

## METHOD FOR PREPARING CITRULLINE

### [BACKGROUND OF THE INVENTION]

#### [0001] Field of Invention

The present invention relates to a method for preparing citrulline. More specifically, the present invention relates to a method for preparing citrulline using microorganisms such as lactic acid bacterium. Further, the present invention relates to a method for preparing a composition (e.g., a milk product) containing citrulline at a high concentration. Furthermore, the present invention relates to a method for preparing a culture solution containing a microorganism having a citrulline producing ability wherein a conversion efficiency from arginine into citrulline is enhanced, and a method for preparing a culture solution that contains a microorganism having a citrulline producing ability and that has an enhanced microbial cell concentration.

#### [0002] Background Art

Citrulline is a functional amino acid and, in Japan, has been approved as foods in 2007 after approval of use as pharmaceuticals. Out of Japan, citrulline has been taken as foods since before 2007 and has a history of sales as supplements because of functions of amelioration in blood flow, prevention of arteriosclerosis, and enhancement in virility or energy. Further, there are reports about additional functions such as an amelioration in cold constitution, an amelioration in skin functions, an alleviation in or recovery from fatigue, growth of muscle, and an improvement in motor functions.

[0003] On the other hand, citrulline is very expensive as food materials, and the intake necessary for exerting the function is relatively large (for example, several hundreds of milligrams). Accordingly, an attempt to incorporate an effective amount of citrulline, for example, in food products poses a problem of a high cost.

[0004] Citrulline is mainly produced by a fermentation method at present. For example, JP S63(1988)-068091A (PTL 1) discloses a process for producing L-citrulline in which a microorganism belonging to the genus *Corynebacterium* or the genus *Brevibacterium* is cultured to produce and accumulate L-citrulline in a culture solution. JP H05(1993)-168486A (PTL 2) discloses a process for producing L-

citrulline in which lactic acid bacterium belonging to the genus *Pediococcus* is infected with bacteriophage to lyse the bacterium and the lysed bacterium is allowed to act on L-arginine to produce L-citrulline. JP H08(1996)-089269A (PTL 3) discloses a process for producing L-citrulline in which a microbial cell that lacks in an L-ornithine producing ability and has been dried over a water-soluble organic solvent is allowed to catalytically act on L-arginine to produce L-citrulline. Bacteria belonging to the genus *Bacillus*, the genus *Pseudomonas*, and the genus *Streptococcus* are exemplified in this document. JP 2010-088301A (PTL 4) discloses a process for producing L-citrulline by fermentation of bacteria belonging to the genus *Vibrio*.

[0005] In these prior art techniques, however, there is room for improvement from the viewpoints of production efficiency and production cost of citrulline, and, thus, it can be said that a process for preparing citrulline that has high production efficiency and cost effectiveness has still been desired.

[0006] As far as the present inventors know, the production of citrulline or ornithine from arginine using *Lactococcus lactis* (*L. lactis*) that is a kind of lactic acid bacterium has not been reported, but on the other hand, there are several reports about metabolization of arginine by *L. lactis* (NPLs 1, 2, and 3). For example, NPLs 1 and 2 describe that arginine has been entirely converted into ornithine by *L. lactis*. Further, transduction of arginine entirely to ornithine in the metabolization of arginine by *L. lactis* is described as a known fact in NPL 3. JP 2009-112205A (PTL 5) describes that L-ornithine is produced in the process of fermentation for the production of cheeses and the like to produce an ornithine-containing composition.

[0007] Further, in studies on the expression of genes on the metabolization of arginine, reports have been made on that a gene encoding arginine deiminase (ADI) that is an enzyme which allows arginine to be converted into citrulline is adjacent to a gene encoding ornithine carbomyltransferase (OTC) that is an enzyme which allows citrulline to be converted into ornithine and that the expression of these two genes is regulated by an identical promotor (NPL 4).

[0008] Furthermore, in studies on the expression of genes on the metabolization of arginine, reports have been made on that, also in

Enterococcus faecalis and Lactobacillus planturum as lactic acid bacterium other than L. lactis, a gene encoding ADI is adjacent to a gene encoding OTC and that the expression of these two genes is regulated by an identical promotor (NPLs 5 and 6).

[0009] In these studies, it has been demonstrated that the expression of the gene encoding ADI and the expression of the gene encoding OTC are simultaneously regulated by real time PCR. Further, in L. lactis, the activity of ADI and the activity of OTC are measured under various culturing conditions (NPL 1), and it is reported that the activity of OTC is higher than that of ADI under any culturing conditions.

[0010] As a result, these studies suggest that citrulline is not accumulated in a culture solution because, in microorganisms having an arginine metabolizing ability, although arginine is converted into citrulline by ADI, citrulline is immediately converted into ornithine by OTC.

