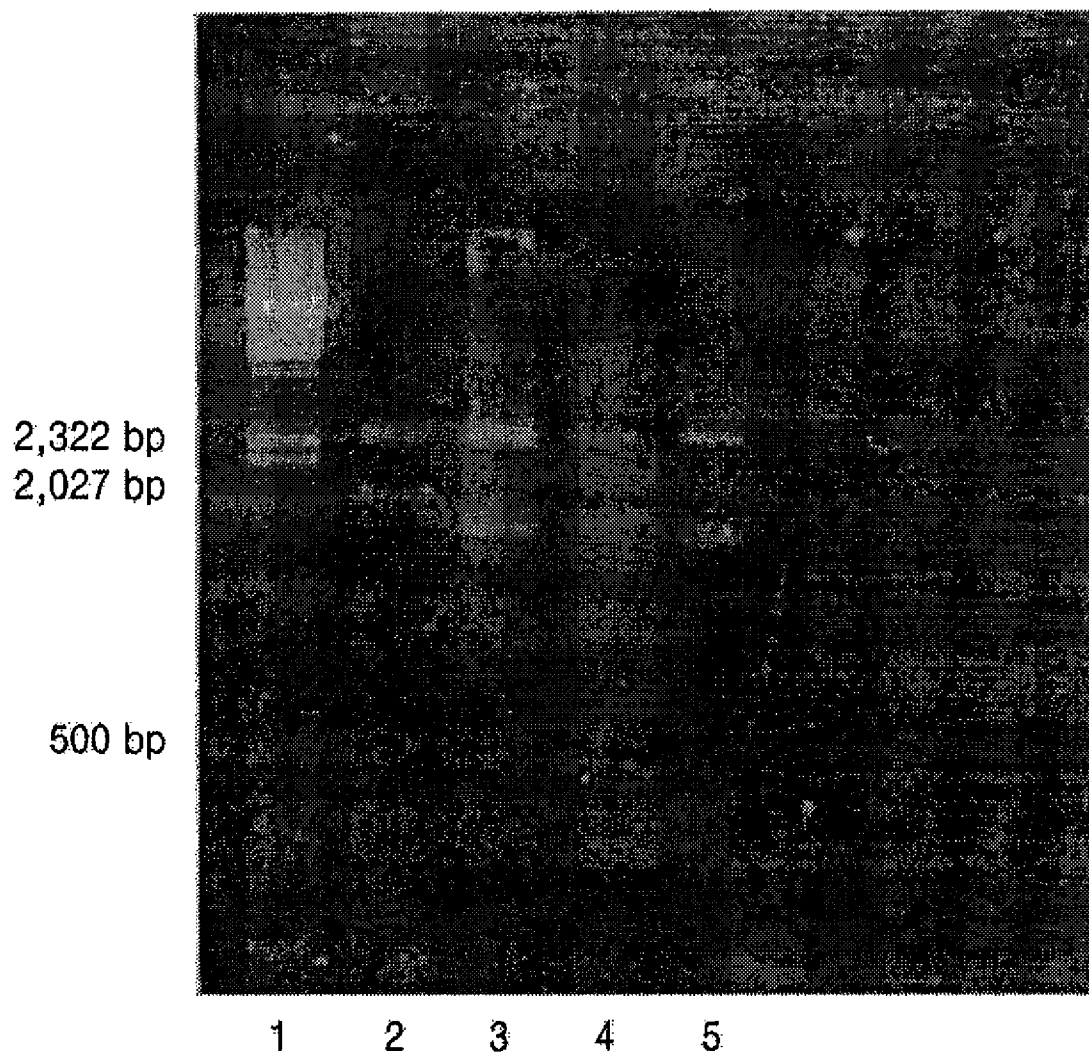
FIG. 13
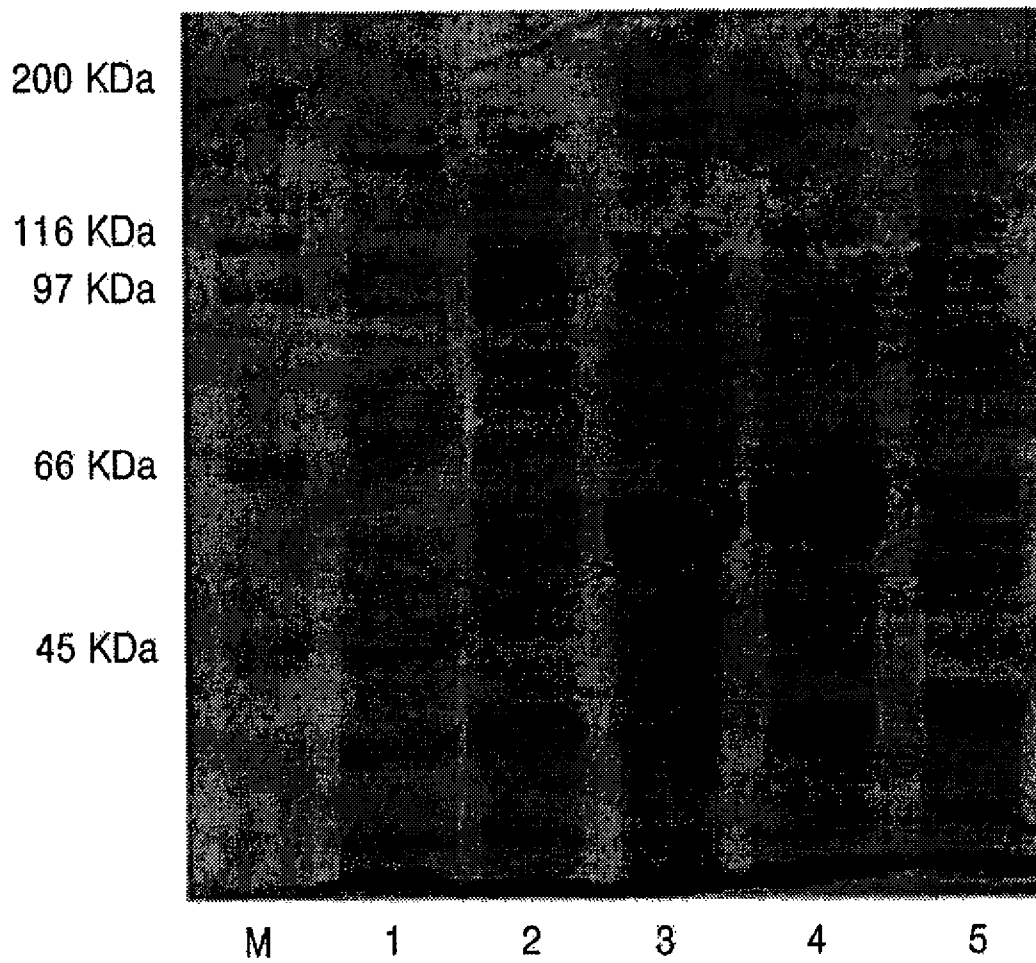
FIG. 14
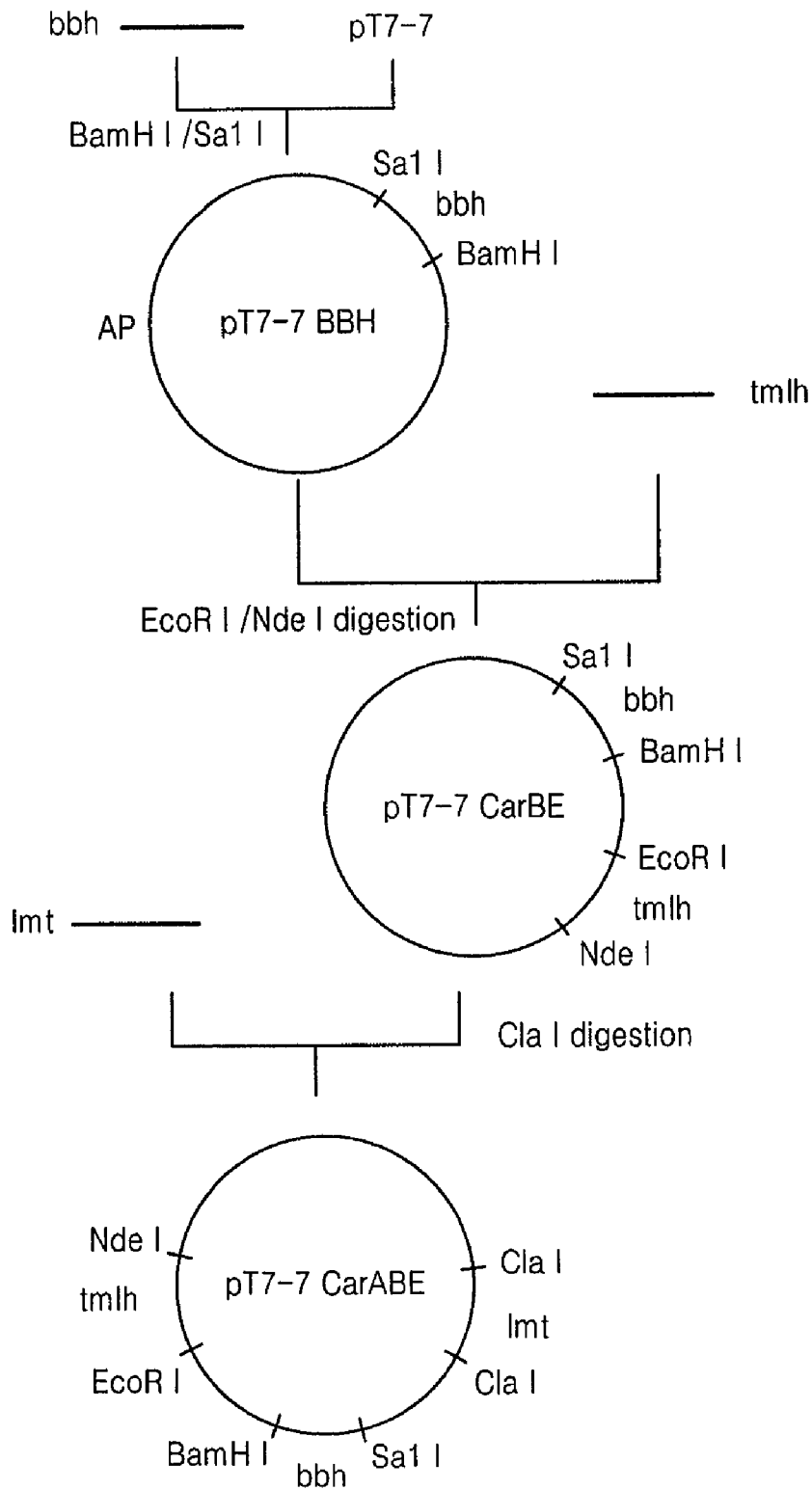
FIG. 15
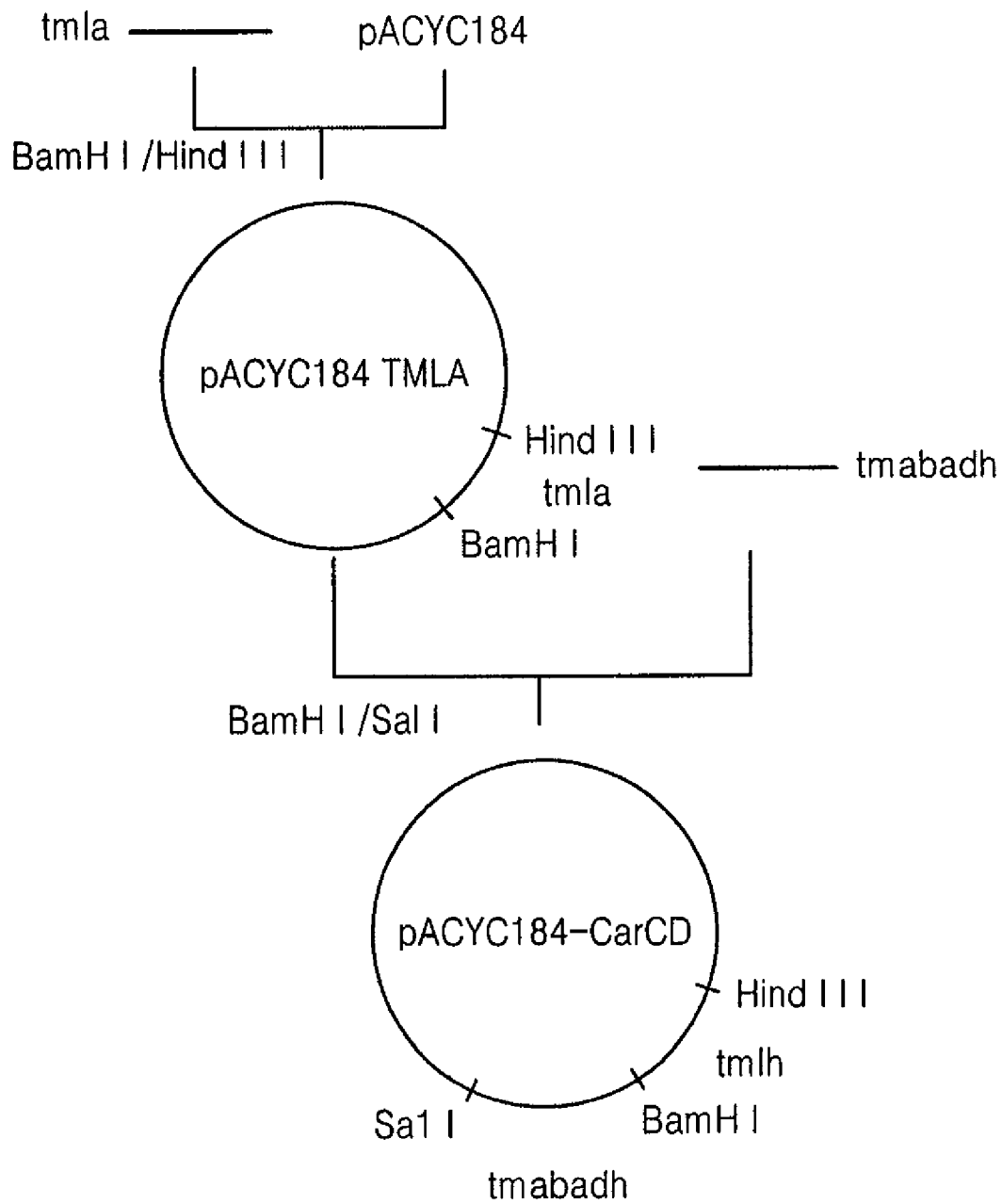
US 7,718,414 B2

1

MICROORGANISM OF *ENTEROBACTERIACAE* GENUS HABORING GENES ASSOCIATED WITH L-CARNITINE BIOSYNTHESIS AND METHOD OF PRODUCING L-CARNITINE USING THE MICROORGANISM

TECHNICAL FIELD

The present invention relates to a microorganism of *Enterobacteriacae* including genes associated with biosynthesis of L-carnitine from *Neurospora crassa* and a method of producing L-carnitine using the microorganism.

BACKGROUND ART

L-carnitine(3-hydroxy-4-trimethylaminobutyrate) generally exists in organisms, and is a zwitterionic compound that carries long-chain activated fatty acids into the mitochondrial matrix across the inner mitochondrial membranes in the mitochondria. It is known that L-carnitine in the human body is synthesized from lysine or protein lysine. Generally, in a mammal, protein lysine is used as a precursor of L-carnitine biosynthesis, but free lysine is used in *Neurospora crassa*. In L-carnitine biosynthesis, ε-N,N,N-trimethyllysine, ε-N,N,N-trimethyl-β-hydroxyllysine, a N,N,N-trimethylamino butyraldehyde intermediate, and γ-butyrobetaine are produced, and γ-butyrobetaine is hydroxylated by γ-butyrobetaine hydroxylase to be L-carnitine. FIG. **1** is a flowchart illustrating a supposed biosynthetic pathway of L-carnitine in *Neurospora crassa*.

L-carnitine can be produced by a chemical synthesis method, a semi-synthesis method using an enzyme reaction, and a method of using a microorganism. However, when the chemical synthesis method is used, there is a problem in that a racemate of DL-carnitine is obtained, and thus this has to be separated. As an example of the semi-synthesis method using an enzyme reaction, U.S. Pat. No. 4,221,869 discloses a method of producing L-carnitine from dehydrocarnitine with carnitine dehydrogenase (EC 1.1.1.108) that uses NAD as a coenzyme. However, dehydrocarnitine is very unstable, and spontaneously decomposes into acetonyl trimethylammonium and carbon dioxide. In addition, DE Patent No. DE-OS-3123975 discloses a method of producing L-carnitine from γ-butyrobetaine with γ-butyrobetaine hydroxylase (EC 1.14.11.1) separated from *Neurospora crassa*. However, there is a disadvantage in that α-ketoglutarate and a reductant (that is, ascorbate) should be added to a reactant during hydroxylation.

As a method of producing L-carnitine using a microorganism, for example, U.S. Pat. No. 5,028,538 discloses a method of collecting L-carnitine from the culture obtained after *E. coli* 044 K 74 is cultured in a medium containing crotonobetaine (4-N,N,N-triethylamino crotonic acid). In addition, U.S. Pat. No. 4,708,936 discloses a method of producing L-carnitine culturing *Achromobacter xylosoxydans* DSM 3225 (HK 1331b) in a medium containing crotonobetain and/or γ-butyrobetaine. However, there are disadvantages in that a precursor of L-carnitine biosynthesis, such as crotonobetain, or a compound that is not an intermediate should be used, and production efficiency of L-carnitine is not high. Therefore, there still remains a need for improving production efficiency in a method of producing L-carnitine using a microorganism.

The inventors of the present invention have tried to produce a microorganism of L-carnitine that uses an inexpensive precursor and also has a high production efficiency, and have found that genes associated with L-carnitine biosynthesis

2

derived from *Neurospora crassa* were well expressed in a microorganism of *Enterobacteriacae*, thereby completing the present invention.

DESCRIPTION OF THE DRAWINGS

FIG. **1** is a flowchart illustrating a supposed biosynthesis pathway of L-carnitine in *Neurospora crassa*.

FIG. **2** is a diagram illustrating results of a natural or SDS-PAGE analysis of an eluting solution obtained after a culture of *Neurospora crassa* is lysed and DEAE column chromatography is performed for the lysed material.

FIG. **3** is a graph illustrating results of measuring trimethyllysine through HPLC after protein bands of a, b and c of FIG. **2** is reacted with lysine and S-adenosylmethionine.

FIG. **4** is a graph showing results of analyzing a sample obtained by reacting with the band of a and a trimethyllysine standard through HPLC.

FIG. **5** is a diagram showing electrophoresis results of a LMT gene amplified by PCR.

FIG. **6** illustrates a producing process of pT7-7 LMT.

FIG. **7** is a diagram showing results of a SDS-PAGE analysis of supernatant of the lysed bacteria obtained when *E. coli* containing S-adenosysmethionine-6-N-lysine-methyltransferase from *Neurospora crassa* is cultured in the presence of IPTG, and bacteria obtained therefrom is lysed.

FIG. **8** illustrates a producing process of pT7-7 TMLH.

FIG. **9** illustrates a producing process of pT7-7 TMLA.

FIG. **10** illustrates a producing process of pT7-7TMA-BADH.

FIG. **11** illustrates a producing process of pT7-7 BBH.

FIG. **12** is a photo showing electrophoresis results of each gene that is inserted onto pT7-7TMLH, pT7-7TMLA, pT7-7TMABADH and pT7-7BBH. In FIG. **12**, lane 1 represents a marker, lane 2 represents pT7-7TMLH, lane 3 represents pT7-7TMLA, lane 4 represents pT7-7TMABADH and lane 5 represents pT7-7BBH.

FIG. **13** is a photo showing SDS-PAGE results of a crude extract obtained from the culture of *E. coli* BL21 (DE3) that is transformed with pT7-7TMLH, pT7-7TMLA, pT7-7TMABADH and pT7-7BBH, respectively. In FIG. **13**, lane 1 represents a marker, lane 2 represents a negative control group, lane 3 represents pT7-7TMLH (52 KDa), lane 4 represents pT7-7TMLA (53 KDa), lane 5 represents pT7-7TMABADH (55 KDa) and lane 6 represents pT7-7BBH (49 KDa).

FIG. **14** illustrates a producing process of pT7-7CarABE.

FIG. **15** illustrates a producing process of pACYC184CarCD.

DETAILED DESCRIPTION OF THE INVENTION

Technical Problem

The present invention provides a microorganism that can produce L-carnitine at high efficiency

The present invention also provides a method of producing L-carnitine using the microorganism.

Technical Solution

According to an aspect of the present invention, there is provided a microorganism that belongs to the *Enterobacteriacae*, the microorganism comprising: polynucleotide encoding activity of S-adenosylmethionine-6-N-lysine methyltransferase (LMT) from *Neurospora crassa*; polynucleotide encoding activity of 3-hydroxy-6-N-trimethyllysine

US 7,718,414 B2

3

aldolase (TMLA); polynucleotide encoding activity of N-tri-methyllysine hydroxylase (TMLH); polynucleotide encoding activity of γ-trimethylaminoaldehyde dehydrogenase (TMABADH); and polynucleotide encoding activity of γ-butyrobetaine hydroxylase (BBH).

The microorganism according to the present invention can be any one including polynucleotide that encodes the five kinds of proteins. Preferably, the microorganism is *Escherichia coli*, and more preferably *Escherichia coli* (Accession number: KCCM-10638)

Polynucleotide that independently encodes five kinds of proteins, that is, LMT, TMLH, TMLA, TMABADH and BBH according to the present invention, can be employed in a microorganism through a vector or by itself. When polynucleotide that independently encodes the five kinds of proteins is employed in a microorganism through a vector, the polynucleotide encoding the five kinds of proteins may be inserted to a single vector and then employed, or may be inserted into at least one vector and then employed. In the present invention, the term "vector" is well-known to those skilled in the art. The vector generally denotes a nucleic acid construct that is used in the introduction of the nucleic acid into a cell. This nucleic acid construct may be a nucleic acid construct derived from a plasmid or a virus genome.

Polynucleotide encoding S-adenosylmethionine-6-N-lysine methyltransferase (LMT) from *Neurospora crassa* according to an embodiment of the present invention encodes S-adenosylmethionine lysinemethyltransferase from *Neurospora crassa*. It is considered that S-adenosylmethionine lysinemethyltransferase catalyzes a reaction of converting lysine into 6-N-trimethyllysine by attaching a methyl group to lysine in a cell of *Neurospora crassa*, but the scope of the present invention is not limited to this specific action mechanism. The polynucleotide encoding S-adenosylmethionine lysinemethyltransferase is preferably polynucleotide encoding an amino acid sequence of SEQ ID NO: 11, and more preferably polynucleotide having a nucleotide sequence of SEQ ID NO: 16.

Polynucleotide encoding N-trimethyllysine hydroxylase (TMLH) from *Neurospora crassa* according to an embodiment of the present invention encodes N-trimethyllysine hydroxylase (TMLH) from *Neurospora crassa*. It is considered that N-trimethyllysine hydroxylase (TMLH) catalyzes a reaction of converting N-trimethyllysine into β-hydroxy-ε-N-trimethyllysine in a cell of *Neurospora crassa*, but the scope of the present invention is not limited to this specific action mechanism. The polynucleotide encoding N-trimethyllysine hydroxylase (TMLH) is preferably polynucleotide encoding an amino acid sequence of SEQ ID NO: 12, and more preferably polynucleotide having a nucleotide sequence of SEQ ID NO: 17.

Polynucleotide encoding 3-hydroxy-6-N-trimethyllysine aldolase (TMLA) from *Neurospora crassa* according to an embodiment of the present invention encodes 3-hydroxy-6-N-trimethyllysine aldolase (TMLA) from *Neurospora crassa*. It is considered that 3-hydroxy-6-N-trimethyllysine aldolase (TMLA) catalyzes a reaction of converting β-hydroxy-ε-N-trimethyllysine into γ-N-trimethylaminobutyraldehyde in a cell of *Neurospora crassa*, but the scope of the present invention is not limited to this specific action mechanism. The polynucleotide encoding 3-hydroxy-6-N-trimethyllysine aldolase (TMLA) is preferably polynucleotide encoding an amino acid sequence of SEQ ID NO: 13, and more preferably polynucleotide having a nucleotide sequence of SEQ ID NO: 18.

Polynucleotide encoding activity of γ-trimethylaminoaldehyde dehydrogenase (TMABADH) from *Neurospora crassa*

4

according to an embodiment of the present invention encodes activity of γ-trimethylaminoaldehyde dehydrogenase (TMABADH) from *Neurospora crassa*. It is considered that γ-trimethylaminoaldehyde dehydrogenase (TMABADH) catalyzes a reaction of converting γ-N-trimethylaminobutyraldehyde into γ-butyrobetaine in a cell of *Neurospora crassa*, but the scope of the present invention is not limited to this specific action mechanism. The polynucleotide encoding γ-trimethylaminoaldehyde dehydrogenase (TMABADH) is preferably polynucleotide encoding an amino acid sequence of SEQ ID NO: 14, and more preferably polynucleotide having a nucleotide sequence of SEQ ID NO: 19.

Polynucleotide encoding activity of γ-butyrobetaine hydroxylase (BBH) from *Neurospora crassa* according to an embodiment of the present invention encodes γ-butyrobetaine hydroxylase (BBH) from *Neurospora crassa*. It is considered that γ-butyrobetaine hydroxylase (BBH) can catalyze a reaction of converting γ-butyrobetaine into L-carnitine in a cell of *Neurospora crassa*, but the scope of the present invention is not limited to this specific action mechanism. The polynucleotide encoding γ-butyrobetaine hydroxylase (BBH) is preferably polynucleotide encoding an amino acid sequence of SEQ ID NO: 15, and more preferably polynucleotide having a nucleotide sequence of SEQ ID NO: 20.

According to another aspect of the present invention, there is provided a method of producing L-carnitine, the method comprising: culturing a microorganism according to the present invention in the presence of a substrate selected from the group consisting of L-lysine, N-trimethyllysine, β-hydroxy-N-trimethyllysine, γ-N-trimethylaminobutyraldehyde, γ-butyrobetaine and mixtures thereof to produce L-carnitine in the culture.

In the method of producing L-carnitine according to the present invention, a concentration of the substrate selected from the group consisting of L-lysine, N-trimethyllysine, β-hydroxy-N-trimethyllysine, γ-N-trimethylaminobutyraldehyde, γ-butyrobetaine and mixtures thereof is preferably 0.1-10 weight % based on the weight of a culture medium, but the present invention is not particularly limited to this range.

In the method according to the present invention, a process of collecting L-carnitine from a culture is well known to those skilled in the art. Examples of such a process include, but are not limited to, ultrafiltration, centrifugal separation, and a method of collecting L-carnitine by recrystallizing the resulting product after cells are separated from a culture such as decantation, and cation exchange chromatography or electrodialysis is performed for the supernatant obtained therefrom.

Advantageous Effects

The microorganism according to the present invention has a good ability of producing L-carnitine so that it can be usefully employed in a method of producing L-carnitine through fermentation.

In the method of producing L-carnitine according to the present invention, L-carnitine can be produced with high efficiency using a microorganism that belongs to the *Enterobacteriacae*.

Best Mode

Hereinafter, the present invention will be described in further detail with reference to the following examples. These examples are for illustrative purposes only and are not intended to limit the scope of the present invention.

US 7,718,414 B2

5

EXAMPLE

Polynucleotide encoding five kinds of proteins associated with L-carnitine biosynthesis from L-lysine in *Neurospora crassa*, and a nucleic acid construct including the same were produced. Next, *E. coli* was transformed with the nucleic acid construct, and the transformed *E. coli* was cultured in a medium including an intermediate product obtained throughout a L-carnitine production pathway to produce L-carnitine and collect L-carnitine.

Example 1

Isolation of Polynucleotide Encoding LMT, TMLH, TMLA, TMABADH and BBH from *Neurospora crassa*

Polynucleotide encoding LMT, TMLH, TMLA, TMABADH and BBH from *Neurospora crassa* was isolated and cloned, and a base sequence thereof was analyzed.

(1) Production of cDNA Library of *Neurospora crassa*

The total mRNA was isolated from a culture including fungal body of *Neurospora crassa* (including a sporophyte) and reverse transcribed using poly T as a primer, and then PCR was performed to amplify cDNA. The amplified cDNA was digested with EcoRI and XhoI, and then the digested cDNA was inserted to a site of EcoRI and XhoI of λAD5 cloning vector to produce cDNA library from *Neurospora crassa*.

Next, the cDNA library was infected into *E. coli* BNN322, and then the infected *E. coli* BNN322 was cultured and amplified. First, *E. coli* BNN322 was cultured overnight in a LB medium including 50 μg/ml of kanamycin and 0.2% of maltose. Then, centrifugal separation was performed for the culture obtained therefrom, a supernatant of the resulting product was then removed, and afterwards cell pellets were resuspended in a solution of 1 ml of 10 mM MgSO₄. The suspension obtained from the resulting product and 5×10^7 PFU of the λ cDNA library was incubated at 30° C. for 30 minutes without shaking, and 2 ml of a LB medium was further added to the culture, and then the resulting culture was shaked in a shaking incubator at 30° C. for 1 hour. The cultured cells were streaked on a LB medium plate including ampicillin (75 μg/ml) and incubated at 37° C. for 8 hours. cDNA library pool was separated from colony of the plate using a Wizard kit. λ including the separated cDNA library pool was used as a template to amplify polynucleotide encoding LMT, TMLH, TMLA, TMABADH and BBH.

(2) Amplification and Cloning of Polynucleotide Encoding LMT (an LMT Gene) and Confirmation of LMT Production

(a) Isolation of an LMT Gene from *Neurospora crassa* and Confirmation of the Functional Expression of the Gene.

Neurospora crassa was cultured and cells were collected. Then, the cells were lysed using 1 M of potassium phosphate buffer pH 7.4 including 2 mM of DTT and 0.2 mM of EDTA, and then protein was extracted. Ammonium sulfate was slowly added to the obtained supernatant to reach a final saturated concentration of 50% to precipitate protein, and then a small amount of 0.1 M of potassium phosphate buffer pH 7.4 was added to the protein precipitated by centrifugation. The resulting solution was desalted using a T1 dialysis membrane and the desalted sample was purified using a DEAE column. At this time, pooling was performed using 0.1 M of potassium phosphate buffer pH 7.4 as a washing buffer and 0.1 M of potassium phosphate buffer pH 7.4 including 0.3 M of NaCl as an eluting buffer. Thereafter, the pooled sample was desalted using a T1 dialysis membrane. The desalted

6

sample was purified by using a CM column. 0.1 M of potassium phosphate buffer pH 7.4 was used as a washing buffer of the column, and a sample that was not adsorbed onto the column and flown out of the column was all pooled.

The protein sample was loaded on the DEAE column again, and then using 0.1 M of potassium phosphate buffer pH 7.4, a concentration gradient elution was performed to reach a NaCl concentration of 0-0.3 M. A protein analysis was performed for the purified sample using natural-PAGE and SDS-PAGE.

FIG. **2** is a diagram illustrating results of a natural-PAGE or SDS-PAGE analysis of an eluting solution obtained after a culture of *Neurospora crassa* was lysed and DEAE column chromatography was performed for the lysed material. In FIG. **2**, lane 1 represents a marker, lane 2 and 3 represent results of a natural-PAGE analysis of DEAE eluting peak 2, and lane 4 and 5 represent results of a natural-PAGE analysis of DEAE eluting peak 3. In FIG. **2**B, lane 1 represents a marker, lane 2 represents a result of a natural-PAGE analysis of DEAE eluting peak 2, lane 3 represents a result of a natural-PAGE analysis of DEAE eluting peak 3, lane 4 and 5 represent results of a SDS-PAGE analysis of DEAE eluting peak 2, and lane 6 and 7 represent results of a SDS-PAGE analysis of DEAE eluting peak 3.

From the results of FIG. **2**, bands of a, b and c were chosen as a LMT candidate protein, and activity of each protein was measured. First, a gel corresponding to each band was cut out, and then the gel was crushed by a homogenizer. Then, 5 ml of 1 g/L lysine (final concentration 500 mg/L) and 2 ml of 1 g/L methyl donor, S-adenosylmethionine (final concentration 200 mg/L) were added thereto and the resulting product was slowly stirred at 28° C. for 24 hours to react, and then a trimethyllysine peak was analyzed using HPLC.

FIG. **3** is a graph representing results of measuring trimethyllysine through HPLC after protein bands of a, b and c are reacted with lysine and S-adenosylmethionine. As illustrated in FIG. **3**, in a sample reacted with the band of a, a peak considered as trimethyllysine was confirmed around at a retention time of 15 minutes. In FIGS. **3**, **1**, **2** and **3** represent results corresponding to each of the bands a, b and c. To exactly confirm the bands, a sample obtained by reacting with the band a was compared with a trimethyllysine standard.

FIG. **4** is a graph representing results of analyzing a sample obtained by reacting with the protein band of a and trimethyllysine standard through HPLC. As illustrated in FIG. **4**, a peak time, a time at which a voltage has the highest value, of the band a is exactly consistent with the standard trimethyllysine sample. Therefore, it is confirmed that the band a includes S-adonosylmethionine-6-N-lysine-methyltransferase, LMT. In FIGS. **4**, **1** and **2** refer to results corresponding to each standard and the band a. Each graph of FIGS. **2** and **3** is a graph into which separate HPLC graphs are integrated.

Next, an N-terminal sequence was analyzed to obtain an amino acid sequence of the LMT protein. First, a protein in SDS-PAGE gel was transferred to a PVDF membrane, and then protein bands were cut out to analyze the N-terminal sequence by Edman method. In particular, phenylisothiocyanate (PTC) was reacted with peptide at pH 8-9 and room temperature, and thus the PTC-peptide in which N-terminal was thiocarbamylated was obtained. Thereafter, the PTC-peptide was reacted under acidic condition to separate only N-terminal amino acid therefrom. The separated amino acid was extracted with ethylacetate, identified with HPLC, and analyzed. As a result, it was confirmed that the N-terminal sequence was AFGKL (SEQ ID NO: 21). Like this, a search for entire genome sequence of known *Neurospora crassa* was conducted based on the confirmed N-terminal amino acid

US 7,718,414 B2

7   8

sequence. As a result, a protein and a gene having an amino acid sequence that is consistent with the N-terminal sequence of the LMT and a nucleotide sequence were confirmed.

(b) Expression Vector Including a LMT Gene and Production of Microorganism

The cultured *Neurospora crassa* was collected and lysed using a liquid nitrogen, and then RNA was purified using a RNA purification kit. A primer of SEQ ID NOS:1 and 2 was produced using information on an amino acid and base sequence of LMT confirmed in (a), and then, using the cDNA library produced in (1), a gene of S-adonosylmethionine-6-N-lysine-methyltransferase was amplified through PCR that uses the primer set as a primer (FIG. **5**). FIG. **5** is a diagram showing electrophoresis results of an LMT gene amplified by PCR.

The obtained PCR product and pT7-7 vector were digested with NdeI and BamHI, respectively, and connected to each other with T4 DNA ligase to produce pT7-7 LMT vector (FIG. **6**). FIG. **6** illustrates a producing process of pT7-7 LMT. *E. coli* BL21 DE3 was transformed with pT7-7 LMT vector using electroporation. 40 μl of *E. coli* BL21 DE3 and 1 μl of pT7-7-LMT vector were mixed, placed in cold cuvettes with a 2 mm gap, and transformed by electroporation under conditions of 2.5 kV, 200Ω, and 25 μF. The obtained transformant was streaked on a solid plating medium containing ampicillin, and then a plasmid was purified from the transformant selected therefrom and digested with NdeI and BamHI. As a result, the introduction of pT7-7LMT into the plasmid was confirmed by confirming the size of the inserted gene and the plasmid; this was referred to as BL21 (DE3)/pT7-7LMT.

(c) Expression of S-adenosylmethionine-6-N-Lysine-Methyltransferase in *E. coli* and Production of Trimethyllysine from Lysine

BL21 (DE3)/pT7-7LMT was cultured to OD_{600} 0.5 in a LB medium, and then cultured for more 4 hours after 1 mM of IPTG was added therein. Centrifugation was performed for the culture, and cells were collected and lysed using an ultrasonic wave. By performing SDS-PAGE for the cell lysate, about 25 kD of S-adenosylmethionine-6-N-lysine-methyltransferase was confirmed (FIG. **7**). FIG. **7** is a diagram representing results of SDS-PAGE analysis of supernatant obtained when *E. coli* containing S-adenosylmethionine-6-N-lysine-methyltransferase from *Neurospora crassa* was cultured in the presence of IPTG and a microorganism obtained therefrom was lysed. In FIG. **7**, lane M refers to a marker, lane 1 refers to a negative control group, lanes 2 and 3 refer to a cell lysate, and a circled part in lane 2 and 3 refers to a band at 25 kD position corresponding to LMT.

E. coli BL21(DE3)/pT7-7LMT was cultured to OD_{600} 0.6 in a 250 ml flask equipped with a baffle in which an LB medium including 50 ml of ampicillin was placed, and then cultured at 28° C. for over 8 hours to form an exact tertiary structure of an enzyme and prevent an inclusion body from forming after 1 mM of IPTG was added therein. During culturing, 500 mg/L of L-lysine and 200 mg/L of S—adenosylmethionine were added as a reaction solution, and a trimethyllysine content of a culture solution was measured. The results are shown in Table 1.

Trimethyllysine was measured by HPLC under the following conditions. SUPELCOSIL LC-DABS from Supelco was used as a column. A buffer was made such that 0.1% of trifluoroacetic acid (TFA) was added to a buffer in which a distilled water and acetonitrile were mixed in a ratio of 2:8, and B buffer was made such that 0.1% of TFA was added to a buffer in which a distilled water and acetonitrile were mixed

in a ratio of 2:8. Trimethyllysine was analyzed using a linear concentration gradient method, maintaining a flow velocity of 0.8 ml/min.

TABLE 1

Assayed materials	Trimethyllysine (μg/ml)
E. coli BL21(DE3)/pT7-7 (IPTG induction) + 500 mg/L lysine + 200 mg/L Ado-Met	0.0
E. coli BL21(DE3)/pT7-7 LMT(IPTG induction) + 500 mg/L lysine + 200 mg/L Ado-Met	20.0

As shown in Table 1, it was confirmed that a gene of S-adenosylmethionine-6-N-lysine-methyltransferase from *Neurospora crassa* was expressed in *E. coli*, and L-lysine was converted into trimethyllysine therefrom.

(3) Amplification and Cloning of Polynucleotide Encoding TMLH (TMLH Gene) and Confirmation of TMLH Production

(a) Amplification and Cloning of Polynucleotide Encoding TMLH (TMLH Gene)

PCR was performed using λ including the cDNA library pool of (1) as a template and using SEQ ID NOS: 3 and 4 as a primer. Then, agarose gel electrophoresis was performed for the PCR product obtained. As a result, about 1.4 kb of a desired product was confirmed. The primers of SEQ ID NOS: 3 and 4 include a sequence that is supposed to encode an initiation codon and termination codon of TMLH from *Neurospora crassa*. A potential TMLH from *Neurospora crassa* was searched by conducting a homology search between an amino acid sequence of the total proteins expressed from *Neurospora crassa* genome and an amino acid sequence of known TMLH from humans and rats, the primer of SEQ ID NOS: 3 and 4 were designed from the amino acid sequence of the potential TMLH.

The PCR product was digested with EcoRI and SalI, and connected to pBS KS$^+$ (Stratagene Inc.) digested with the same enzyme, and then *E. coli* DH5α was transformed with pBS KS$^+$ (TMLH) to which the obtained PCR product was inserted. The transformed *E. coli* DH5α was incubated at 37° C. for 8 hours, and then pBS KS$^+$ (TMLH) was isolated and digested with EcoRI and SalI to determine whether a PCR product was properly inserted. Next, the isolated pBS KS$^+$ (TMLH) was digested with NdeI and SalI, and then a segment of NdeI and SalI was isolated after agarose gel electrophoresis. The segment was connected to expression vector pT7-7 that was digested with the same enzyme to obtain pT7-7 TMLH (refer to FIG. **8**). pT7-7 (TMLH) was transformed into the *E. coli* BL21 (DE3).

(b) Confirmation of TMLH Production

E. coli BL21 (DE3) that was transformed with the obtained pT7-7 (TMLH) was incubated to OD_{600} 0.6 at 37° C. in a 250 ml flask equipped with a baffle in which 50 ml of LB medium including 100 μg/ml of ampicillin was placed therein, and incubated for more 4 hours after 1 mM of IPTG was added thereto. pT7-7 (TMLH) was isolated from the culture and digested with NdeI and SalI, and then agarose gel electrophoresis was performed. The results are shown in FIG. **12**. As shown in FIG. **12**, a band corresponding to a segment of NdeI and SalI was confirmed (lane 2). Next, pT7-7 (TMLH) was isolated and a nucleotide sequence of TMLH was analyzed. As a result, the nucleotide sequence of TMLH was confirmed to be the same sequence as that stored in a database of *Neurospora crassa* genome of NCBI (SEQ ID NO: 17).

In addition, an expressed TMLH protein was confirmed in cultures of *E. coli* BL21 (DE3) that was transformed with

US 7,718,414 B2

<table>
<tr><td>9</td><td>10</td></tr>
</table>

pT7-7 (TMLH). First, centrifugal separation was performed for the culture at 4,000×g for 15 minutes and cell pellets were collected. The obtained cell pellets were added to 1 ml of a lysis buffer (140 mM NaCl, 200 g/l glycerol, and 1 mM DTT in 10 mM of pH 7.4 sodium phosphate buffer solution) and resuspended. The cell suspension was placed in an ice bath and cells were lysed using an ultrasonic disintegrator by propagating an ultrasonic wave five times for 10 seconds each time. Centrifugal separation was performed for the cell lysate with 10,000 g at 4° C. for 20-30 minutes, and then cell debris was removed and the supernatant was collected to obtain a cell crude extract. 8% SDS-PAGE was performed by collecting a sample from the obtained cell crude extract (refer to FIG. **13**, lane 2). As a result of performing SDS-PAGE, about 52 KDa of a band corresponding to TMLH was confirmed.

(3) Amplification and Cloning of Polynucleotide Encoding 3-hydroxy-6-N-trimethyllysine aldolase (TMLA) and Confirmation of TMLA Production

(a) Amplification and Cloning of Polynucleotide Encoding 3-hydroxy-6-N-trimethyllysine aldolase (TMLA)

PCR was performed using λ including the cDNA library pool of (1) as a template and using SEQ ID NOS: 5 and 6 as a primer. Then, agarose gel electrophoresis was performed for the PCR product obtained. As a result, about 1.4 kb of a desired product was confirmed. The primer of SEQ ID NOS: 5 and 6 included a sequence that encoded an initiation codon and termination codon of TMLA from *Neurospora crassa*. A potential TMLA from *Neurospora crassa* was searched by conducting homology search between an amino acid sequence of total proteins expressed from *Neurospora crassa* genome and an amino acid sequence of known TMLA from humans and rats, the primers SEQ ID NOS: 5 and 6 were designed from the amino acid sequence of the potential TMLA.