[Citation List]

[Patent Literature]

[0011] [PTL 1] JP S63(1988)-068091A

[PTL 2] JP H05(1993)-168486A

[PTL 3] JP H08(1996)-089269A

[PTL 4] JP 2010-088301A

[PTL 5] JP 2009-112205A

[Non Patent Literature]

[0012] [NPL 1] Crow V. L., T. D. Thomas. 1982. Arginine metabolism in lactic streptococci. J. Bacteriol. 150:1024-1032.

[NPL 2] Poolman B., A. J. M. Driessen, W. N. Konings. 1987. Regulation of arginine-ornithine exchange and arginine deiminase pathway in Streptococcus lactis. Bacteriol., 169: 5597-5604

[NPL 3] Larsen R., G. Burist, O. P. Kuipers, J. Kok. 2004. ArgR and AhrC are required for regulation of arginine metabolism in Lactococcus lactis. J. Bacteriol. 186: 1147-1157

[NPL 4] Budin-Verneuil A., E. Maguin, Y. Auffray, D. S. Ehrlich, V. Pichereau. 2006. Genetic structure and trascriptional analysis of the arginine deiminase (ADI) cluster in Lactococcus lactis MG1363. Can. J.Microbiol. 52: 617-622

[NPL 5] Barcelona B., A. Marina, V. Rubio. 2002. Gene structure, organization, expression, and potential regulatory mechanisms of

arginine catabolism in *Enterococcus faecalis*. J. Bacteriol. 184: 6289-6300

[NPL 6] Spano G., G. Chieppa, L. Beneduce, S. Massa, 2004. Expression analysis of putative *arcA*, *arcB* and *arcC* genes partially cloned from *Lactobacillus plantarum* isolated from wine. J. Appl. Microbiol. 96 185-193

#### [SUMMARY OF THE INVENTION]

##### [Technical Problem]

[0013] It has been considered that the inhibition of transduction from citrulline to ornithine is effective to efficiently convert a large portion of arginine into citrulline using microorganisms such as lactic acid bacterium. As far as the present inventors know, up to now, there is no report on a method for the transduction of a large portion of arginine to citrulline in an efficient manner.

[0014] The present inventors have now found that citrulline can be microbiologically efficiently produced by culturing a medium at a temperature above a commonly used temperature (an optimal temperature for culturing of microorganisms) using microorganism having a citrulline producing ability. Further, the present inventors have found that a composition (e.g., foods and drinks such as fermented milk products) containing citrulline at a high concentration can be produced by culturing a starting material (e.g., starting milk) at a temperature above a commonly used temperature (an optimal temperature for fermentation of microorganisms) using microorganism having a citrulline producing ability.

[0015] The present inventors have further found that the citrulline producing ability of microorganisms can be enhanced by performing culturing or fermentation after the addition of arginine to a medium or a starting material. The present inventors have also found that the concentration (number) of microorganisms can be increased by performing culturing while adjusting pH of a medium (a culture solution). The present invention has been made based on such finding.

[0016] Accordingly, an object of the present invention is to provide a method for preparing citrulline that can efficiently produce citrulline. Another object of the present invention is to provide a

process for preparing a citrulline-containing composition including citrulline at a high concentration.

[0017] Still another object of the present invention is to provide a method for culturing and a method for fermentation of microorganisms that can realize an enhanced efficiency of conversion from arginine to citrulline. A further object of the present invention is to provide a method for culturing that can enhance the concentration of microorganism having a citrulline producing ability.

[Solution to Problem]

[0018] Thus, the present invention relates to the following embodiments (1) to (18).

(1) A method for preparing citrulline, comprising the steps of inoculating a microorganism having a citrulline producing ability into a culture medium and culturing the microorganism at a temperature from 40°C to 70°C.

(2) The method for preparing citrulline according to (1), wherein the microorganism having a citrulline producing ability is lactic acid bacterium, bifidobacteria, or propionibacteriaceae.

(3) The method for preparing citrulline according to (2), wherein the lactic acid bacterium is one or more members selected from the group consisting of *Lactococcus lactis*, *Lactobacillus fermentum*, and *Lactobacillus buchneri*.

(4) The method for preparing citrulline according to (2), wherein the lactic acid bacterium is one or more members selected from the group consisting of *Lactococcus lactis* spp. *lactis* strain OLS3789, strain OLS3797, and strain OLS3818.

[0019] (5) A method for preparing a citrulline-containing composition, comprising the steps of adding a microorganism having a citrulline producing ability to a starting material and culturing the microorganism at a temperature from 40°C to 70°C.

(6) The method for preparing a citrulline-containing composition according to (5), wherein the microorganism having a citrulline producing ability is lactic acid bacterium, bifidobacteria, or propionibacteriaceae.

(7) The method for preparing a citrulline-containing composition according to (6), wherein the lactic acid bacterium is one or more members selected from the group consisting of *Lactococcus lactis*,

*Lactobacillus fermentum*, and *Lactobacillus buchneri*.