The PCR product was digested with EcoRI and SalI, and connected to pBS KS$^+$ (Stratagene Inc.) digested with the same enzyme, and then *E. coli* DH5α was transformed with pBS KS$^+$ (TMLA) to which the obtained PCR product was inserted. The transformed *E. coli* DH5α was incubated at 37° C. for 8 hours, and then pBS KS$^+$ (TMLA) was isolated therefrom and digested with EcoRI and SalI to determine whether the PCR product was properly inserted. Next, the isolated pBS KS$^+$ (TMLA) was digested with NdeI and SalI, and then a segment of NdeI and SalI was isolated after agarose gel electrophoresis. The segment was connected to expression vector pT7-7 that was digested with the same enzyme to obtain pT7-7(TMLA) (refer to FIG. **9**). *E. coli* BL21 (DE3) was transformed with pT7-7 (TMLA).

(b) Confirmation of TMLA Production

E. coli BL21 (DE3) that was transformed with the obtained pT7-7 (TMLA) was incubated to OD$_{600}$ 0.6 at 37° C. in a 250 ml flask equipped with a baffle in which 50 ml of LB medium including 100 μg/ml of ampicillin was placed therein, and then incubated for more 4 hours after 1 mM of IPTG was added thereto. pT7-7 (TMLA) was isolated from the culture and digested with NdeI and SalI, and then agarose gel electrophoresis was performed. The results are shown in FIG. **12**. As shown in FIG. **12**, a band corresponding to a segment of NdeI and SalI was confirmed (lane 3). Next, pT7-7 (TMLA) was isolated and a nucleotide sequence of TMLA was analyzed. As a result, the nucleotide sequence of TMLA was confirmed to be the same sequence as that stored in a database of *Neurospora crassa* genome of NCBI (SEQ ID NO: 18).

In addition, an expressed TMLA protein was confirmed in cultures of *E. coli* BL21(DE3) that was transformed with pT7-7 (TMLA). First, centrifugal separation was performed for the culture at 4,000×g for 15 minutes and cell pellets were

collected. The obtained cell pellets was added to 1 ml of a lysis buffer (140 mM NaCl, 200 g/l glycerol, and 1 mM DTT in 10 mM of sodium phosphate buffer solution pH 7.4) and resuspended. The cell suspension was placed in an ice bath and cells were lysed using an ultrasonic disintegrator by propagating an ultrasonic wave five times for 10 seconds each time. Centrifugal separation was performed for the cell lysate with 10,000 g at 4° C. for 20-30 minutes, and then cell debris was removed and the supernatant was collected to obtain a cell crude extract. 8% SDS-PAGE was performed by collecting a sample from the obtained cell crude extract, (refer to FIG. **13**, lane 3). As a result of performing SDS-PAGE, about 53 KDa of a band corresponding to TMLA was confirmed.

(4) Amplification and Cloning of Polynucleotide Encoding γ-trimethylaminoaldehyde Dehydrogenase (TMABADH) and Confirmation of TMABADH Production

(a) Amplification and Cloning of Polynucleotide Encoding γ-trimethylaminoaldehyde dehydrogenase (TMABADH)

PCR was performed using A including the cDNA library pool of (1) as a template and using SEQ ID NOS: 7 and 8 as a primer. Then, agarose gel electrophoresis was performed for the PCR product obtained. As a result, about 1.5 kb of a desired product was confirmed. The primer of SEQ ID NOS: 7 and 8 included a sequence that encoded an initiation codon and termination codon of TMABDH from *Neurospora crassa*. A potential TMABADH from *Neurospora crassa* was searched by conducting homology search between an amino acid sequence of total proteins expressed from *Neurospora crassa* genome and an amino acid sequence of known TMABADH from humans and rats, and the primer of SEQ ID NOS: 7 and 8 were designed from the amino acid sequence of the potential TMABADH. The PCR product was digested with EcoRI and SalI, and connected to pBS KS$^+$ (Stratagene Inc.) digested with the same enzyme, and then *E. coli* DH5α was transformed with pBS KS$^+$ (TMABADH) in which the obtained PCR product was inserted. The transformed *E. coli* DH5α was incubated at 37° C. for 8 hours, and then pBS KS$^+$ (TMABADH) was isolated therefrom and digested with EcoRI and SalI to determine whether the PCR product was properly inserted. Next, the isolated pBS KS$^+$ (TMABADH) was digested with NdeI and SalI, and then a segment of NdeI and SalI was isolated after agarose gel electrophoresis. The segment was connected to expression vector pT7-7 that was digested with the same enzyme to obtain pT7-7(TMABADH) (refer to FIG. **10**). *E. coli* BL21 (DE3) was transformed with pT7-7 (TMABADH).

(b) Confirmation of TMABADH Production

E. coli BL21 (DE3) that was transformed with the obtained pT7-7 (TMABADH) was incubated to OD$_{600}$ 0.6 at 37° C. in a 250 ml flask equipped with a baffle in which 50 ml of LB medium including ampicillin was placed, and then incubated for more 4 hours after 1 mM of IPTG was added thereto. pT7-7 (TMABADH) was isolated from the culture and digested with NdeI and SalI, and then agarose gel electrophoresis was performed. The results are shown in FIG. **12**. As shown in FIG. **12**, a band corresponding to a segment of NdeI and SalI was confirmed (lane 4). Next, pT7-7 (TMABADH) was isolated and a nucleotide sequence of TMLH was analyzed. As a result, the nucleotide sequence of TMABADH was confirmed to be the same sequence as that stored in a database of *Neurospora crassa* genome of NCBI (SEQ ID NO: 19).

In addition, an expressed TMABADH protein was confirmed in cultures of *E. coli* BL21 (DE3) that was transformed with pT7-7 (TMABADH). First, centrifugal separation was performed for the culture at 4,000×g for 15 minutes and cell pellets were collected. The obtained cell pellets was added to

US 7,718,414 B2

| 11 | 12 |

1 ml of a lysis buffer (140 mM NaCl, 200 g/l glycerol, and 1 mM DTT in 10 mM of sodium phosphate buffer solution pH 7.4) and resuspended. The cell suspension was placed in an ice bath and cells were lysed using an ultrasonic disintegrator by propagating an ultrasonic wave five times for 10 seconds each time. Centrifugal separation was performed for the cell lysate with 10,000 g at 4° C. for 20-30 minutes, and then cell debris was removed and the supernatant was collected to obtain a cell crude extract. 8% SDS-PAGE was performed by collecting a sample from the obtained cell crude extract (refer to FIG. **13**). As a result of performing SDS-PAGE, about 55 kD of a band corresponding to TMABADH was confirmed.

(5) Amplification and Cloning of Polynucleotide Encoding γ-Butyrobetaine Hydroxylase (BBH) and Confirmation of BBH Production

(a) Amplification and Cloning of Polynucleotide Encoding γ-Butyrobetaine Hydroxylase (BBH)

PCR was performed using λ including the cDNA library pool of (1) as a template and using SEQ ID NOS: 9 and 10 as a primer. Then, agarose gel electrophoresis was performed for the PCR product obtained. As a result, about 1.3 kb of a desired product was confirmed. The primers of SEQ ID NOS: 9 and 10 include a sequence that is supposed to encode an initiation codon and termination codon of BBH from *Neurospora crassa*. A potential BBH from *Neurospora crassa* was searched by conducting homology search between an amino acid sequence of the total proteins expressed from *Neurospora crassa* genome and an amino acid sequence of known BBH from humans and rats, and the primer of SEQ ID NOS: 9 and 10 were designed from the amino acid sequence of the potential BBH.

The PCR product was digested with EcoRI and SalI, and connected to pUC19 digested with the same enzyme, and then *E. coli* DH5α was transformed with pUC19 (BBH) to which the obtained PCR product was inserted. The transformed *E. coli* DH5α was incubated at 37° C. for 8 hours in a LB medium including 100 μg/ml of ampicillin and then pUC19 (BBH) was isolated therefrom and digested with EcoRI and SalI to determine whether the PCR product was properly inserted. Next, the isolated pUC19 (BBH) was digested with NdeI and SalI, and then a segment of NdeI and SalI was isolated after agarose gel electrophoresis. The segment was connected to expression vector pT7-7 that was digested with the same enzyme to obtain pT7-7(BBH) (refer to FIG. **11**). *E. coli* BL21 (DE3) was transformed with pT7-7 (BBH).

E. coli BL21 (DE3) that was transformed with the obtained pT7-7 (BBH) was incubated to OD$_{600}$ 0.6 at 37° C. in a 250 ml flask equipped with a baffle in which 50 ml of LB medium including 100 μg/ml of ampicillin was placed therein, and then incubated for more 4 hours after 1 mM of IPTG was added thereto. pT7-7 (BBH) was isolated from the culture and digested with NdeI and SalI, and then 0.8% agarose gel electrophoresis was performed. The results are shown in FIG. **12**. As shown in FIG. **12**, a band corresponding to a segment of NdeI and SalI was confirmed (lane 5). Next, pT7-7 (BBH) was isolated and a nucleotide sequence of BBH was analyzed. As a result, the nucleotide sequence of BBH was confirmed to be the same sequence as that stored in a database of *Neurospora crassa* genome of NCBI (SEQ ID NO: 20).

(b) Confirmation of Production of BBH Protein

An expressed BBH protein was confirmed in cultures of *E. coli* BL21(DE3) that was transformed with pT7-7 (BBH). First, centrifugal separation was performed for the culture at 4,000×g for 15 minutes and cell pellets were collected. The obtained cell pellets was added to 1 ml of a lysis buffer (140 mM NaCl, 200 g/l glycerol, and 1 mM DTT in 10 mM of sodium phosphate buffer solution pH 7.4) and resuspended.

The cell suspension was placed in an ice bath and cells were lysed using an ultrasonic disintegrator by propagating an ultrasonic wave five times for 10 seconds each time. Centrifugal separation was performed for the cell lysate with 10,000 g at 4° C. for 20-30 minutes, and then cell debris was removed and the supernatant was collected to obtain a cell crude extract. 8% SDS-PAGE was performed by collecting a sample from the obtained cell crude extract, (refer to FIG. **13**, lane 5). As a result of performing SDS-PAGE, about 49 kDa of a band corresponding to BBH was confirmed.

Example 2

Production of Host Cell Including all of LMT, TMLH, TMLA, TMABADH and BBH Gene

Genes of LMT, TMLH and BBH from cDNA library of *Neurospora crassa* that was produced in Example 1 were amplified, and pT7-7 ABE having all of the three genes was produced. In addition, genes of TMLA and TMABADH from cDNA library of *Neurospora crassa* that was produced in Example 1 were produced, and pACYC184-CarCD having all of the two genes was produced. The produced pT7-7-CarABE and pACYC184-CarCD were employed in *E. coli* to produce a transformed microorganism having all of the genes of LMT, TMLH, TMLA, TMABADH and BBH. The transformed microorganism was referred to as *E. coli* BL21 (DE3) CJ2004-2, and deposited on Dec. 13, 2004, in Korean Culture Center of Microorganisms (KCCM), an International Depositary Authority (Accession number KCCM-10638).

(1) Production of pT7-7-CarABE Having all of the Three Genes of LMT, TMLH and BBH

First, lmt including termination codon was amplified from T7 promoter using cDNA library of *Neurospora crassa* as a template and using oligonucleotide of SEQ ID NOS: 1 and 2 as a primer. Next, TMLH including termination codon was amplified from T7 promoter using cDNA library of *Neurospora crassa* as a template and using oligonucleotide of SEQ ID NOS: 3 and 4 as a primer. Then, BBH including termination codon was amplified from T7 promoter using oligonucleotide of SEQ ID NOS: 9 and 10 as a primer. The amplified product of LMT, TMLH and BBH was introduced into pT7-7. First, the amplified product of BBH was digested with a restriction enzyme, such as BamHI and SalI, and segments of BamHI and SalI were obtained therefrom, and then the segments were connected to pT7-7 that was digested with the same enzyme to obtain pT7-7 BBH. Next, the amplified product of TMLH was digested with NdeI and EcoRI, and segments of NdeI and EcoRI were obtained therefrom, and then the segments were connected to pT7-7 BBH that was digested with the same enzyme to obtain pT7-7 CarBE. Then, the amplified product of LMT was digested with ClaI, and a segment of ClaI was obtained therefrom, and then the segment was connected to lmt that was digested with the same enzyme to obtain pT7-7 CarABE (refer to FIG. **14**).

(2) Production of pACYC184 CarCD Having all of the Genes of TMLA and TMABADH

First, TMLA including termination codon was amplified from T7 promoter using cDNA library of *Neurospora crassa* as a template and using oligonucleotide of SEQ ID NOS: 5 and 6 as a primer. Next, TMABADH including termination codon was amplified from T7 promoter using cDNA library of *Neurospora crassa* as a template and using oligonucleotide of SEQ ID NOS: 7 and 8 as a primer. The amplified products of TMLA and TMABADH were introduced into pACYC184. First, the amplified product of TMLA was digested with BamHI and HlndIII, and segments of BamHI and HindIII

US 7,718,414 B2

13

were obtained therefrom, and then the segments were connected to pACYC184 that was digested with the same enzyme to obtain pACYC184 TMLA. Next, the amplified product of TMABADH was digested with BamHI and SalI, and segments of BamHI and SalI were obtained therefrom, and then the segments were connected to pACYC184 TMLA that was digested with the same enzyme to obtain pACYC184 CarCD.

Example 3

Production of L-Carnitine Using a Microorganism Including Polynucleotide Encoding LMT, TMLH, TMLA, TMABADH and BBH

E. coli BL21(DE3) in which both pT7-7-CarABE and pACYC184-CarCD produced in Example 2 were introduced was cultured in a medium including L-Iysine, and a production amount of L-carnitine was determined. The introduction of pT7-7-CarABE and pACYC184-CarCD into *E. coli* BL21 (DE3) was performed using electroporation as described in Example 1.

(1) Production of L-Carnitine by Culturing *E. coli* BL21 (DE3) that was Transformed with Both PT7-7-CarABE and pACYC184-CarCD that were Produced in Example 2

First, *E. coli* BL21 (DE3) that was transformed with both pT7-7-CarABE and pACYC184-CarCD was plated in a LB solid plating medium including ampicillin (100 μg/ml) and chloramphenicol (50 μg/ml), and cultured. Colonies of the microorganism in the solid plating medium were incubated to OD_{600} 1.0 at 37° C. for 12 hours in a flask including 20 ml of a LB medium to which ampicillin (100 μg/ml) and chloramphenicol (50 μg/ml) were added. 0.1 ml of a culture of the

14

incubated microorganism was placed in a 250 ml flask equipped with a baffle including 20 ml of a LB medium that has 2 mM of L-lysine, and then incubated to OD_{600} 0.6 at 37° C. When IPTG was added, 1 mM of IPTG was added after a value of OD_{600} reached 0.6 and then the microorganism was incubated for more 4 hours. A group of incubating the microorganism in a LB medium without L-lysine that was induced with IPTG using the same method described above and group of culturing the microorganism in a LB medium including L-lysine that was not induced with IPTG were used as a control group. After incubation was terminated, a L-carnitine content of the culture was determined using in the same manner as in (1). The results are shown in Table 2.

TABLE 2

Production of L-carnitine by single culture

Culture condition	Concentration (mg/l)
LB medium(IPTG induction)	0
2 mM LB medium including lysine(IPTG no induction)	0.14
2 mM LB medium including lysine(IPTG induction)	19.81

As shown in Table 2, by culturing a microorganism including all of the polynucleotides encoding LMT, TMLH, TMLA, TMABADH and BBH in a medium containing L-lysine, L-carnitine can be produced at high efficiency. In addition, production amounts of L-carnitine shown in Table 2 were compared to one another, and it was confirmed that L-carnitine has higher producing efficiency when cultured in a medium including lysine.

```
                    SEQUENCE LISTING


<160> NUMBER OF SEQ ID NOS: 21

<210> SEQ ID NO 1
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 1

ggaattccat atggccttcg gaaagcttta cac                          33


<210> SEQ ID NO 2
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 2

cgggatcctt agacgttggt caacttgggg                             30


<210> SEQ ID NO 3
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 3
```

US 7,718,414 B2

15   16

-continued

```
atgaattcca tatgagaccg caagtggtag gg                              32


<210> SEQ ID NO 4
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 4

atgaattctc attttccgct ggtttctttc cg                              32


<210> SEQ ID NO 5
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 5

atggatccta atacgactca ctataggga                                 29


<210> SEQ ID NO 6
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 6

attaagcttt tagagaccgg catcgtatct                                30


<210> SEQ ID NO 7
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 7

atggatccta atacgactca ctataggga                                 29


<210> SEQ ID NO 8
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 8

attgtcgact catgccgcca ggtttacatg gat                            33


<210> SEQ ID NO 9
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 9

atggatccta atacgactca ctataggga                                 29


<210> SEQ ID NO 10
<211> LENGTH: 31
<212> TYPE: DNA
```

US 7,718,414 B2

17   18

-continued

<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 10

attagtcgac tcaatacctct cccccacccct g   31

<210> SEQ ID NO 11
<211> LENGTH: 216
<212> TYPE: PRT
<213> ORGANISM: Neurospora crassa

<400> SEQUENCE: 11

Met Ala Phe Gly Lys Leu Tyr Thr Tyr Glu Ala Asn Pro Arg Ser Thr
1   5   10   15

Ala Ile Leu Ala Val Ala Lys Ala Asn Asn Leu Asp Leu Glu Val Ile
                20   25   30

Lys Val Asp Leu Glu Ala Ala Ile Glu Glu Tyr Lys Lys Val Asn Pro
            35   40   45

Leu Gly Lys Val Pro Thr Phe Val Gly Ala Asp Gly Tyr Thr Leu Phe
        50   55   60

Glu Cys Ile Ala Ile Ala Ile Tyr Val Ala Ser Gln Asn Glu Lys Thr
65   70   75   80

Thr Leu Leu Gly Lys Thr Lys Gln Asp Tyr Ala Ser Ile Leu Lys Trp
                85   90   95

Leu Ser Phe Phe Asn Thr Glu Val Leu Pro Pro Leu Ala Gly Trp Tyr
                100   105   110

Arg Pro Leu Leu Gly Lys Ala Pro Tyr Asn Lys Lys Ala Val Glu Asp
            115   120   125

Ala Gln Ala Thr Ala Leu Lys Ala Ile Ser Val Ala Glu Ala His Leu
        130   135   140

Lys Asn Asn Thr Phe Pro Val Gly Glu Arg Ile Thr Leu Ala Asp Leu
145   150   155   160

Phe Ala Thr Gly Ile Ile Ala Arg Gly Phe Glu Phe Phe Asp Lys
                165   170   175

Ala Trp Arg Glu Gln Tyr Pro Asn Val Thr Arg Trp Tyr Thr Thr Val
                180   185   190

Tyr Asn Gln Pro Ile Tyr Ser Ala Val Ala Pro Pro Phe Ala Leu Leu
                195   200   205

Asp Thr Pro Lys Leu Thr Asn Val
    210   215

<210> SEQ ID NO 12
<211> LENGTH: 471
<212> TYPE: PRT
<213> ORGANISM: Neurospora crassa

<400> SEQUENCE: 12

Met Arg Pro Gln Val Val Gly Ala Ile Leu Arg Ser Arg Ala Val Val
1   5   10   15

Ser Arg Gln Pro Leu Ser Arg Thr His Ile Phe Ala Ala Val Thr Val
                20   25   30

Ala Lys Ser Ser Ser Pro Ala Gln Asn Ser Arg Arg Thr Phe Ser Ser
            35   40   45

Ser Phe Arg Arg Leu Tyr Glu Pro Lys Ala Glu Ile Thr Ala Glu Gly
        50   55   60

Leu Glu Leu Ser Pro Pro Gln Ala Val Thr Gly Gly Lys Arg Thr Val

US 7,718,414 B2

19   20

-continued

```
65              70              75              80

Leu Pro Asn Phe Trp Leu Arg Asp Asn Cys Arg Cys Thr Lys Cys Val
                85              90              95

Asn Gln Asp Thr Leu Gln Arg Asn Phe Asn Thr Phe Ala Ile Pro Ser
                100             105             110

Asp Ile His Pro Thr Lys Val Glu Ala Thr Lys Glu Asn Val Thr Val
                115             120             125

Gln Trp Ser Asp Asn His Thr Ser Thr Tyr Pro Trp Pro Phe Leu Ser
                130             135             140

Phe Tyr Leu Thr Ser Asn Ala Arg Gly His Glu Asn Asp Gln Ile Ser
145             150             155             160

Leu Trp Gly Ser Glu Ala Gly Ser Arg Pro Pro Thr Val Pro Phe Pro
                165             170             175

Arg Val Met Ala Ser Asp Gln Gly Val Ala Asp Leu Thr Ala Met Ile
                180             185             190

Lys Glu Phe Gly Phe Cys Phe Val Lys Asp Thr Pro His Asp Asp Pro
                195             200             205

Asp Val Thr Arg Gln Leu Leu Glu Arg Ile Ala Phe Ile Arg Val Thr
                210             215             220

His Tyr Gly Gly Phe Tyr Asp Phe Thr Pro Asp Leu Ala Met Ala Asp
225             230             235             240

Thr Ala Tyr Thr Asn Leu Ala Leu Pro Ala His Thr Asp Thr Thr Tyr
                245             250             255

Phe Thr Asp Pro Ala Gly Leu Gln Ala Phe His Leu Leu Glu His Lys
                260             265             270

Ala Ala Pro Ser Arg Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro
                275             280             285

Ser Glu Glu Lys Glu Ala Ala Gly Ser Ala Ala Gly Glu Ala Ala Ala
        290             295             300

Ala Ala Glu Gly Gly Lys Ser Leu Leu Val Asp Gly Phe Asn Ala Ala
305             310             315             320

Arg Ile Leu Lys Glu Glu Asp Pro Arg Ala Tyr Glu Ile Leu Ser Ser
                325             330             335

Val Arg Leu Pro Trp His Ala Ser Gly Asn Glu Gly Ile Thr Ile Ala
                340             345             350

Pro Asp Lys Leu Tyr Pro Val Leu Glu Leu Asn Glu Asp Thr Gly Glu
                355             360             365

Leu His Arg Val Arg Trp Asn Asn Asp Asp Arg Gly Val Val Pro Phe
                370             375             380

Gly Glu Lys Tyr Ser Pro Ser Glu Trp Tyr Glu Ala Ala Arg Lys Trp
385             390             395             400

Asp Gly Ile Leu Arg Arg Lys Ser Ser Glu Leu Trp Val Gln Leu Glu
                405             410             415

Pro Gly Lys Pro Leu Ile Phe Asp Asn Trp Arg Val Leu His Gly Arg
                420             425             430

Ser Ala Phe Ser Gly Ile Arg Arg Ile Cys Gly Gly Tyr Ile Asn Arg
                435             440             445

Asp Asp Phe Ile Ser Arg Trp Arg Asn Thr Asn Tyr Pro Arg Ser Glu
        450             455             460

Val Leu Pro Arg Val Thr Gly
465             470
```

<210> SEQ ID NO 13

US 7,718,414 B2

21   22

-continued

```
<211> LENGTH: 480
<212> TYPE: PRT
<213> ORGANISM: Neurospora crassa

<400> SEQUENCE: 13

Met Ser Thr Tyr Ser Leu Ser Glu Thr His Lys Ala Met Leu Glu His
 1               5                  10                  15

Ser Leu Val Glu Ser Asp Pro Gln Val Ala Glu Ile Met Lys Lys Glu
             20                  25                  30

Val Gln Arg Gln Arg Glu Ser Ile Ile Leu Ile Ala Ser Glu Asn Val
         35                  40                  45

Thr Ser Arg Ala Val Phe Asp Ala Leu Gly Ser Pro Met Ser Asn Lys
     50                  55                  60

Tyr Ser Glu Gly Leu Pro Gly Ala Arg Tyr Tyr Gly Gly Asn Gln His
 65                  70                  75                  80

Ile Asp Glu Ile Glu Val Leu Cys Gln Asn Arg Ala Leu Glu Ala Phe
                 85                  90                  95

His Leu Asp Pro Lys Gln Trp Gly Val Asn Val Gln Cys Leu Ser Gly
             100                 105                 110

Ser Pro Ala Asn Leu Gln Val Tyr Gln Ala Ile Met Pro Val His Gly
             115                 120                 125

Arg Leu Met Gly Leu Asp Leu Pro His Gly Gly His Leu Ser His Gly
     130                 135                 140

Tyr Gln Thr Pro Gln Arg Lys Ile Ser Ala Val Ser Thr Tyr Phe Glu
145                 150                 155                 160

Thr Met Pro Tyr Arg Val Asn Ile Asp Thr Gly Leu Ile Asp Tyr Asp
                 165                 170                 175

Thr Leu Glu Lys Asn Ala Gln Leu Phe Arg Pro Lys Val Leu Val Ala
             180                 185                 190

Gly Thr Ser Ala Tyr Cys Arg Leu Ile Asp Tyr Glu Arg Met Arg Lys
             195                 200                 205

Ile Ala Asp Ser Val Gly Ala Tyr Leu Val Val Asp Met Ala His Ile
     210                 215                 220

Ser Gly Leu Ile Ala Ser Glu Val Ile Pro Ser Pro Phe Leu Tyr Ala
225                 230                 235                 240

Asp Val Val Thr Thr Thr Thr His Lys Ser Leu Arg Gly Pro Arg Gly
                 245                 250                 255

Ala Met Ile Phe Phe Arg Arg Gly Val Arg Ser Val Asp Ala Lys Thr
             260                 265                 270

Gly Lys Glu Thr Leu Tyr Asp Leu Glu Asp Lys Ile Asn Phe Ser Val
             275                 280                 285

Phe Pro Gly His Gln Gly Gly Pro His Asn His Thr Ile Thr Ala Leu
     290                 295                 300

Ala Val Ala Leu Lys Gln Ala Ala Ser Pro Glu Phe Lys Glu Tyr Gln
305                 310                 315                 320

Gln Lys Val Val Ala Asn Ala Lys Ala Leu Glu Lys Lys Leu Lys Glu
             325                 330                 335

Leu Gly Tyr Lys Leu Val Ser Asp Gly Thr Asp Ser His Met Val Leu
             340                 345                 350

Val Asp Leu Arg Pro Ile Gly Val Asp Gly Ala Arg Val Glu Phe Leu
             355                 360                 365

Leu Glu Gln Ile Asn Ile Thr Cys Asn Lys Asn Ala Val Pro Gly Asp
     370                 375                 380

Lys Ser Ala Leu Thr Pro Gly Gly Leu Arg Ile Gly Thr Pro Ala Met
```

US 7,718,414 B2

23   24

-continued

```
385              390              395              400

Thr Ser Arg Gly Phe Gly Glu Ala Asp Phe Glu Lys Val Ala Val Phe
            405              410              415

Val Asp Glu Ala Val Lys Leu Cys Lys Glu Ile Gln Ala Ser Leu Pro
            420              425              430

Lys Glu Ala Asn Lys Gln Lys Asp Phe Lys Ala Lys Ile Ala Thr Ser
            435              440              445

Asp Ile Pro Arg Ile Asn Glu Leu Lys Gln Glu Ile Ala Ala Trp Ser
        450              455              460

Asn Thr Phe Pro Leu Pro Val Glu Gly Trp Arg Tyr Asp Ala Gly Leu
465              470              475              480


<210> SEQ ID NO 14
<211> LENGTH: 495
<212> TYPE: PRT
<213> ORGANISM: Neurospora crassa

<400> SEQUENCE: 14

Met Glu Val Glu Leu Thr Ala Pro Asn Gly Lys Lys Trp Met Gln Pro
  1              5               10               15

Leu Gly Leu Phe Ile Asn Asn Glu Phe Val Lys Ser Ala Asn Glu Gln
            20               25               30

Lys Leu Ile Ser Ile Asn Pro Thr Thr Glu Glu Glu Ile Cys Ser Val
            35               40               45

Tyr Ala Ala Thr Ala Glu Asp Val Asp Ala Ala Val Ser Ala Ala Arg
        50               55               60

Lys Ala Phe Arg His Glu Ser Trp Lys Ser Leu Ser Gly Thr Glu Arg
65               70               75               80

Gly Ala Leu Met Arg Lys Leu Ala Asp Leu Val Ala Glu Asn Ala Glu
                85               90               95

Ile Leu Ala Thr Ile Glu Cys Leu Asp Asn Gly Lys Pro Tyr Gln Thr
            100              105              110

Ala Leu Asn Glu Asn Val Pro Glu Val Ile Asn Val Leu Arg Tyr Tyr
            115              120              125

Ala Gly Tyr Ala Asp Lys Asn Phe Gly Gln Val Ile Asp Val Gly Pro
        130              135              140

Ala Lys Phe Ala Tyr Thr Val Lys Glu Pro Leu Gly Val Cys Gly Gln
145              150              155              160

Ile Ile Pro Trp Asn Tyr Pro Leu Asp Met Ala Ala Trp Lys Leu Gly
                165              170              175

Pro Ala Leu Cys Cys Gly Asn Thr Val Val Leu Lys Leu Ala Glu Gln
            180              185              190

Thr Pro Leu Ser Val Leu Tyr Leu Ala Lys Leu Ile Lys Glu Ala Gly
            195              200              205

Phe Pro Pro Gly Val Ile Asn Ile Ile Asn Gly His Gly Arg Glu Ala
        210              215              220

Gly Ala Ala Leu Val Gln His Pro Gln Val Asp Lys Ile Ala Phe Thr
225              230              235              240

Gly Ser Thr Thr Thr Gly Lys Glu Ile Met Lys Met Ala Ser Tyr Thr
                245              250              255

Met Lys Asn Ile Thr Leu Glu Thr Gly Gly Lys Ser Pro Leu Ile Val
            260              265              270

Phe Glu Asp Ala Asp Leu Glu Leu Ala Ala Thr Trp Ser His Ile Gly
            275              280              285
```

US 7,718,414 B2

25   26

-continued

```
Ile Met Ser Asn Gln Gly Gln Ile Cys Thr Ala Thr Ser Arg Ile Leu
    290                 295                 300

Val His Glu Lys Ile Tyr Asp Glu Phe Val Glu Lys Phe Lys Ala Lys
305                 310                 315                 320

Val Gln Glu Val Ser Val Leu Gly Asp Pro Phe Glu Glu Ser Thr Phe
                325                 330                 335

His Gly Pro Gln Val Thr Lys Ala Gln Tyr Glu Arg Val Leu Gly Tyr
            340                 345                 350

Ile Asn Val Gly Lys Glu Glu Gly Ala Thr Val Met Met Gly Gly Glu
            355                 360                 365

Pro Ala Pro Gln Asn Gly Lys Gly Phe Phe Val Ala Pro Thr Val Phe
    370                 375                 380

Thr Asn Val Lys Pro Thr Met Lys Ile Phe Arg Glu Glu Ile Phe Gly
385                 390                 395                 400

Pro Cys Val Ala Ile Thr Thr Phe Lys Thr Glu Glu Glu Ala Leu Thr
                405                 410                 415

Leu Ala Asn Asp Ser Met Tyr Gly Leu Gly Ala Ala Leu Phe Thr Lys
                420                 425                 430

Asp Leu Thr Arg Ala His Arg Val Ala Arg Glu Ile Glu Ala Gly Met
            435                 440                 445

Val Trp Val Asn Ser Ser Asn Asp Ser Asp Phe Arg Ile Pro Phe Gly
    450                 455                 460

Gly Val Lys Gln Ser Gly Ile Gly Arg Glu Leu Gly Glu Ala Gly Leu
465                 470                 475                 480

Ala Pro Tyr Cys Asn Val Lys Ser Ile His Val Asn Leu Ala Ala
                485                 490                 495


<210> SEQ ID NO 15
<211> LENGTH: 425
<212> TYPE: PRT
<213> ORGANISM: Neurospora crassa

<400> SEQUENCE: 15

Met Ala Thr Ala Ala Val Gln Val Ser Val Pro Ala Pro Val Gly Gln
    1               5                   10                  15

Pro Asp Ile Gly Tyr Ala Pro Asp His Asp Lys Tyr Leu Ala Arg Val
                20                  25                  30

Lys Arg Arg Arg Glu Asn Glu Lys Leu Glu Ser Ser Leu Pro Pro Gly
            35                  40                  45

Phe Pro Arg Arg Leu Asp Ser Asp Leu Val Trp Asp Gly Asn Thr Leu
    50                  55                  60

Ala Glu Thr Tyr Asp Trp Thr Tyr Arg Leu Thr Glu Glu Ala Ile Asp
65                  70                  75                  80

Glu Ile Glu Ala Ala Leu Arg His Phe Lys Ser Leu Asn Lys Pro Leu
                85                  90                  95

Gly Tyr Ile Asn Gln Glu Thr Phe Pro Leu Pro Arg Leu His His Thr
            100                 105                 110

Leu Arg Ser Leu Ser His Glu Leu His His Gly His Gly Phe Lys Val
            115                 120                 125

Leu Arg Gly Leu Pro Val Thr Ser His Thr Arg Glu Glu Asn Ile Ile
    130                 135                 140

Ile Tyr Ala Gly Val Ser Ser His Val Ala Pro Ile Arg Gly Arg Gln
145                 150                 155                 160

Asp Asn Gln His Asn Gly His Pro Ala Asp Val Val Leu Ala His Ile
                165                 170                 175
```

US 7,718,414 B2

27   28

-continued

```
Lys Asp Leu Ser Thr Thr Val Ser Asp Val Ser Lys Ile Gly Ala Pro
            180                 185                 190

Ala Tyr Thr Thr Glu Lys Gln Val Phe His Thr Asp Ala Gly Asp Ile
            195                 200                 205

Val Ala Leu Phe Cys Leu Gly Glu Ala Ala Glu Gly Gly Gln Ser Tyr
        210                 215                 220

Leu Ser Ser Ser Trp Lys Val Tyr Asn Glu Leu Ala Ala Thr Arg Pro
225                 230                 235                 240

Asp Leu Val Arg Thr Leu Ala Glu Pro Trp Val Ala Asp Glu Phe Gly
                245                 250                 255

Lys Glu Gly Arg Lys Phe Ser Val Arg Pro Leu Leu His Phe Gln Ser
            260                 265                 270

Thr Ala Ala Ala Ala Ser Arg Glu Ala Lys Pro Glu Ser Glu Arg Leu
        275                 280                 285

Ile Ile Gln Tyr Ala Arg Arg Thr Phe Thr Gly Tyr Trp Gly Leu Pro
        290                 295                 300

Arg Ser Ala Asp Ile Pro Pro Ile Thr Glu Ala Gln Ala Glu Ala Leu
305                 310                 315                 320

Asp Ala Leu His Phe Thr Ala Glu Lys Tyr Ala Val Ala Leu Asp Phe
                325                 330                 335

Arg Gln Gly Asp Val Gln Phe Val Asn Asn Leu Ser Val Phe His Ser
            340                 345                 350

Arg Ala Gly Phe Arg Asp Glu Gly Glu Lys Gln Arg His Leu Val Arg
        355                 360                 365

Leu Trp Leu Arg Asp Pro Glu Asn Ala Trp Glu Thr Pro Glu Ala Leu
        370                 375                 380

Lys Glu Arg Trp Glu Arg Val Tyr Gly Gly Val Ser Pro Glu Arg Glu
385                 390                 395                 400

Val Phe Pro Leu Glu Pro Gln Ile Arg Ser Ala Ser Lys Gly Glu Ser
                405                 410                 415

Val Gly Thr Gln Gly Gly Gly Gly Tyr
            420                 425


<210> SEQ ID NO 16
<211> LENGTH: 651
<212> TYPE: DNA
<213> ORGANISM: Neurospora crassa

<400> SEQUENCE: 16

atggccttcg gaaagcttta cacctacgag gcgaacccccc gctccacggc catcttggct        60

gtcgcgaagg ccaacaacct cgacctcgag gttatcaagg tcgaccttga ggctgccatc       120

gaggagtaca agaaggtcaa ccctctcggc aaggtccccca ccttcgttgg tgccgacggc       180

tacactctct tcgagtgcat cgccatcgcc atctatgtcg cttcccagaa cgagaagacc       240

actctcctcg gcaagaccaa gcaggactat gcctccatcc tgaagtggct ctctttcttc       300

aacaccgagg tccttccccc tcttgctggc tggtaccgcc ctctccttgg caaggctccc       360

tacaacaaga aggctgttga ggacgctcag gctactgccc tcaaggccat ctctgtcgcc       420

gaggcccacc tcaagaacaa caccttcccc gttggcgagc gcatcaccct tgccgatctc       480

ttcgccactg gcatcattgc ccgcggcttc gagttcttct tcgacaaggc ctggcgcgag       540

cagtacccca acgtcacccg ttggtacacc actgtctaca accagcccat ctactcggcc       600

gttgctcctc ccttcgctct ccttgatacc cccaagttga ccaacgtcta a              651
```

US 7,718,414 B2

29   30

-continued

```
<210> SEQ ID NO 17
<211> LENGTH: 1407
<212> TYPE: DNA
<213> ORGANISM: Neurospora crassa

<400> SEQUENCE: 17

atgagaccgc aagtggtagg ggcaatcctc cgctctagag ctgttgtcag cagacaacct      60

ctttcgagga cccatatctt tgctgccgtc actgttgcaa agtcctcatc acctgcccag     120

aactcgagaa gaaccttttc atcctctttc cgacggttgt atgagccaaa ggcggagata     180

acagctgagg gacttgagtt gagccctcca caggctgtta cgggtggaaa gcggactgtt     240

ttacccaact tctggctacg tgacaactgc cggtgtacga aatgcgtgaa ccaagatact     300

ctccagagaa acttcaacac ttttgccatc ccctccgaca tccacccaac aaaggttgaa     360

gccaccaagg agaacgtcac cgtccaatgg tccgacaacc acacatccac ctacccctgg     420

cccttcctct ctttctacct cacctccaac gcgcgcgggc acgaaaacga ccagatctcc     480

ctctggggct ccgaagccgg ctcccgcccg ccaaccgtct ccttccctcg cgtgatggca     540

tcagaccagg gcgtcgccga cctaaccgcc atgatcaaag agttcggctt ctgtttcgtc     600

aaagacacac cccatgacga cccggacgtg acccgccagc ttctggagag aatcgccttt     660

atccgagtga cccattacgg cggctttttac gatttcacgc ccgacctcgc gatggccgac     720

acggcgtaca cgaacctggc gctgccggcg catacggata cgacgtactt cacggacccg     780

gcggggttgc aggctttttca cttgttggag cataaggccg ctccttctcg tcctcctcct     840

cctcctcctc ctcctcctcc tccttctgaa gaaaaagaag ctgcaggctc agcagcaggg     900

gaggcggcgg cggcagcaga aggggggaaag tcgttgttgg tcgatgggtt caacgccgcg     960

aggattctga aggaggagga tccccgggct tatgagatct tgagcagcgt gagactgccg    1020

tggcatgcga gtggaaacga aagggatcacg attgcgcccg ataagcttta tccggtgctg    1080

gaactgaatg aggataccgg ggaactgcat agggttaggt ggaataatga tgatagggggt    1140

gtggtgccgt ttgggggagaa gtacagcccg tcagagtggt atgaggcggc gaggaagtgg    1200

gatgggattt tgaggaggaa gagcagcgag ttgtggggtgc agttggagcc ggggaagccg    1260

ttgaggttct tcatggacgg agcgcgttct cgggtattag gaggatttgt ggagggtata    1320

tcaaccgcga tgacttcatc tctcggtgga ggaacacgaa ttacccaagg agcgaggttc    1380

ttccgagggt tactggttaa ggactga                                        1407


<210> SEQ ID NO 18
<211> LENGTH: 1443
<212> TYPE: DNA
<213> ORGANISM: Neurospora crassa

<400> SEQUENCE: 18

atgtctacct actccctctc cgagactcac aaggccatgc tcgagcatag cttggtcgag      60

tccgaccccc aggtcgccga gatcatgaag aaggaggttc agcgccagcg cgagtccatc     120

atcctcatcg cctccgagaa cgtcacctcg cgtgccgtct cgatgcccct cggctccccc     180

atgtccaaca agtactcgga gggtcttccc ggcgcccgct actatggtgg caaccagcac     240

atcgacgaga tcgaggttct ctgccagaac cgtgcccttg aggccttcca cctcgacccc     300

aagcagtggg gtgtcaatgt tcagtgcttg tccggcagcc ctgccaacct ccaggtctac     360

caggccatca tgcccgtcca cggcagactc atgggtcttg acctccccca cggtggccat     420

ctttcccacg gttaccagac cccccagcgc aagatctctg ctgtctctac ctacttcgag     480
```

US 7,718,414 B2

31   32

-continued

```
accatgccct accgcgtcaa cattgacact ggtctcatcg actacgatac cctcgagaag        540

aacgcccagc tcttccgccc caaggtcctc gtcgccggta cctctgccta ctgccgtctg        600

attgactacg agcgcatgcg caagattgcc gactccgttg gcgcttacct tgtcgtcgat        660

atggctcaca tttccggcct cattgcctcc gaggttatcc cctcgccctt cctctacgcc        720

gatgtcgtca ccaccaccac tcacaagtct ctccgtggcc ctcgtggcgc catgatcttc        780

ttccgccgcg gtgtccgctc cgttgacgcc aagaccggca aggagaccct ctacgacctt        840

gaggacaaga tcaacttctc cgtcttccct ggtcaccagg gtggcccccа caaccacacc        900

atcaccgccc ttgccgttgc cctcaagcag gctgcctccc ccgagttcaa ggagtaccag        960

cagaaggtcg ttgccaacgc caaggctctc gagaagaagc tcaaggagct cggctacaag       1020

ctcgtctctg acggcactga ctctcacatg gtcctcgttg accttcgccc catcggcgtc       1080

gatggtgccc gtgttgagtt cctccttgag cagatcaaca ttacctgcaa caagaacgcc       1140

gttcccggcg acaagagcgc cctcaccccc ggcggtctcc gtattggtac ccccgctatg       1200

acctcccgtg gcttcggcga ggccgacttc gagaaggtcg ccgtcttcgt cgatgaggct       1260

gtcaagctct gcaaggagat ccaggcttcc ctccccaagg aggctaacaa gcagaaggac       1320

ttcaaggcca agatcgccac cagcgatatt ccccgcatca acgagctcaa gcaggagatt       1380

gccgcctgga gcaacacctt ccccctcccc gttgagggct ggagatacga tgccggtctc       1440

taa                                                                     1443
```

<210> SEQ ID NO 19
<211> LENGTH: 1488
<212> TYPE: DNA
<213> ORGANISM: Neurospora crassa

<400> SEQUENCE: 19

```
atggaagtcg agcttacggc ccccaacggc aagaagtgga tgcagccact gggcttgttc         60

attaataacg agtttgtcaa aagtgccaat gagcagaagt tgatttccat caacccaact        120

accgaagagg agatctgctc ggtatacgcc gcaaccgccg aggatgttga cgccgcagta        180

tcagcagccc gcaaggcctt taggcacgaa tcatggaagt cgctatccgg cactgagcgc        240

ggcgccctga tgcgcaagct ggccgaccta gtgccgagaa tgccgaaat cctagccacc         300

atcgagtgcc tggacaacgg caagccgtat cagacagccc ttaacgagaa cgtgcccgaa        360

gtgatcaacg tcctcaggta ctatgccggc tatgcggaca agaactttgg ccaagtgatt        420

gacgttggcc ccgccaagtt tgcctacacg gtcaaggagc tctcggcgt atgtggccag        480

atcatcccct ggaactaccc gctagatatg gccgcctgga gctgggggcc agctctctgc        540

tgcggcaaca ccgtggtcct caagctggcc gagcagactc ccctgtccgt gttgtacttg        600

gctaagctca ttaaggaggc cggcttccct cccggtgtga tcaatatcat caacggacac        660

ggcagggaag cgggtgccgc acttgtgcaa catcctcagg tggacaagat tgcctttacc        720

ggcagcacca ctacgggcaa ggagatcatg aagatggctt cctataccat gaagaacatc        780

accctggaga ctggcggcaa gtcaccgttg atcgtgtttg aggatgccga ccttgagctg        840

gcggcgacat ggtcacacat cggcatcatg agcaaccagg ccaaatctg cacagccact        900

tcacgcattc tcgtgcacga aagatctac gacgagtttg tcgaaaaatt caaggccaaa         960

gtccaggagg tttcggtact cggcgacccc ttcgaggaga gcacgttcca cggacctcag       1020

gtcaccaaag cgcagtatga gcgtgttctg ggctatatca atgtcggaaa ggaagagggt       1080
```

US 7,718,414 B2

<table>
<tr><td>33</td><td>34</td></tr>
</table>

-continued

```
gccacggtga tgatgggtgg tgagccggct ccgcagaacg gtaaaggttt ctttgtggcc    1140

ccgactgtct tcacgaacgt caagccgacc atgaagatct tcagggagga gatctttggg    1200

ccctgcgtgg ccattaccac gttcaaaacg gaggaggagg cgttgacgct ggccaacgac    1260

agcatgtatg gcctgggagc ggctctgttc accaaggacc taaccagggc acacagagtg    1320

gcgcgggaga tcgaggccgg catggtctgg gtcaacagca gcaacgattc agactttagg    1380

attccatttg gaggcgtgaa gcagtctggt attgggaggg agttgggaga ggcaggtctg    1440

gcaccttatt gcaacgtcaa gagtatccat gtaaacctgg cggcatga              1488
```

```
<210> SEQ ID NO 20
<211> LENGTH: 1278
<212> TYPE: DNA
<213> ORGANISM: Neurospora crassa

<400> SEQUENCE: 20

atggccacgg cagcggttca ggtttcagtc ccagctccgg ttggacaacc agatatcggg      60

tacgctcctg accacgacaa gtacctcgca agagtcaaaa gacgacgaga aaacgagaag     120

ctggagtcgt ctcttccgcc aggtttccct cgaagactag actcggacct tgtgtgggac     180

ggcaacaccc tcgccgagac gtacgactgg acctacagac tgacagaaga ggccattgat     240

gaaatcgagg ccgcgcttcg tcattttaag agcctcaaca agcccctagg ctacatcaac     300

caagaaacct tcccccttcc ccgcctacac cacactctcc gctccctctc ccacgagctc     360

caccacggcc acggcttcaa agtcctccgc gggctccccg tcacctccca tacacgcgag     420

gaaaacatca tcatctacgc cggcgtctcc tcgcatgtcg ctcctatccg cggccgccag     480

gacaaccagc acaacggcca cccagccgac gtagtcctag cacacatcaa agacctgtcc     540

acgactgttt ctgacgtgag caaaatcggt gcacccgcct acaccaccga gaaacaagtc     600

ttccacaccg acgcaggcga catcgtcgcc ctcttttgct tgggagaggc cgccgagggc     660

ggacagagtt acctgtccag cagctggaag gtgtacaacg agctggcagc cactcggccc     720

gatctggttc gcacgctggc ggagccgtgg gtggcggacg agtttggcaa ggaagggagg     780

aagtttctg tgcgaccgct tttgcatttt cagtctactg ctgctgctgc ttctagggaa     840

gcaaagcccg agtctgaacg gctcatcatc cagtacgccc gccgcacgtt tacggggtat     900

tggggattac cgaggtcggc ggatatcccg cccattacgg aggcgcaggc ggaggcgttg     960

gatgcgctgc actttacggc ggagaagtac gcggtggcgc tggatttcag gcaggggat    1020

gtccagtttg tgaataactt gagtgtgttc cattcgaggg cggggtttag agatgagggg    1080

gagaagcaga ggcatttggt taggttgtgg ttgagagatc cggagaatgc gtgggagacg    1140

cccgaggcgt tgaaggaacg gtgggaacgc gtgtatggcg gggtgagtcc ggagagggag    1200

gtgtttccgc ttgagccgca gattaggagc gcgagtaagg gggagagcgt ggggacgcag    1260

ggtgggggag ggtattga                                                1278
```

```
<210> SEQ ID NO 21
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Neurospora crassa

<400> SEQUENCE: 21

Ala Phe Gly Lys Leu
 1               5
```

US 7,718,414 B2

35

36

The invention claimed is:

1. A transformed microorganism that belongs to the *Enterobacteriacae* genus, the microorganism comprising:

a polynucleotide encoding a polypeptide having S-adenosylmethionine-6-N-lysine methyltransferase activity from *Neurospora crassa*, wherein the polynucleotide is a polynucleotide encoding the amino acid sequence of SEQ ID NO: 11;

a polynucleotide encoding a polypeptide having 6-N-trimethyllysine hydroxylase activity wherein the polynucleotide is a polynucleotide encoding the amino acid sequence of SEQ ID NO: 12;

a polynucleotide encoding a polypeptide having 3-hydroxy-6-N-trimethyllysine aldolase activity wherein the polynucleotide is a polynucleotide encoding the amino acid sequence of SEQ ID NO: 13;

a polynucleotide encoding a polypeptide having γ-trimethylaminoaldehyde dehydrogenase activity wherein the polynucleotide is a polynucleotide encoding the amino acid sequence of SEQ ID NO: 14, and

a polynucleotide encoding a polypeptide having γ-butyrobetaine hydroxylase activity wherein the polynucleotide is a polynucleotide encoding the amino acid sequence of SEQ ID NO: 15.

2. The microorganism of claim 1, wherein the microorganism is *Escherichia coli*.

3. The microorganism of claim 1, wherein the microorganism is *Escherichia coli* (accession number: KCCM-10638).

4. A method of producing L-carnitine, the method comprising:

culturing a microorganism according to claim 1 in the presence of a substrate selected from the group consisting of L-lysine, N-trimethyllysine, β-hydroxy-N-trimethyllysine, γ-N- trimethylaminobutyraldehyde, γ-butyrobetaine and mixtures thereof to produce L-carnitine in the culture.

5. The method of claim 4, wherein the concentration of the substrate selected from the group consisting of L-lysine, N-trimethyllysine, β-hydroxy-N-trimethyllysine, γ-N-trimethylaminobutyraldehyde, γ-butyrobetaine and mixtures thereof is 0.1-10 weight % based on the weight of a culture medium.

* * * * *

Levocarnitine
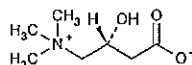
$C_7H_{15}NO_3$ 161.20

(*R*)-3-Carboxy-2-hydroxy- *N, N, N*-trimethyl-1-propanaminium hydroxide, inner salt.
(*R*)-(3-Carboxy-2-hydroxypropyl)trimethylammonium hydroxide, inner salt   [*541-15-1*].

» Levocarnitine contains not less than 97.0 percent and not more than 103.0 percent of $C_7H_{15}NO_3$, calculated on the anhydrous basis.

Packaging and storage— Preserve in tight containers.

USP Reference standards ⟨ 11 ⟩ — *USP Levocarnitine RS* .

Identification, *Infrared Absorption* ⟨ 197K ⟩ — The test specimen and the Reference Standard are dried previously in vacuum at 50° for 5 hours.

Specific rotation ⟨ 781S ⟩ : between −29° and −32°.

Test solution: 100 mg per mL, in water.

pH ⟨ 791 ⟩ : between 5.5 and 9.5 in a solution (1 in 20).

Water content ⟨ 921 ⟩ : not more than 4.0%.

Residue on ignition ⟨ 281 ⟩ : not more than 0.5%.

Change to read:
Chloride ⟨ 221 ⟩ ¹ — A 0.090-g portion shows no more chloride than corresponds to 0.50 mL of 0.020 N hydrochloric acid (0.4%).₁₁

Heavy metals ⟨ 231 ⟩ : not more than 0.002%.

$$\frac{0.002 g}{100 g} \times \frac{10^6 mcg}{} = 20 ppm$$

Limit of potassium— [N OTE — The *Standard solution* and the *Test solutions* may be modified, if necessary, to obtain solutions, of suitable concentrations, adaptable to the linear or working range of the instrument.]

Standard solution— Transfer 5.959 g of potassium chloride, previously dried at 105° for 2 hours and accurately weighed, to a 250-mL volumetric flask, dilute with water to volume, and mix. This solution contains 12.5 mg of potassium per mL. Dilute an accurately measured volume of this solution quantitatively, and stepwise, if necessary, with water to obtain a solution containing 31.25 μg of potassium per mL.

Test solutions— Transfer 62.5 mg of Levocarnitine to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix to obtain a stock solution. To three separate 25-mL volumetric flasks add 0, 2.0, and 4.0 mL of the *Standard solution.* To each flask add 20.0 mL of the stock solution, dilute with

Similac® formulas for every baby's nutritional needs

Use as directed by your baby's doctor.

Advance	Sensitive	Expert Care
Complete nutrition for the 1st year	For babies with fussiness, gas, & spit-up	For babies needing extra TLC
Advance®	Sensitive	Alimentum®
Organic	Spit-Up	NeoSure®
	Soy	Diarrhea

And **Similac Go & Grow®** for toddlers learning to eat table food

Our **Feeding Expert hotline** is available to help you with feeding questions: **800-986-8800**

Breast milk is recommended. If you choose to use infant formula, the makers of Similac have a formula that's right for your baby.

Filled by volume to 1 Quart. Extra space in bottle allows for shaking and easy pouring.

Have product-related questions? Call 1-800-515-7677, 8:30 am–5:00 pm, Eastern time, weekdays.

www.Similac.com

SAMPLE UPC
0 70074 59884 0

U.S. Patent Nos. 5,700,590; 6,136,858; 6,596,767; 7,090,879; D416,801 and D419,455
© 2011 Abbott Laboratories
#59883 40072 8954-01

Abbott

READY TO FEED | DO NOT ADD WATER

DHA/ARA
BIRTH TO 12 MONTHS
MILK-BASED

BRAND FED #1 IN HOSPITALS

Similac® ADVANCE®

IMMUNE SUPPORT
Early Shield®
BRAIN & EYE · STRONG BONES

LUTEIN & DHA

Organic
Complete Nutrition

USDA ORGANIC

For Your Baby's 1st Year

Infant Formula with Iron

1 QT (946 mL)

NUTRIENTS PER 100 CALORIES (5 FL OZ)

PROTEIN	2.07	G	WATER	133	G
FAT	5.40	G	LINOLEIC ACID	860	MG
CARBOHYDRATE	10.9	G			

VITAMINS

VITAMIN A	300	IU	NIACIN	1050	MCG
VITAMIN D	60	IU	FOLIC ACID (FOLACIN)	15	MCG
VITAMIN E	1.5	IU	PANTOTHENIC ACID	450	MCG
VITAMIN K	8	MCG	BIOTIN	4.4	MCG
THIAMIN (VIT. B₁)	100	MCG	VITAMIN C		
RIBOFLAVIN (VIT. B₂)	150	MCG	(ASCORBIC ACID)	9	MG
VITAMIN B₆	60	MCG	CHOLINE	16	MG
VITAMIN B₁₂	0.25	MCG	INOSITOL	4.7	MG

MINERALS

CALCIUM	78	MG	COPPER	90	MCG
PHOSPHORUS	42	MG	IODINE	6	MCG
MAGNESIUM	6	MG	SELENIUM	1.8	MCG
IRON	1.8	MG	SODIUM	24	MG
ZINC	0.75	MG	POTASSIUM	105	MG
MANGANESE	5	MCG	CHLORIDE	65	MG

INGREDIENTS: WATER, ORGANIC NONFAT MILK, ORGANIC MALTODEXTRIN, ORGANIC SUGAR, ORGANIC HIGH OLEIC SUNFLOWER OIL, ORGANIC SOY OIL, ORGANIC COCONUT OIL; LESS THAN 0.5% OF: C. COHNII OIL*, M. ALPINA OIL†, BETA-CAROTENE, LUTEIN, LYCOPENE, FRUCTOOLIGOSACCHARIDES, POTASSIUM CITRATE, CALCIUM CARBONATE, ASCORBIC ACID, SOY LECITHIN, CARRAGEENAN, MAGNESIUM CHLORIDE, SALT, FERROUS SULFATE, CHOLINE CHLORIDE, CHOLINE BITARTRATE, TAURINE, m-INOSITOL, d-ALPHA-TOCOPHERYL ACETATE, L-CARNITINE, ZINC SULFATE, NIACINAMIDE, CALCIUM PANTOTHENATE, RIBOFLAVIN, VITAMIN A PALMITATE, CUPRIC SULFATE, THIAMINE CHLORIDE HYDROCHLORIDE, PYRIDOXINE HYDRO-CHLORIDE, FOLIC ACID, MANGANESE SULFATE, PHYLLOQUINONE, BIOTIN, POTASSIUM IODIDE, SODIUM SELENATE, VITAMIN D₃, CYANOCOBALAMIN, POTASSIUM HYDROXIDE AND NUCLEOTIDES (ADENOSINE 5'-MONOPHOSPHATE, CYTIDINE 5'-MONOPHOSPHATE, DISODIUM GUANOSINE 5'-MONOPHOSPHATE, DISODIUM URIDINE 5'-MONOPHOSPHATE).
CONTAINS MILK AND SOY INGREDIENTS.

*SOURCE OF DOCOSAHEXAENOIC ACID (DHA) †SOURCE OF ARACHIDONIC ACID (ARA)

USE AS DIRECTED BY A DOCTOR
Directions for Preparation and Use

USE BY DATE ON BOTTLE • DO NOT ADD WATER
DO NOT USE IF BAND AROUND CAP
OR INNER FOIL SEAL IS DAMAGED.

Your baby's health depends on carefully following these directions. Failure to follow these directions could result in severe harm. Ask your baby's doctor if you need to boil (sterilize) bottles, nipples and rings before use.

Use

1. Shake very well before each use
Remove protective band; twist off and clean cap

2. Invert cap; press down to pierce foil, then turn cap a half turn
Remove foil

3. Pour formula into bottle; attach nipple
Once feeding begins, use within 1 hour or discard

Prepare and feed all formula in this container within 3 days after opening.

Storage

Once opened, store quart bottle in refrigerator. Store prepared bottles in refrigerator and **feed to baby within 48 hours.** Store unopened containers at room temperature; avoid extreme temperatures. Do not reuse container.

Warning

Do not use this container to warm formula. **Never use a microwave to warm formula.** Serious burns can result.

Abbott Nutrition, Abbott Laboratories, Columbus, Ohio 43219-3034 USA

CERTIFIED ORGANIC BY QUALITY ASSURANCE INTERNATIONAL

Abbott

DHA/ARA
BIRTH TO **12** MONTHS
MILK-BASED | POWDER

BRAND FED #1 IN HOSPITALS

Similac®
ADVANCE®

IMMUNE SUPPORT
Early Shield®
BRAIN & EYE · STRONG BONES

LUTEIN & DHA

Organic
Complete Nutrition

USDA ORGANIC

For Your
Baby's 1st Year

Infant
Formula
with Iron

NET WT. 1.45 LB (658 g)

NUTRIENTS PER 100 CALORIES (5 FL OZ, PREPARED AS DIRECTED)

PROTEIN	2.07	G	WATER	133	G
FAT	5.63	G	LINOLEIC ACID	860	MG
CARBOHYDRATE	10.4	G			

VITAMINS

VITAMIN A	300	IU	NIACIN	1050	MCG
VITAMIN D	60	IU	FOLIC ACID (FOLACIN)	15	MCG
VITAMIN E	1.5	IU	PANTOTHENIC ACID	450	MCG
VITAMIN K	8	MCG	BIOTIN	4.4	MCG
THIAMIN (VIT. B_1)	100	MCG	VITAMIN C		
RIBOFLAVIN (VIT. B_2)	150	MCG	(ASCORBIC ACID)	9	MG
VITAMIN B_6	60	MCG	CHOLINE	16	MG
VITAMIN B_{12}	0.25	MCG	INOSITOL	4.7	MG

MINERALS

CALCIUM	78	MG	COPPER	90	MCG
PHOSPHORUS	42	MG	IODINE	6	MCG
MAGNESIUM	6	MG	SELENIUM	1.8	MCG
IRON	1.8	MG	SODIUM	24	MG
ZINC	0.75	MG	POTASSIUM	105	MG
MANGANESE	5	MCG	CHLORIDE	65	MG

INGREDIENTS: ORGANIC NONFAT MILK, **ORGANIC** MALTODEXTRIN, **ORGANIC** SUGAR, **ORGANIC** HIGH OLEIC SUNFLOWER OIL, **ORGANIC** SOY OIL, **ORGANIC** COCONUT OIL; **LESS THAN 2% OF:** C. COHNII OIL*, M. ALPINA OIL†, BETA-CAROTENE, LUTEIN, LYCOPENE, FRUCTOOLIGOSACCHARIDES, POTASSIUM CITRATE, CALCIUM CARBONATE, ASCORBIC ACID, SOY LECITHIN, ASCORBYL PALMITATE, FERROUS SULFATE, SALT, CHOLINE CHLORIDE, CHOLINE BITARTRATE, TAURINE, m-INOSITOL, MAGNESIUM CHLORIDE, ZINC SULFATE, MIXED TOCOPHEROLS, d-ALPHA-TOCOPHERYL ACETATE, NIACINAMIDE, CALCIUM PANTOTHENATE, L-CARNITINE, VITAMIN A PALMITATE, CUPRIC SULFATE, THIAMINE CHLORIDE HYDROCHLORIDE, RIBOFLAVIN, PYRIDOXINE HYDROCHLORIDE, FOLIC ACID, MANGANESE SULFATE, PHYLLOQUINONE, BIOTIN, SODIUM SELENATE, VITAMIN D_3, CYANOCOBALAMIN, POTASSIUM IODIDE, POTASSIUM HYDROXIDE AND NUCLEOTIDES (CYTIDINE 5'-MONOPHOSPHATE, DISODIUM GUANOSINE 5'-MONOPHOSPHATE, DISODIUM URIDINE 5'-MONOPHOSPHATE, ADENOSINE 5'-MONOPHOSPHATE).
CONTAINS MILK AND SOY INGREDIENTS.
*SOURCE OF DOCOSAHEXAENOIC ACID (DHA) †SOURCE OF ARACHIDONIC ACID (ARA)

Abbott Nutrition, Abbott Laboratories
Columbus, Ohio 43219-3034 USA

CERTIFIED ORGANIC BY QUALITY ASSURANCE INTERNATIONAL

USE BY DATE ON CONTAINER · **USE AS DIRECTED BY A DOCTOR**
Directions for Preparation and Use

Your baby's health depends on carefully following these directions. Proper hygiene, handling and storage are important when preparing infant formula. Failure to follow these directions could result in severe harm. Ask your baby's doctor if you need to use cooled, boiled water for mixing and if you need to boil (sterilize) bottles, nipples and rings before use.

1 Wash your hands, surfaces and utensils

Pour water into clean bottle (see mixing guide)

2 Add 1 unpacked level scoop (8.6 g) to each 2 fl oz of water

Return dry scoop to holder in lid

3 Cap bottle; shake well; attach nipple

Once feeding begins, use within 1 hour or discard

Storage: Once mixed, store bottles in refrigerator and **feed to baby within 24 hours.** Store unopened or opened container at room temperature; avoid extreme temperatures. **Use opened container contents within 1 month.** Do not reuse container.

Warning: Powdered infant formulas are not sterile and should not be fed to premature infants or infants who might have immune problems unless directed and supervised by your baby's doctor. **Never use a microwave to warm formula.** Serious burns can result.

DO NOT USE IF OUTER QUALITY SEAL OR INNER FOIL SEAL IS DAMAGED.

U.S. Patent Nos. 5,700,590; 6,136,858; 6,596,767; 7,090,879; D576,035 and D578,401

POSITION ONLY

0 70074 50822 1

© 2011 Abbott Laboratories #50821 40075 8954-01

MIXING GUIDE
GUÍA DE MEZCLA

PEEL HERE
Desprenda aquí

IMPORTANT NOTICE: BREAST MILK IS BEST FOR BABIES. Before using an infant formula, ask the advice of your health-care professional. If you choose to supplement breastfeeding with formula or to formula feed exclusively, there is no better formula than Vermont Organics™ ORGANIC Infant Formula DHA and ARA. **Powdered infant formulas are NOT sterile and should NOT be fed to premature infants or infants who might have immune problems unless directed and supervised by your baby's doctor.**

DIRECTIONS FOR PREPARATION AND USE

Your baby's health depends on carefully following these preparation, use and storage instructions; changes could affect your baby's nutrition and safety. Before preparing the infant formula, make sure to always wash your hands. Clean can top before opening. **Ask your baby's doctor about the need to boil or sterilize water for formula and the proper preparation of bottle and feeding utensils.**

Storage: Store unused prepared bottled formula in the refrigerator at 35-40°F (2-4°C); **use within 24 hours.** DO NOT FREEZE. Warm infant formula to room temperature and shake well before feeding. Prepared formula should not be without refrigeration more than 2 hours. Store open and unopened cans in a dry area at room temperature. Cover the opened can tightly with plastic cap; use contents within 1 month. Avoid any extreme temperatures. **Warning: Do not use microwave to prepare or warm formula. Serious burns may occur.** Filled by weight, not by volume. Contents may settle during shipment.

Contents yield approximately 190 fl oz of formula

1. Pour desired amount of warm water (approx. 100°F/40°C) into bottle. (See Feeding Chart below.)
2. Add powder. Always add powder to water.
3. Cap bottle and shake well until powder is dissolved. Feed immediately. Discard any remaining formula in bottle after 1 hour from start of feeding.

Use this Feeding Chart for proper amount of water and powder.

To Make	Water	Powder (Use scoop enclosed to measure)
2 fl oz bottle	2 fl oz	1 unpacked level **scoop** (8.7 g)
4 fl oz bottle	4 fl oz	2 unpacked level **scoops** (17.4 g)
6 fl oz bottle	6 fl oz	3 unpacked level **scoops** (26.1 g)
8 fl oz bottle	8 fl oz	4 unpacked level **scoops** (34.8 g)
1 quart	29 fl oz	1 unpacked level standard measuring **cup & 2** unpacked level **scoops** (123 g)

Easy to Digest
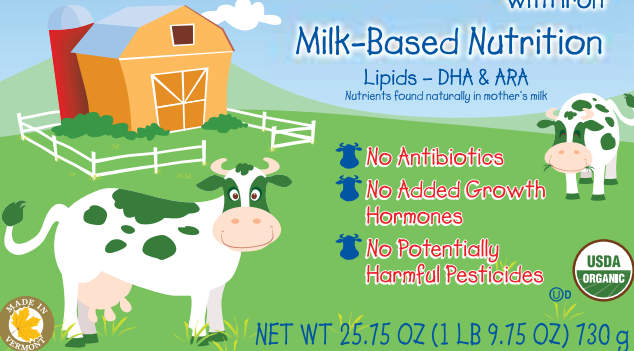Powder • Add Water

For Babies
0–12 Months

Vermont Organics™

organic infant formula
with iron

Milk-Based Nutrition
Lipids – DHA & ARA
Nutrients found naturally in mother's milk

- No Antibiotics
- No Added Growth Hormones
- No Potentially Harmful Pesticides

USDA ORGANIC

NET WT 25.75 OZ (1 LB 9.75 OZ) 730 g

INGREDIENTS: ORGANIC REDUCED MINERALS WHEY, **ORGANIC** VEGETABLE OILS (PALM OR PALM OLEIN, HIGH OLEIC (SAFFLOWER OR SUNFLOWER), COCONUT, SOY), **ORGANIC** NONFAT MILK, **ORGANIC** LACTOSE, **AND LESS THAN 1%:** MORTIERELLA ALPINA OIL*, CRYPTHECODINIUM COHNII OIL**, VITAMIN A PALMITATE, BETA-CAROTENE, VITAMIN D (CHOLECALCIFEROL), VITAMIN E (dl-ALPHA TOCOPHERYL ACETATE), MIXED TOCOPHEROL CONCENTRATE, VITAMIN K (PHYTONADIONE), ASCORBYL PALMITATE, THIAMINE HYDROCHLORIDE, RIBOFLAVIN, PYRIDOXINE HYDROCHLORIDE, CYANOCOBALAMIN, NIACINAMIDE, FOLIC ACID, CALCIUM PANTOTHENATE, BIOTIN, ASCORBIC ACID, CHOLINE CHLORIDE, INOSITOL, CALCIUM CHLORIDE, CALCIUM HYDROXIDE, FERROUS SULFATE, ZINC SULFATE, MANGANESE SULFATE, CUPRIC SULFATE, POTASSIUM BICARBONATE, POTASSIUM CHLORIDE, POTASSIUM IODIDE, POTASSIUM HYDROXIDE, SODIUM SELENITE, SODIUM CITRATE, TAURINE, SOY LECITHIN, NUCLEOTIDES (ADENOSINE-5'-MONOPHOSPHATE, CYTIDINE-5'-MONOPHOSPHATE, DISODIUM GUANOSINE-5'- MONOPHOSPHATE, DISODIUM INOSINE-5'-MONOPHOSPHATE, DISODIUM URIDINE-5'MONOPHOSPHATE). **CONTAINS MILK AND SOY INGREDIENTS.**

Diluted: Each 5 fl oz (150 mL) contains 100 Calories

NUTRIENTS:	PER 100 CALORIES:
PROTEIN g	2.2
FAT g	5.3
CARBOHYDRATE g	10.6
WATER g	134
LINOLEIC ACID mg	750

VITAMINS:	PER 100 CALORIES:
VITAMIN A IU	300
VITAMIN D IU	60
VITAMIN E IU	2
VITAMIN K mcg	8
THIAMINE (VITAMIN B₁) mcg	100
RIBOFLAVIN (VITAMIN B₂) mcg	150
VITAMIN B₆ mcg	63
VITAMIN B₁₂ mcg	0.2
NIACIN mcg	750
FOLIC ACID (FOLACIN) mcg	7.5
PANTOTHENIC ACID mcg	315

VITAMINS:	PER 100 CALORIES:
BIOTIN mcg	2.3
VITAMIN C (ASCORBIC ACID) mg	9
CHOLINE mg	15
INOSITOL mg	4.1

MINERALS:	
CALCIUM mg	63
PHOSPHORUS mg	42
MAGNESIUM mg	7
IRON mg	1.8
ZINC mg	0.75
MANGANESE mcg	15
COPPER mcg	71
IODINE mcg	9
SELENIUM mcg	2.1
SODIUM mg	23
POTASSIUM mg	84
CHLORIDE mg	56

DISTRIBUTED BY:
VERMONT ORGANICS INFANT FORMULA
147 IND. PARK RD. GEORGIA, VT 05468

CERTIFIED ORGANIC BY QUALITY ASSURANCE INTERNATIONAL 02FTP

* A SOURCE OF ARACHIDONIC ACID (ARA)
** A SOURCE OF DOCOSAHEXAENOIC ACID (DHA)

Questions or Comments:
1-800-538-7615
9:00 a.m. to 5:00 p.m. EST
Monday – Friday

Produced under an ISO-9001 Quality System.

All infant formula sold in the U.S. are required to be manufactured in accordance with, and meet the nutritional requirements of, the Federal Food, Drug and Cosmetic Act for infant formula under the regulation of the U.S. Food and Drug Administration.

life's DHA•ARA
LIFE'SDHA+ARA™ is a trademark of Martek Biosciences Corporation.

6 83744 13001 3
USE BEFORE DATE ON BOTTOM OF CAN

Breast milk is best. But if you decide to supplement breastfeeding with formula or formula-feed exclusively, Vermont Organics™ ORGANIC Infant Formula is a wholesome choice for your baby.

Made in the heart of the Green Mountains, Vermont Organics™ ORGANIC meets all USDA organic certification requirements. In addition to containing sources of organic fat, protein and carbohydrate, Vermont Organics™ ORGANIC is produced without the use of antibiotics, added growth hormones or potentially harmful pesticides.

MADE IN VERMONT

Las instrucciones en Español se encuentran al dorso de esta etiqueta.

IMPORTANT NOTICE: BREAST MILK IS BEST FOR BABIES. Before using an infant formula, ask the advice of your health-care professional. This organic soy infant formula powder is free of lactose and cow's milk protein. It is intended to meet the nutritional needs of term infants and babies who are sensitive to lactose or those who do not consume milk protein or milk products. **Powdered infant formulas are NOT sterile and should NOT be fed to premature infants or infants who might have immune problems unless directed and supervised by your baby's doctor.**

DIRECTIONS FOR PREPARATION AND USE

Your baby's health depends on carefully following these preparation, use and storage instructions; changes could affect your baby's nutrition and safety. Before preparing the infant formula, make sure to always wash your hands. Clean can top before opening. **Ask your baby's doctor about the need to boil or sterilize water for formula and the proper preparation of bottle and feeding utensils.**

Storage: Store unused prepared bottled formula in the refrigerator at 35-40°F (2-4°C); **use within 24 hours.** DO NOT FREEZE. Warm infant formula to room temperature and shake well before feeding. Prepared formula should not be without refrigeration more than 2 hours. Store open and unopened cans in a dry area at room temperature. Cover the opened can tightly with plastic cap; use contents within 1 month. Avoid any extreme temperatures.
Warning: Do not use microwave to prepare or warm formula. Serious burns may occur.
Filled by weight, not by volume. Contents may settle during shipment.
Contents yield approximately 187 fl oz of formula

1. Pour desired amount of warm water (approx. 100°F/40°C) into bottle. (See Feeding Chart below.)

2. Add powder. Always add powder to water.

3. Cap bottle and shake well until powder is dissolved. Feed immediately. Discard any remaining formula in bottle after 1 hour from start of feeding.

Use this Feeding Chart for proper amount of water and powder

To Make	Water	Powder (Use scoop enclosed to measure)
2 fl oz bottle	2 fl oz	1 unpacked level **scoop** (8.8 g)
4 fl oz bottle	4 fl oz	2 unpacked level **scoops** (17.6 g)
6 fl oz bottle	6 fl oz	3 unpacked level **scoops** (26.4 g)
8 fl oz bottle	8 fl oz	4 unpacked level **scoops** (35.2 g)
1 quart	29 fl oz	1 unpacked level standard measuring **cup &** 2 unpacked level **scoops** (125 g)

Martek DHA

Easy to Digest
Powder • Add Water

For Babies
0-12 Months

Vermont Organics™

soy organic infant formula
with iron
Soy-Based Nutrition
Lipids – DHA & ARA
Nutrients found naturally in mother's milk

● Milk-Free
● Lactose-Free
● No Potentially Harmful Pesticides

soy

MADE IN VERMONT

Ⓤ Pareve

USDA ORGANIC

NET WT 25.75 OZ (1 LB 9.75 OZ) 730 g

INGREDIENTS: **ORGANIC** CORN SYRUP SOLIDS, **ORGANIC** SOY PROTEIN, **ORGANIC** PALM OLEIN OR PALM OIL, **ORGANIC** COCONUT OIL, **ORGANIC** HIGH OLEIC (SAFFLOWER OR SUNFLOWER) OIL, **ORGANIC** SOY OIL, **AND LESS THAN 1%:** MORTIERELLA ALPINA OIL*, CRYPTHECODINIUM COHNII OIL**, ASCORBYL PALMITATE, L-CARNITINE, L-METHIONINE, MIXED TOCOPHEROL CONCENTRATE, SOY LECITHIN, TAURINE, CALCIUM CHLORIDE, CALCIUM PHOSPHATE, CUPRIC SULFATE, FERROUS SULFATE, MAGNESIUM CHLORIDE, MANGANESE SULFATE, POTASSIUM BICARBONATE, POTASSIUM CHLORIDE, POTASSIUM CITRATE, POTASSIUM HYDROXIDE, POTASSIUM IODIDE, SODIUM CITRATE, SODIUM SELENITE, ZINC SULFATE, ASCORBIC ACID, BETA-CAROTENE, BIOTIN, CALCIUM PANTOTHENATE, CHOLINE BITARTRATE, CYANOCOBALAMIN, FOLIC ACID, INOSITOL, NIACINAMIDE, PYRIDOXINE HYDROCHLORIDE, RIBOFLAVIN, THIAMINE HYDROCHLORIDE, VITAMIN A PALMITATE, VITAMIN D (CHOLECALCIFEROL), VITAMIN E (dl-ALPHA TOCOPHERYL ACETATE), VITAMIN K (PHYTONADIONE).
CONTAINS SOY INGREDIENTS.

Diluted: Each 5 fl oz (150 mL) contains 100 Calories

NUTRIENTS:	PER 100 CALORIES:		VITAMINS:	PER 100 CALORIES:
PROTEIN g	2.5		BIOTIN mcg	3
FAT g	5.3		VITAMIN C (ASCORBIC ACID) mg	12
CARBOHYDRATE g	10.6		CHOLINE mg	24
WATER g	134		INOSITOL mg	6
LINOLEIC ACID mg	750		**MINERALS:**	
VITAMINS:			CALCIUM mg	105
VITAMIN A IU	300		PHOSPHORUS mg	69
VITAMIN D IU	60		MAGNESIUM mg	11
VITAMIN E IU	2		IRON mg	1.8
VITAMIN K mcg	8		ZINC mg	1.2
THIAMINE (VITAMIN B₁) mcg	80		MANGANESE mcg	25
RIBOFLAVIN (VITAMIN B₂) mcg	90		COPPER mcg	75
VITAMIN B₆ mcg	60		IODINE mcg	15
VITAMIN B₁₂ mcg	0.3		SELENIUM mcg	2.8
NIACIN mcg	1000		SODIUM mg	36
FOLIC ACID (FOLACIN) mcg	16		POTASSIUM mg	120
PANTOTHENIC ACID mcg	500		CHLORIDE mg	80

DISTRIBUTED BY:
VERMONT ORGANICS INFANT FORMULA
141 IND. PARK RD, GEORGIA, VT 05468

CERTIFIED ORGANIC BY QUALITY ASSURANCE INTERNATIONAL 02FTP

* A SOURCE OF ARACHIDONIC ACID (ARA) ** A SOURCE OF DOCOSAHEXAENOIC ACID (DHA)

Questions or Comments:
1-800-538-7615
9:00 a.m. to 5:00 p.m. EST
Monday – Friday

Produced under
an ISO-9001
Quality System.

All infant formulas sold in the U.S. are required to be manufactured in accordance with, and meet the nutritional requirements of, the Federal Food, Drug and Cosmetic Act for infant formula under the regulation of the U.S. Food and Drug Administration.

6 83744 13002 0

USE BEFORE DATE ON BOTTOM OF CAN

Breast milk is best. But if you decide to supplement breastfeeding with formula or formula-feed exclusively, and lactose-sensitivity is a concern, *Vermont Organics™ Soy ORGANIC Infant Formula* is a wholesome choice for your baby.

Made in the heart of the Green Mountains, Vermont Organics™ Soy ORGANIC meets all USDA organic certification requirements. A milk-free, lactose-free baby formula containing organic soy protein.

Las instrucciones en Español se encuentran al dorso de esta etiqueta.

Lonza

Abbott Nutrition

Lonza Sales Ltd
Muenchensteinerstrasse 38
CH-4002 Basel

Richard Sasse
Lonza Life Science Ingredients

Tel +31 76 5480018
richard.sasse@lonza.com

RS
November 2, 2011

Environmental statement

Dear Sirs,

We herewith conform that the products Carnipure™ crystalline and Carnipure™ tartrate as produced by Lonza are produced at production sites which have a documented Environmental Management System in place.

The sites have environmental roles and responsibilities defined and documented and objectives and targets are defined and environmental training is in place.

The sites maintain an annual reporting of progress and performance in which resource consumption, air emissions, waste water and waste products are registered and evaluated on a site level.

The production is in closed systems. All materials used are registered and all material output is registered. Side products are as far as possible re-used in production. Material which can not be re-used is treated where possible on site in order to reduce the amount of waste material. Final waste material is disposed to qualified external contractors who treat the material further.

Both sites are also registered with Sedex.

Kind regards,
Lonza Sales Ltd.

Richard Sasse
Global Business Manager Carnipure

Michael DeGennaro
Global VP Marketing and Sales Human Nutrition

Lonza

Policy on Safety
Health and Environment

Lonza is committed to operations and practices, which prevent harm to people and damage to environment or property. The following principles for safety, health and environment (SHE) resulting from the company's ethical conviction take precedence and apply throughout the group.

We actively manage SHE as an integral part of our business and operations practices, and apply comprehensive SHE management systems.

We require our regional and local managements to comply in their area of responsibility with the applicable laws and the internal and external SHE requirements.

We require our regional and local managements to integrate security measures into their SHE management systems. Security measures must at least encompass the protection of facilities, equipment, know-how and data, and the transportation and storage of dangerous goods.

We strive to minimize the environmental impact of our operations and business practices as well as to optimize the use of natural resources by our processes and products.

We will maintain a safe workplace environment for our employees and provide appropriate information and training to increase their skills and promote their safety, health and environmental awareness. All employees have a fundamental responsibility for SHE matters at work.

We are committed to continual improvement of our SHE performance and measure the progress by specific SHE performance indicators. We promote measures to increase the ecoefficiency of our investments and optimize our SHE costs, while maintaining technological competitiveness.

We operate only processes, apply technologies and develop and handle products, which are assessed for their SHE risks. Safe handling and use of our products is to be guaranteed internally as well as communicated externally.

New or modified processes and installations must be subjected to a systematic risk assessment prior to introduction. All raw materials, intermediates and products are assessed for their regulatory compliance.

We have emergency procedures in place and emergency response organizations at all our sites to control and to limit the impact of incidents and threats. All internal and external communication channels in case of an emergency are defined and operable.

We periodically audit our operations, business and management practices at all our sites with regard to SHE performance and compliance through independent experts.

We openly communicate and provide information on our SHE performance to our employees, customers, shareholders and investors, governmental authorities and the public at large.

Lonza wants to ensure that its business practices conform to its ethical conviction and the principles of sustainable development.

Stefan Borgas
Chief Executive Officer

April 2010

The legal structure of the subsidiaries and affiliates, as well as the legal structure of their organs and employees will remain unaffected by this policy.

Material Safety Data Sheet

Creation Date 19-Apr-2010   **Revision Date** 19-Apr-2010   **Revision Number** 1

1. PRODUCT AND COMPANY IDENTIFICATION

Product Name   **L(-)-Carnitine**

Cat No.   **AC241040000; AC241040010; AC241040100; AC241040500**

Synonyms   3-Hydroxy-4-(trimethylammonio)butanoate; Vitamin BT

Recommended Use   Laboratory chemicals

Company	**Entity / Business Name**	**Emergency Telephone Number**
Fisher Scientific	Acros Organics	For information in the US, call: 800-ACROS-01
One Reagent Lane	One Reagent Lane	For information in Europe, call: +32 14 57 52 11
Fair Lawn, NJ 07410	Fair Lawn, NJ 07410	
Tel: (201) 796-7100		Emergency Number, Europe: +32 14 57 52 99
		Emergency Number, US: 201-796-7100
		CHEMTREC Phone Number, US: 800-424-9300
		CHEMTREC Phone Number, Europe: 703-527-3887

2. HAZARDS IDENTIFICATION

WARNING!

Emergency Overview
Irritating to eyes, respiratory system and skin. Hygroscopic.

Appearance White   **Physical State** Solid   **odor** odorless

Target Organs   Skin, Respiratory system, Eyes

Potential Health Effects

Acute Effects
Principle Routes of Exposure

_____

Thermo Fisher Scientific - **L(-)-Carnitine**   **Revision Date** 19-Apr-2010
_____

Eyes	Irritating to eyes.
Skin	Irritating to skin. May be harmful in contact with skin.
Inhalation	Irritating to respiratory system. May be harmful if inhaled.
Ingestion	May be harmful if swallowed. Ingestion may cause gastrointestinal irritation, nausea, vomiting and diarrhea.

Chronic Effects   None known.

See Section 11 for additional Toxicological information.

Aggravated Medical Conditions   No information available.

3. COMPOSITION/INFORMATION ON INGREDIENTS

Haz/Non-haz

Component	CAS-No	Weight %
(R)-(3-Carboxy-2-hydroxypropyl)trimethylammonium hydroxide	541-15-1	>95

4. FIRST AID MEASURES

Eye Contact   Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Obtain medical attention.

Skin Contact   Wash off immediately with plenty of water for at least 15 minutes. Obtain medical attention.

Inhalation   Move to fresh air. If breathing is difficult, give oxygen. Obtain medical attention.

Ingestion   Do not induce vomiting. Obtain medical attention.

Notes to Physician   Treat symptomatically.

5. FIRE-FIGHTING MEASURES

Flash Point   No information available.
   Method   No information available.

Autoignition Temperature   No information available.
Explosion Limits
   Upper   No data available
   Lower   No data available

Suitable Extinguishing Media   Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.

Unsuitable Extinguishing Media   No information available.

Hazardous Combustion Products   No information available.

   Sensitivity to mechanical impact   No information available.
   Sensitivity to static discharge   No information available.

Specific Hazards Arising from the Chemical
Keep product and empty container away from heat and sources of ignition

_____

_____

Protective Equipment and Precautions for Firefighters
As in any fire, wear self-contained breathing apparatus pressure-demand, MSHA/NIOSH (approved or equivalent) and full protective gear. Thermal decomposition can lead to release of irritating gases and vapors.

NFPA	**Health** 2	**Flammability** 1	**Instability** 0	**Physical hazards** N/A

6. ACCIDENTAL RELEASE MEASURES

Personal Precautions   Ensure adequate ventilation. Use personal protective equipment. Avoid dust formation.

Environmental Precautions   Should not be released into the environment.

Methods for Containment and Clean   Sweep up or vacuum up spillage and collect in suitable container for disposal. Avoid dust
Up   formation.

7. HANDLING AND STORAGE

Handling   Wear personal protective equipment. Ensure adequate ventilation. Do not get in eyes, on skin, or on clothing. Avoid ingestion and inhalation. Avoid dust formation.

Storage   Keep containers tightly closed in a dry, cool and well-ventilated place. Keep refrigerated.

8. EXPOSURE CONTROLS / PERSONAL PROTECTION

Engineering Measures   Ensure that eyewash stations and safety showers are close to the workstation location. Ensure adequate ventilation, especially in confined areas.

Exposure Guidelines   This product does not contain any hazardous materials with occupational exposure limits established by the region specific regulatory bodies.

NIOSH IDLH: Immediately Dangerous to Life or Health

Personal Protective Equipment
  Eye/face Protection   Wear appropriate protective eyeglasses or chemical safety goggles as described by OSHA's eye and face protection regulations in 29 CFR 1910.133 or European Standard EN166.
  Skin and body protection   Wear appropriate protective gloves and clothing to prevent skin exposure.
  Respiratory Protection   Follow the OSHA respirator regulations found in 29 CFR 1910.134 or European Standard EN 149. Use a NIOSH/MSHA or European Standard EN 149 approved respirator if exposure limits are exceeded or if irritation or other symptoms are experienced.

9. PHYSICAL AND CHEMICAL PROPERTIES

Physical State   Solid
Appearance   White
odor   odorless
Odor Threshold   No information available.
pH   5-9 5% aq. sol.
Vapor Pressure   No information available.
Vapor Density   No information available.
Viscosity   No information available.
Boiling Point/Range   No information available.
Melting Point/Range   208 - 212°C / 406.4 - 413.6°F
Decomposition temperature   > 365°C

_____

Thermo Fisher Scientific - **L(-)-Carnitine**   **Revision Date** 19-Apr-2010
_____

9. PHYSICAL AND CHEMICAL PROPERTIES

Flash Point	No information available.
Evaporation Rate	No information available.
Specific Gravity	No information available.
Solubility	Soluble in water
log Pow	No data available
Molecular Weight	161.2
Molecular Formula	C7 H15 N O3

10. STABILITY AND REACTIVITY

Stability	Hygroscopic.
Conditions to Avoid	Incompatible products. Excess heat. Avoid dust formation. Exposure to moist air or water.
Incompatible Materials	Strong oxidizing agents
Hazardous Decomposition Products	Carbon monoxide (CO), Carbon dioxide (CO_2), Nitrogen oxides (NOx)
Hazardous Polymerization	Hazardous polymerization does not occur.
Hazardous Reactions .	None under normal processing..

11. TOXICOLOGICAL INFORMATION

Acute Toxicity

Component Information

Component	LD50 Oral	LD50 Dermal	LC50 Inhalation
(R)-(3-Carboxy-2-hydroxypropyl)trimethylammonium hydroxide	>5g/kg (rat)	Not listed	Not listed

Irritation	Irritating to eyes, respiratory system and skin
Toxicologically Synergistic Products	No information available.

Chronic Toxicity

Carcinogenicity	There are no known carcinogenic chemicals in this product
Sensitization	No information available.
Mutagenic Effects	No information available.
Reproductive Effects	No information available.

_____

_____

Developmental Effects	No information available.
Teratogenicity	No information available.
Other Adverse Effects	See actual entry in RTECS for complete information. The toxicological properties have not been fully investigated..
Endocrine Disruptor Information	No information available

12. ECOLOGICAL INFORMATION

Ecotoxicity
No information available..

Persistence and Degradability	No information available
Bioaccumulation/ Accumulation	No information available
Mobility	No information available

13. DISPOSAL CONSIDERATIONS

Waste Disposal Methods	Chemical waste generators must determine whether a discarded chemical is classified as a hazardous waste. Chemical waste generators must also consult local, regional, and national hazardous waste regulations to ensure complete and accurate classification.

14. TRANSPORT INFORMATION

DOT	Not regulated
TDG	Not regulated
IATA	Not regulated
IMDG/IMO	Not regulated

15. REGULATORY INFORMATION

International Inventories

Component	TSCA	DSL	NDSL	EINECS	ELINCS	NLP	PICCS	ENCS	AICS	CHINA	KECL

_____

Thermo Fisher Scientific - L(-)-Carnitine   **Revision Date** 19-Apr-2010

_____

15. REGULATORY INFORMATION

(R)-(3-Carboxy-2-hydroxypropyl)trimethylammonium hydroxide	-	-	-	208-768-0	-		X	X	X	X	-

Legend:
X - Listed
E - Indicates a substance that is the subject of a Section 5(e) Consent order under TSCA.
F - Indicates a substance that is the subject of a Section 5(f) Rule under TSCA.
N - Indicates a polymeric substance containing no free-radical initiator in its inventory name but is considered to cover the designated polymer made with any free-radical initiator regardless of the amount used.
P - Indicates a commenced PMN substance
R - Indicates a substance that is the subject of a Section 6 risk management rule under TSCA.
S - Indicates a substance that is identified in a proposed or final Significant New Use Rule
T - Indicates a substance that is the subject of a Section 4 test rule under TSCA.
XU - Indicates a substance exempt from reporting under the Inventory Update Rule, i.e. Partial Updating of the TSCA Inventory Data Base Production and Site Reports (40 CFR 710(B).
Y1 - Indicates an exempt polymer that has a number-average molecular weight of 1,000 or greater.
Y2 - Indicates an exempt polymer that is a polyester and is made only from reactants included in a specified list of low concern reactants that comprises one of the eligibility criteria for the exemption rule.

U.S. Federal Regulations

TSCA 12(b) Not applicable

SARA 313
Not applicable

SARA 311/312 Hazardous Categorization
Acute Health Hazard	Yes
Chronic Health Hazard	No
Fire Hazard	No
Sudden Release of Pressure Hazard	No
Reactive Hazard	No

Clean Water Act
Not applicable

Clean Air Act
Not applicable

OSHA
Not applicable

CERCLA
Not Applicable

California Proposition 65
This product does not contain any Proposition 65 chemicals.

State Right-to-Know
Not applicable

U.S. Department of Transportation
Reportable Quantity (RQ):	N
DOT Marine Pollutant	N

_____

_____

DOT Severe Marine Pollutant   N

U.S. Department of Homeland Security
This product does not contain any DHS chemicals.

Other International Regulations

Mexico - Grade   No information available

Canada

This product has been classified in accordance with the hazard criteria of the Controlled Products Regulations (CPR) and the MSDS contains all the information required by the CPR.

WHMIS Hazard Class
D2B Toxic materials

16. OTHER INFORMATION

Prepared By   Regulatory Affairs
                                         Thermo Fisher Scientific
                                         Tel: (412) 490-8929

Creation Date   19-Apr-2010

Print Date   19-Apr-2010

Revision Summary   "***", and red text indicates revision

Disclaimer
The information provided on this Safety Data Sheet is correct to the best of our knowledge, information and belief at the date of its publication. The information given is designed only as a guide for safe handling, use, processing, storage, transportation, disposal and release and is not to be considered as a warranty or quality specification. The information relates only to the specific material designated and may not be valid for such material used in combination with any other material or in any process, unless specified in the text.

End of MSDS

_____

glanbia
NUTRITIONALS

Glanbia Nutritionals (NA), Inc   Tel: +1 760 438 0089
5927 Geiger Court   Fax: +1 760 438 0336
Carlsbad, CA 92008   www.glanbianutritionals.com

MATERIAL SAFETY DATA SHEET

NAME: L-Carnitine Base	PRODUCT NUMBER: 031402-C
REVISED DATE: December 23, 2009	SUPERCEDES: January 8, 2007

GLANBIA NUTRITIONALS (NA), INC.
5927 GEIGER COURT
CARLSBAD, CA 92008-7305
PHONE 760-438-0089
FAX 760-438-0336

I. PRODUCT INFORMATION

TRADE NAMES/SYNONYMS: L-Carnitine Base

CHEMICAL NAME: L-β-Hydroxy-Trimethylammonium-Butyric Acid (Carnitine Base)

CAS NUMBER: Carnitine Base (541-15-1)

CHEMICAL FAMILY: Vitamins

CHEMICAL FORMULA: (Carnitine base) $C_7H_{15}NO_3$

II. HAZARDOUS INGREDIENTS

None.

III. COMPONENTS

L-Carnitine Base

IV. CHEMICAL AND PHYSICAL PROPERTIES

BOILING POINT: NA	VAPOR PRESSURE: NA
MELTING POINT: NA	VAPOR DENSITY: NA
SOL. IN WATER: Soluble	SPECIFIC GRAVITY: NA
PH (1:20): 5.5-9.5	EVAPORATION RATE: NA

APPEARANCE/ODOR: White crystalline powder with characteristic odor.

1

glanbia
NUTRITIONALS

Glanbia Nutritionals (NA), Inc   Tel: +1 760 438 0089
5927 Geiger Court   Fax: +1 760 438 0336
Carlsbad, CA 92008   www.glanbianutritionals.com

EXTINGUISHING MEDIA: Water fog, dry chemical, foam.

FIRE FIGHTING INSTRUCTIONS: Flush the closed containers with a cold water fog of fine spray.
Implement proper electrical grounding within facility so as to minimize the accumulation of
electrostatic charges. Remaining product may be disposed of according to local ordinances.

UNUSUAL FIRE AND EXPLOSION HAZARDS: Use proper ventilation so as minimize dust formation.
Implement proper electrical grounding within facility so as to minimize the accumulation of
electrostatic charges.

VIII. REACTIVITY DATA

STABILITY: Stable (If protected from humidity)

INCOMPATIBILITY: NA

HAZARDOUS PRODUCTS OF DECOMPOSITION: NA

HAZARDOUS POLYMERIZATION: Will not occur

IX. ENVIRONMENTAL AND DISPOSAL INFORMATION

GENERAL:

ACTION TO TAKE FOR SPILLS/LEAKS: Use proper personal protective clothing per section IX-Shut off
the source of the spill or leak if it is safe to do so-Scoop of shovel spilled material for disposal Mop of
flush the area with water.

DISPOSAL METHOD: To be performed in compliance with all Federal, State & Local Regulations.

X. PRECAUTIONS FOR SAFE HANDLING, STORAGE AND USE

Handle and store under normal conditions at room temperature protected from humidity.

XI. SHIPPING INFORMATION

Not regulated for shipping purposes.

XII. ADDITIONAL INFORMATION

None.

3

glanbia
NUTRITIONALS

Glanbia Nutritionals (NA), Inc   Tel: +1 760 438 0089
5927 Geiger Court   Fax: +1 760 438 0336
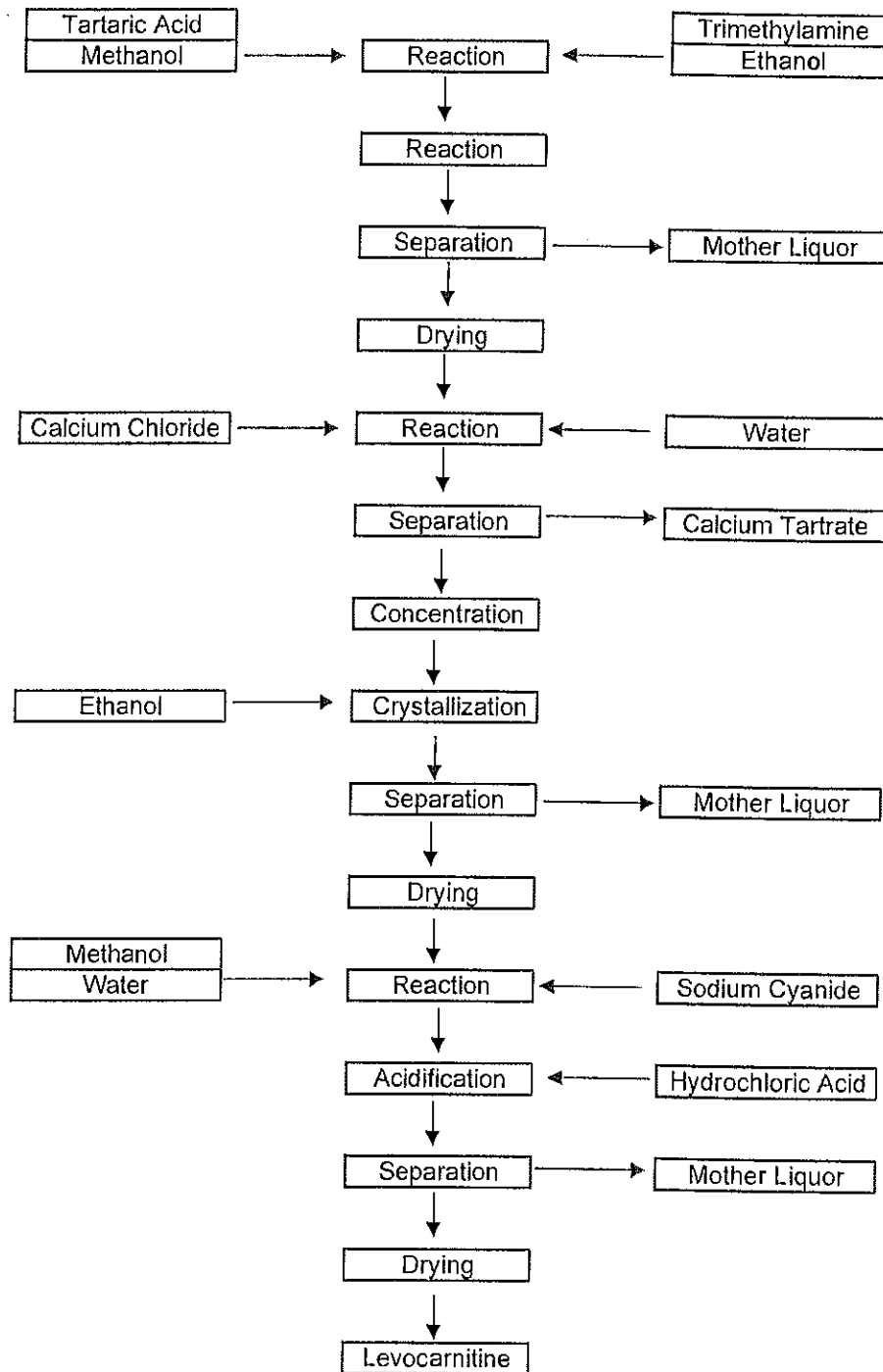Carlsbad, CA 92008   www.glanbianutritionals.com

---

NOTICE

The data contained herein is based on information that Glanbia Nutritionals (NA), Inc. believes to be reliable, but no expressed or implied warranty is made with regard to the accuracy of such data or its suitability for a given situation. Such data relates only to the specific product described and not to such product in combination with any other product and no agent of Glanbia Nutritionals (NA), Inc. is authorized to vary any of such data. Glanbia Nutritionals (NA), Inc. and its agent disclaim all liability for any actions taken or foregone on reliance upon such data.

L-Carnitine Base Flow Chart

```
   Tartaric Acid                                      Trimethylamine
   Methanol    ────────►    Reaction    ◄────────       Ethanol
                                │
                                ▼
                             Reaction
                                │
                                ▼
                            Separation   ────────►   Mother Liquor
                                │
                                ▼
                             Drying
                                │
                                ▼
Calcium Chloride  ────────►   Reaction    ◄────────      Water
                                │
                                ▼
                            Separation   ────────►   Calcium Tartrate
                                │
                                ▼
                           Concentration
                                │
                                ▼
   Ethanol     ────────►   Crystallization
                                │
                                ▼
                            Separation   ────────►   Mother Liquor
                                │
                                ▼
                             Drying
                                │
                                ▼
   Methanol
    Water      ────────►    Reaction    ◄────────    Sodium Cyanide
                                │
                                ▼
                           Acidification ◄────────  Hydrochloric Acid
                                │
                                ▼
                            Separation   ────────►   Mother Liquor
                                │
                                ▼
                             Drying
                                │
                                ▼
                          Levocarnitine
```

A thorough examination of the toxicological effects of bovine lactoferrin supplementation of infant formulas has not been done. However, studies conducted in newborn pigs have demonstrated that hepatic protein synthesis activity is higher in pigs fed formula supplemented with bovine lactoferrin compared with those fed unsupplemented formula or human colostrum (Burrin et al., 1996). Clearly, more research is required before drawing conclusions about the safety of lactoferrin supplementation for infants.

Recent advances in recombinant DNA methodologies, including the cloning of the human cDNA for lactoferrin (Powell & Ogden, 1990; Rey et al., 1990), have resulted in interest in the utilization of recombinant lactoferrin. This interest has been heightened by the feasibility of expression of human lactoferrin in cow milk (Krimpenfort, 1993). The technical aspects of transgenic lactoferrin sources applied to infant nutrition will require extensive evaluation to address efficacy and safety. Applications from other transgenic proteins will likely serve as good models in which the appropriate studies can be designed (Clark, 1996; Colman, 1996).

Conclusions and recommendations. **The Expert Panel did not recommend the addition of lactoferrin to infant formulas at this time.** Although it is technically feasible to add bovine lactoferrin or transgenic human transferrin to infant formulas, bovine lactoferrin does not bind consistently to human lactoferrin receptors and has not been shown to increase iron absorption. The efficacy and safety of adding human lactoferrin to infant formulas has not been adequately evaluated.

Given the emerging knowledge of the biological importance of human lactoferrin in infant nutrition, the Expert Panel regarded the notion of lactoferrin supplementation as worthy of consideration. However, clinical studies will be essential to demonstrate the efficacy and safety of such addition. Consistent with this position, the Expert Panel concluded that any consideration of the use of commercial formula manufactured using cow milk with transgenic sources of human lactoferrin be preceded by thorough evaluation of the scientific evidence of its nutrient value and safety. For a further discussion of issues and recommendations about the testing of new proteins for infant formulas see Chapter X.

Carnitine

Background. L-carnitine (β-hydroxy-γ-trimethylaminobutyrate) is a quaternary amine which is an integral component of the transport of long-chain fatty acids into the matrix of the mitochondria for β-oxidation (Borum, 1986). Carnitine plays an important role in the oxidation of medium-chain fatty acids in skeletal and cardiac muscle and in the transport of potentially toxic acylated metabolites outside the cell (Borum, 1995).

Dietary sources for carnitine are provided by mature human milk, where it is predominantly found in the whey fraction, in concentrations that range from 45 to 80 µmol/L (7.2 to 12.9 mg/L or 1.1 to 1.9 mg/100 kcal) (Borum, 1983; Mitchell & Snyder, 1991; Hromadová et al., 1994; Penn et al., 1987). In general, cow milk has approximately twice the carnitine concentrations as human milk. Consequently, free L-carnitine levels in commercial milk-based formulas vary from 6.7 to 23 mg/L (1 to 3.4 mg/100 kcal) (Borum et al., 1979; Indyk & Wollard, 1995; Novak et al., 1987; Rebouche, 1986). Soybean-based formulas manufactured prior to 1985 contained less than 2 µmol carnitine/L (48 µg/100 kcal) (Rebouche, 1992). Since 1986, soy-based formulas have been fortified to provide a level similar to that of human milk (Penn et al., 1987, Rebouche, 1992).

Based on the documented capacity of adults to produce carnitine endogenously from lysine and methionine, no RDA was established for carnitine in the 1989 revisions (NRC, 1989). Carnitine is currently not included in CFR 107.100. ESPGAN (1991) recommended a minimum level of 7.5 µmol/100 kcal (1.2 mg/100 kcal) (see Appendix A).

Review of extant data. Despite the absence of any direct evidence of its dietary essentiality, some have suggested that carnitine could be classified as a conditionally essential nutrient, i.e., required under defined circumstances (Borum, 1995). Carnitine is accumulated in adipose tissues shortly after birth and has been shown to increase the utilization of fatty acids for energy (Olson & Rebouche, 1987). Low plasma carnitine concentrations, along with limited biosynthetic capacity, support the possibility that infants have a dietary requirement for carnitine (Borum, 1986; Giovanni et al., 1991).

In part, the contention of conditional essentiality is buttressed by evidence that the terminal enzyme in the biosynthetic pathway for carnitine, γ-butyrobetaine hydroxylase, is developmentally regulated in the liver, but not the kidney (Olson & Rebouche, 1987). Rebouche (1992) has documented γ-butyrobetaine hydroxylase activity in newborn infants. Although adults are capable of biosynthesis in the liver and the kidney from lysine and methionine in the presence of adequate amounts of vitamin C, nicotinic acid, and vitamin B_6, both adults and infants have limited biosynthetic capacity (Rebouche, personal communication, 1997). It is conceivable that the infant is incapable of producing sufficient quantities to meet metabolic demands. Consequently, rather than an absolute requirement, it may be the case that infants require more carnitine than can be accommodated by endogenous production.

Additional support for the importance of carnitine in infant nutrition comes from studies of inborn errors of carnitine metabolism. In their review of these disorders, Feller & Rudman (1988) noted that the clinical manifestations are due to disruptions in fatty acid metabolism.

As noted previously, prior to 1986, soybean-based formulas contained less than 2 µmol carnitine/L. Several investigators reported clinical manifestations of carnitine deficiency in infants fed soy-based formulas. These manifestations included failure to thrive, nonketotic hypoglycemia, hypotonia, and cardiomyopathy (Slonim et al., 1981; Winter et al., 1987). Based in part on this evidence, the 1986 revision of the IFA mandated that carnitine be added to soy-based formulas at a level similar to that found in human milk (Penn et al., 1987; Rebouche, 1992).

The focus of studies of carnitine requirements in infants has been on the biochemical response to various dietary interventions, rather than an assessment of endogenous production (Rebouche, 1992). The impact of carnitine supplementation of soy-based formulas on growth and metabolism has been evaluated in several reports (Novak et al., 1983, 1987; Olson & Rebouche, 1989).

Novak et al. (1983) compared two small groups of healthy full-term infants receiving either commercial soy-based formula without carnitine (n=5) or the same formula supplemented with 50 µmol L-carnitine/ml (1.2 mg/100 kcal; n=7), an amount comparable to the levels found in human milk. The test diets served as the sole source of nutrition for the first five months of life. Blood samples were collected monthly throughout the trial and analyzed for free carnitine and lipid metabolites, i.e., free fatty acids, triglycerides, and relative amounts of lipoproteins. Novak et al. (1983) reported lower plasma carnitine levels, higher plasma triglyceride levels, and higher very low density lipoprotein (VLDL) levels in the unsupplemented group. They concluded that their results confirmed the importance of carnitine in fat metabolism and that the absence of a dietary source of carnitine could be metabolically significant. Generalization of the results of this study should be tempered by the small sample size.

In a follow-up study, Novak et al. (1987) compared plasma and urine concentrations of free carnitine and acylcarnitine in three groups of infants (age three- to seven-days-old). One group received a carnitine-free soy-based formula (n=13), and the other two groups received the same formula supplemented with either 50 µmol/ml (1.2 mg/100 kcal; n=13) or 250 µmol/ml (6.0 mg/100 kcal; n=6) of L-carnitine. All infants received a standard cow milk-based formula from birth to the beginning of the trial and the experimental diets until three months of age.

Novak et al. (1987) reported that, with the exception of greater concentrations of urine acylcarnitine in the group receiving 250 µmol/ml, no significant differences were found among the three groups at any time during the study.

Olson et al. (1989) compared growth and markers of fat metabolism between infants fed unsupplemented (n=11) and L-carnitine (86 µmol/L; 2.1 mg/100 kcal) supplemented (n = 11) soy-based formula for 112 days. At 56 and 112 days, serum carnitine levels were lower and serum free fatty acids were significantly higher in the unsupplemented group, confirming the Novak et al. (1983) study. The excretion rates of medium-chain dicarboxylic acids were significantly higher in the unsupplemented group although triglyceride levels were similar. No differences in growth rates were observed between these study groups.

<u>Conclusions and recommendations</u>. **The Expert Panel recommended a minimum carnitine content of infant formulas of 1.2 mg/100 kcal (7.5 µmol/100 kcal), a level similar to that found in human milk.** Although the evidence that dietary carnitine is essential for the term infant is not convincing, biochemical changes are noted when infants are fed a carnitine-free diet and there are several anecdotal reports of abnormal clinical manifestations associated with diets low in carnitine. Infants nourished with soy protein-based formula with low carnitine content had lower plasma and urine carnitine levels and evidence of altered lipid metabolism, but no significant differences in rates of growth compared with supplemented infants. The functional significance of these metabolic differences in normal term infants is not known.

The Expert Panel recommended a maximum carnitine content of infant formulas of 2.0 mg/100 kcal (12.4 µmol/100 kcal), a value similar to the upper limit reported for human milk. The Expert Panel was unaware of any studies in which a NOAEL or LOAEL had been identified for carnitine exposure in infants. Consequently, in the absence of data the Expert Panel concluded that the maximum should be set at a level comparable to the upper ranges of carnitine concentrations reported for human milk.

Taurine

<u>Background</u>. Taurine (2-aminoethanesulfonic acid), a small, sulfur-containing β-amino acid with a sulfonic acid group, is an intracellular amino acid found in most tissues. Long considered as simply a by-product of the catabolism of methionine and cysteine, taurine is unique among amino acids in that it is not incorporated into proteins. Numerous biochemical roles have been identified for taurine, including detoxification of retinol, iron and xenobiotics (Emudianughe et al., 1983), calcium transport (Dolara et al., 1973; Huxtable et al., 1980), myocardial contractility (Grosso & Bressler, 1976), and osmotic regulation

SUPPLEMENT

Proposed amino acid levels for infant formulas (mg/100 kcal) (*from Chapter V, Table 5-3*).

Amino Acid	Content (mg/100 kcal)	
	Minimum	Maximum[a]
isoleucine	88	176
leucine	171	342
lysine	124	248
methionine + cysteine[b]	58	116
phenylalanine + tyrosine[b]	133	266
threonine	77	154
tryptophan	26	52
valine	90	180

[a]   The maximum IAA levels were calculated on the basis of twice the minimum levels based upon the ratio of the Expert Panel's minimum and maximum total protein recommendations, i.e., 1.7:3.4 g total protein/100 kcal. When an individual amino acid is added to a formula, the maximum for that amino acid is not to exceed 1.5 times the minimum value.

[b]   Although the sulfur containing amino acids (methionine and cysteine) and the aromatic amino acids (phenylalanine and tyrosine) are listed in combination, it should be noted that in no case should the requirement be met with only one of the respective constituents. Because the ratio of each of these combinations of amino acids is approximately 1:1 in human milk, ratios that exceed 2:1 or 1:2 are probably unbalanced and should not be used without appropriate testing for adequacy.

Along with the indispensable amino acid composition, the Expert Panel concluded that additional measures were needed to reflect the digestibility and bioavailability of the amino acids from these sources. The assessment of protein quality of novel sources is an issue closely aligned with the general consideration of novel proteins in infant formula. The Expert Panel recommended that the issue of protein quality assessment for novel protein sources be reviewed in conjunction with the general consideration of the use of novel proteins in infant formulas within the next five years. For a further discussion of the issues related to the use of novel substances in infant formulas see Chapter X.

INDIVIDUAL PROTEINS, AMINO ACIDS, AND OTHER NITROGENOUS SUBSTANCES

Lactoferrin

The Expert Panel did not recommend the addition of lactoferrin to infant formulas at this time. Although it is technically feasible to add bovine lactoferrin or transgenic human transferrin to infant formulas, bovine lactoferrin does not bind consistently to human lactoferrin receptors and has not been shown to increase iron absorption. The efficacy and safety of adding human lactoferrin to infant formulas has not been adequately evaluated.

Given the emerging knowledge of the biological importance of human lactoferrin in infant nutrition, the Expert Panel regarded the notion of lactoferrin supplementation as worthy of consideration. However, clinical studies will be essential to demonstrate the efficacy and safety of such addition. Consistent with this position, the Expert Panel concluded that any consideration of the use of commercial formula manufactured using cow milk with transgenic sources

of human lactoferrin be preceded by thorough evaluation of the scientific evidence of its nutrient value and safety. For a further discussion of issues and recommendations about the testing of new proteins for infant formulas see Chapter X.

Carnitine

Minimum: **The Expert Panel recommended a minimum carnitine content of infant formulas of 1.2 mg/100 kcal, a level similar to that found in human milk.** Although the evidence that dietary carnitine is essential for the term infant is not convincing, biochemical changes are noted when infants are fed a carnitine-free diet and there are several anecdotal reports of abnormal clinical manifestations associated with diets low in carnitine. Infants nourished with soy protein-based formula with low carnitine content had lower plasma and urine carnitine levels and evidence of altered lipid metabolism, but no significant differences in rates of growth compared with supplemented infants. The functional significance of these metabolic differences in normal term infants is not known.

Maximum: **The Expert Panel recommended a maximum carnitine content of infant formulas of 2.0 mg/100 kcal, a value similar to the upper limit reported for human milk.** The Expert Panel was unaware of any studies in which a NOAEL or LOAEL had been identified for carnitine exposure in infants. Consequently, in the absence of data the Expert Panel concluded that the maximum should be set at a level comparable to the upper ranges of carnitine concentrations reported for human milk.

3. McCarten KM, Borden S, Rosenberg HK, and Spackman TJ: Delayed onset of right diaphragmatic hernia associated with group B streptococcal infection in the newborn (abstr), Am J Roentgenol **133**:435, 1979.
4. Wiseman NE, and MacPherson RI: "Acquired" congenital diaphragmatic hernia, J Pediatr Surg **12**:657, 1977.
5. Blank E, and Campbell JR: Congenital posterolateral defect in the right side of the diaphragm, Pediatrics **57**:807, 1976.
6. Riggs W, and Herschman A: Absent liver sign in congenital diaphragmatic hernia, South Med J **63**:265, 1970.

7. White JJ, Oh KS, and Haller JA: Positive-contrast peritoneography for accurate delineation of diaphragmatic abnormalities, Surgery **76**:398, 1974.
8. Meyers MA: Peritoneography, Am J Roentgenol **117**:353, 1973.
9. Oh, KS, Dorst JP, White JJ, et al: Positive-contrast peritoneography and herniography, Radiology **108**:647, 1973.

Comparison of serum carnitine and ketone body concentrations in breast- and in formula-fed newborn infants

Joseph B. Warshaw, M.D.,* and **Edward Curry, M.D.,** *New Haven, Conn.*

THE HUMAN NEWBORN INFANT undergoes major metabolic changes during the transition from fetal to extrauterine life. Although glucose appears to be the major energy fuel during fetal development and an important precursor for glycogen and lipid depots, the newborn infant rapidly develops the capacity to oxidize fatty acids and ketone bodies as fuels alternative to glucose.

Fatty acids derived from the high fat–milk diet and endogenous fat stores become the preferred fuel of heart and other tissues with high-energy demands. In the human neonate, there is a dramatic increase in serum levels of free fatty acids during the first hours of life.[1, 2] This is followed by increased concentrations of ketone bodies in the serum. Animal studies have shown that tissues of neonates, including brain, can actively utilize fatty acids and ketone bodies as oxidative substrates.[3] Important regulatory influences may include fatty acid availability, increased oxygen supply, and increases in the availability of carnitine. Carnitine serves as an essential carrier for acyl groups across the mitochondrial membrane to sites of oxidation, and therefore has a central role in the mitochondrial oxidation of fatty acids. In the present study we have examined relationships between breast and formula feeding and serum levels of carnitine

and ketone bodies generated as a consequence of fatty acid oxidation.

METHODS AND MATERIALS

Eleven breast-fed and 11 formula-fed infants were admitted to the study. Study patients were singleton births with a gestational age of at least 37 weeks and birth weight of over 3,000 gm. Three infants were delivered by cesarean section without postnatal complication. Cord blood was retained for subsequent analysis, and capillary blood obtained by heel prick was obtained at 6, 24, and 42 hours for analysis of carnitine, free fatty acids, ketone bodies, and glucose. This study was approved by the Human Investigation Committee, and informed consent was acquired from the parents of all infants in the study.

Analytical procedures. Blood, colostrum, breast milk, and formula were deproteinized with 6% perchloric acid. The colostrum and breast milk were centrifuged for 30 minutes at $10,000 \times g$ and blood for 10 minutes at $10,000 \times g$ to obtain a supernatant fraction. The supernatant was neutralized with $1M\ K_2CO_3$- $3M\ KOH$ to pH 7.0 at $0°$ and centrifuged at $8,000\ g \times 10$ minutes to remove the insoluble potassium perchlorate. The supernatant from the colostrum and breast milk were also filtered through a 0.45μ Millipore filter.

Free-carnitine concentrations were assayed spectrophotometrically as reported by Marquis and Fritz.[4] Free fatty acids were determined by a colorimetric assay described by Kvam et al[5] except heptane was used instead of pentane because of its lower boiling point.

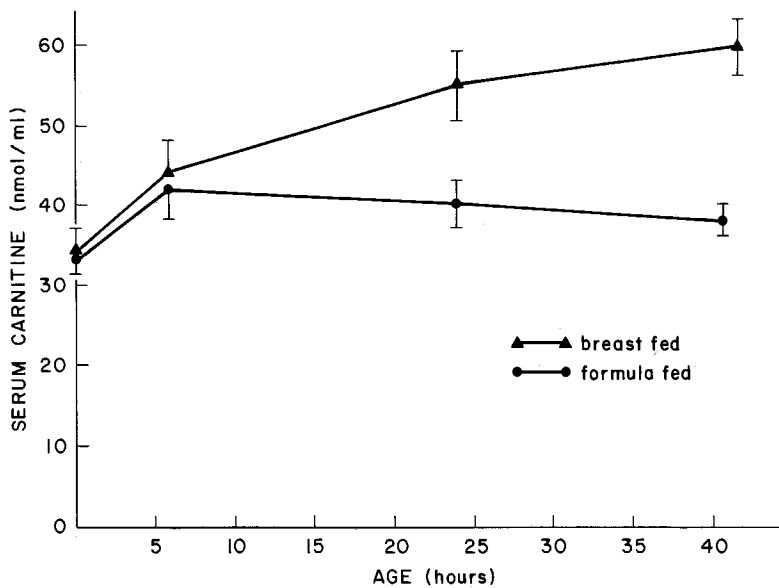β-Hydroxybutyrate and acetoacetate were determined

Fig. 1. Comparison of serum carnitine concentrations in breast- and formula-fed infants. Blood samples were obtained at birth (cord blood) and at 6, 24, and 42 hours of age by heel stick. Values given are means ± SEM for 11 infants in each group. Other conditions are as in the text.

fluorometrically following a minor modification of the procedure described by Mellanby and Williamson.[6] Plasma glucose was determined by the YSI model 23A glucose analyzer (Yellow Springs, Instrument Co., Yellow Springs, Ohio).

The carnitine acetyltransferase and β-hydroxybutyrate dehydrogenase was obtained from Boehringer-Mannheim Biochemical Co. (Indianapolis). Other reagents were obtained from commercial sources.

RESULTS

Carnitine concentration in the cord blood of both breast- and formula-fed infants in the study was 34(±5) nmoles/ml. As is shown in Fig. 1, by 42 hours after birth the serum carnitine concentration in the breast-fed group increased to 60(±8) nmoles/ml, as compared to 38(±4) nmoles/ml in the formula-fed group ($P < 0.05$). Carnitine content of breast milk collected from nursing mothers from one to five days postpartum ranged from 70 to 95 nmoles/ml, with higher values of up to 115 nmoles/ml in colostrum. The carnitine content of commercial formula (Enfamil) was between 40 to 80 nmoles/ml. There was considerable variation in both human milk and formula.

As a reflection of fatty acid oxidation and because of possible relationships to serum carnitine levels, ketone body concentration (β-hydroxybutyrate + acetoacetate) was measured during the 42-hour study period. The mean ketone body concentration in the cord blood of both the

breast- and formula-fed groups was similar—1.35(±0.3) and 1.50(±0.6) μmoles/ml, respectively. As shown in Fig. 2, by 42 hours after birth, ketone body concentration in the breast-fed infants increased to 5.9(±0.5) μmoles/ml, as compared with 2.9(±0.6) μmoles/ml in the formula-fed group. The differences between the two groups is significant at 42 hours of life ($P < 0.01$). This pattern is similar to that observed for the increase in serum carnitine concentrations seen in Fig. 1.

The free fatty acid concentrations in cord blood of breast- and formula-fed groups were not significantly different, 121(±35) and 97(±20) μmoles/ml, respectively. At 42 hours after birth the mean fatty acid concentrations in formula- and breast-fed groups were similar; they were 144(±40) versus 159(±37) μmoles/ml, respectively.

Glucose values over the first 42 hours of life were not significantly different in breast- and formula-fed infants.

The mean birth weight for the formula fed-infants was 3,613 ± 118 gm, and 3,868 (±280) gm for the breast-fed infants; the difference was not statistically significant. The mean weight loss over the first 42 hours of life in formula- and breast-fed infants was 215 and 200 gm, respectively, again not significantly different.

DISCUSSION

Results reported here show higher plasma carnitine levels in breast-fed as compared with formula-fed infants.
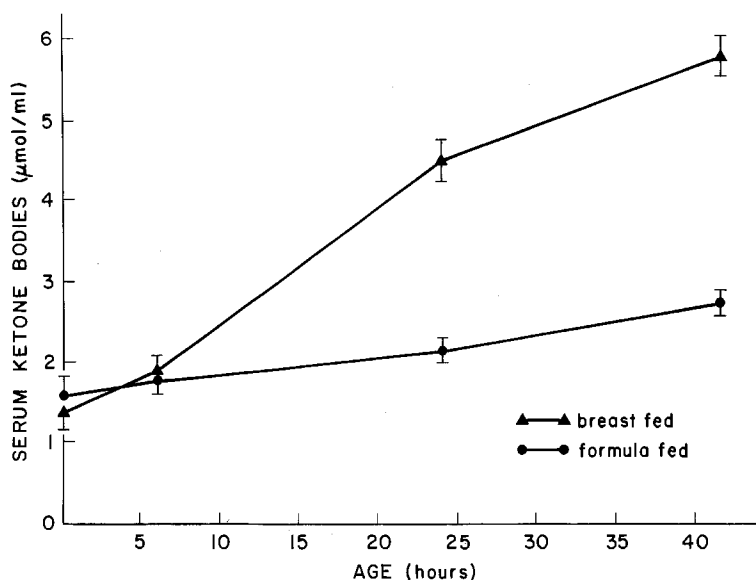
Fig. 2. Comparison of serum ketone body concentrations in breast- and formula-fed infants. Blood samples were obtained at birth (cord blood) and at 6, 24, and 42 hours of age by heel stick. Values given are means ± SEM for 11 infants in each group. Other conditions are as in the text.

This was associated with increased plasma ketone concentrations in the breast-fed group. Carnitine plays a central role in the mitochondrial oxidation of long-chain fatty acids.

Previous work has suggested that developmental increases in tissue carnitine levels as well as in enzymes associated with acylcarnitine formation (acylcarnitine transferase) are important for postnatal increases in fatty acid oxidation.[7, 8] Several investigators have shown that ketone body oxidation is also of importance in the newborn period, particularly in brain where ketone bodies may be an important substrate alternative to glucose. Ketone bodies may also serve as lipogenic precursors in brain and other tissues during development.[4, 9, 10]

Carnitine content of newborn rat liver increases dramatically with the onset of suckling, and a major regulatory influence of fatty acid oxidation and ketogenesis in the newborn rat appears to be related to the abundant supply of carnitine in rat's milk.[11] In human infants, Novak et al[12] have reported that oxygen consumption and lipolysis in adipose tissue are increased by carnitine so that carnitine may have a regulatory role in the postnatal release of fatty acids from adipose tissue. These investigators have further shown that carnitine and β-hydroxybutyrate levels in blood of infants fed carnitine-free soy-based formulas were less than in normally fed infants.[13]

In the present study we found somewhat lower carnitine levels in commercial formulas as compared with breast milk. The differences, however, do not appear to be of sufficient magnitude to account for the changes

observed. Although differences in ketone body concentration in breast-fed infants may in part relate to the higher concentrations of carnitine found in colostrum and breast milk as compared with formula, it can also be speculated that the bioavailability of carnitine in breast milk may be greater than from commercial formulas, as has been found with other nutritional components in milk such as iron. Possible differences in the concentrations of free and bound carnitines in breast milk and formula may also influence absorption. Further work is required to clarify these relationships.

Although formula-fed infants had lower blood carnitine and ketone body levels, the plasma concentrations of free fatty acids and of glucose were similar, suggesting that the hyperketonemia observed was not due to caloric deprivation in the breast-fed group. Moreover, weight loss was similar in the two groups during the first 42 hours of life. Our data suggest, therefore, that breast feeding results in higher serum carnitine levels than found in newborn infants fed commercial formulas, and that this results in enhanced ketone body production. Further work will be required to better define relationships between carnitine and postnatal metabolic adaptation in the human, but this study again emphasizes possible advantages of breast milk in nutrition of newborn infants.

REFERENCES

1. Novak M, Melichar V, Hahn P, et al: Release of free fatty acids from adipose tissue obtained from newborn infants, J Lipid Res **6**:91, 1965.

2. Blazquez E, Sagase T, Blazquez M, et al: Neonatal changes in the concentration of rat liver cyclic AMP and serum glucose. FFA, insulin pancreatic glucagon and total glucagon in man and the rat, J Lab Clin Med **83:**957, 1974.

3. Warshaw JB, and Terry ML: Cellular energy metabolism during fetal development. VI. Fatty acid oxidation by developing brain, Dev Biol **52:**161, 1976.

4. Marquis N, and Fritz I: Enzymological determination of free carnitine concentration in rat tissue, J Lipid Res **5:**184, 1964.

5. Kvam P, Schmidt J, and Riggilo D: Colormetric microdetermination of plasma free fatty acids, J Pharm Sci **53:**988, 1964.

6. Mellanby J, and Williamson D; Methods of enzymatic analysis, *in* Bergmeyer HU, editor: New York 1965, Academic Press, Inc., p 459.

7. Wittels B, and Bressler R: Lipid metabolism in the newborn heart, J Clin Invest **44:**1638, 1965.

8. Warshaw JB: Cellular energy metabolism during fetal development. IV. Fatty acid activation, acyl transfer and fatty acid oxidation during development of the chick and rat, Dev Biol **28:**537, 1972.

9. Edmund J: Ketone bodies as precursors of sterols and fatty acids in the developing rat, J Biol Chem **249:**72, 1974.

10. Seccombe DW, Harding PGR, and Possmayer F: Fetal utilization of maternally derived ketone bodies for lipogenesis in the rat, Biochem Biophys Acta **488:**402, 1977.

11. Robles-Valdes, McGary D and Foster D: Maternal-fetal carnitine relationships and neonatal ketosis in the rat, J Biol Chem **251:**6007, 1976.

12. Novak M, Penn-Walker D, and Hahn P: Effect of carnitine on lipolysis in subcutaneous adipose tissue of newborns, Biol Neonate **25:**85, 1975.

13. Weiser PB, Buch M, and Novak M: Effect of carnitine on ketone body production in human newborns, Pediatr Res **12:**401, 1978 (abstr).