(8) The method for preparing a citrulline-containing composition according to (6), wherein the lactic acid bacterium is one or more members selected from the group consisting of *Lactococcus lactis* spp. *lactis* strain OLS3789, strain OLS3797, and strain OLS3818.

(9) The method for preparing a citrulline-containing composition according to any one of (5) to (8), wherein the starting material is a raw milk.

(10) The method for preparing a citrulline-containing composition according to any one of (5) to (9), wherein the citrulline-containing composition is food and drink.

[0020] (11) A method for preparing a culture solution containing a microorganism that has a citrulline producing ability wherein a conversion efficiency from arginine into citrulline at a culturing temperature from 40°C to 70°C is enhanced, the method comprising the step of culturing a microorganism having a citrulline producing ability in a culture medium to which arginine is added.

(12) The method for preparing a culture solution according to (11), wherein arginine is added to the medium before the culturing and/or is intermittently added during culturing, or is continuously added during culturing.

(13) A method for preparing a culture solution containing a microorganism having a citrulline producing ability and having an increased concentration of the microorganism contained, the method comprising the step of culturing a microorganism having a citrulline producing ability in a culture medium of which pH is adjusted between 5 to 7 thereby increasing a concentration of the microorganism.

(14) The method for preparing a culture solution according to (13), wherein pH of the medium is adjusted by the addition of an alkali.

[0021] (15) A method for preparing a culture solution containing a microorganism having a citrulline producing ability wherein a conversion efficiency from arginine into citrulline at a temperature from 35°C to 70°C is enhanced and a concentration of the microorganism contained therein being increased, the method comprising the step of culturing a microorganism having a citrulline producing ability in a culture medium to which arginine is added and of which pH is set between 5 to 7

thereby increasing a concentration of the microorganism.

(16) The method for preparing a culture solution according to (15), wherein arginine is added to the medium before the culturing and/or is intermittently added during culturing, or is continuously added during culturing.

(17) A method for preparing a microorganism having a citrulline producing ability wherein a conversion efficiency from arginine into citrulline is enhanced, the method comprising the step of subjecting a culture solution obtained by a method according to any one of (11) to (16) to centrifugation and/or membrane separation.

(18) A method for preparing citrulline according to any one of (1) to (4), comprising the steps of inoculating into a medium a culture solution obtained by a method for preparing a culture solution according to any one of claims (11) to (16) or a microorganism obtained by a method for preparing a microorganism according to (17), and culturing the culture solution or the microorganism, or a method for preparing a citrulline-containing composition according to any one of (5) to (10), the method comprising the step of adding the culture solution or the microorganism to a starting material and fermenting the mixture.

[Advantageous effect]

[0022] The process for preparing citrulline according to the present invention can efficiently produce citrulline. Further, nutritional compositions such as milk products containing citrulline at a high concentration can be obtained by the process for preparing a citrulline-containing composition according to the present invention. Furthermore, according to the method for culturing a microorganism and a method for fermentation according to the present invention, the efficiency of transduction from arginine to citrulline can be enhanced. Furthermore, according to a method for culturing a microorganism according to the present invention, the concentration of the microorganism can be enhanced. Furthermore, the process for preparing citrulline and the process for preparing a citrulline-containing composition according to the present invention can efficiently produce citrulline using a microorganism with a citrulline producing ability wherein a conversion efficiency from arginine into citrulline is enhanced and/or having an enhanced concentration of the microorganism and/or using a culture solution

containing the microorganism, and, further, can provide nutritional compositions such as milk products containing citrulline at a high concentration.

#### [BRIEF DESCRIPTION OF THE DRAWINGS]

[0023] [Fig. 1] Fig. 1 is a graph illustrating a change in the concentration of microbial cells of L. lactis OLS3797 strain with the elapse of time when the L. lactis OLS3797 strain is cultured while regulating pH of a medium (culture solution) at 5.5.

[Fig. 2] Fig. 2 is a graph illustrating a comparison in the efficiency of transduction from arginine to citrulline between a culture solution after the elapse of 10, 11, and 12 hr from the start of culturing when L. lactis OLS3797 strain is cultured while regulating pH of a medium (a culture solution), and a culture solution when L. lactis OLS3797 strain is subjected to stationary culturing.

[Fig. 3] Fig. 3 is a graph illustrating a change in pH with the elapse of time, a change in microbial cell concentration with the elapse of time, and a change in arginine concentration, citrulline concentration, and ornithine concentration with the elapse of time when arginine is added to a MRS medium before L. lactis OLS3797 strain is cultured in the medium.

[Fig. 4] Fig. 4 is a graph illustrating a change in pH with the elapse of time, a change in microbial cell concentration with the elapse of time, and a change in arginine concentration, citrulline concentration, and ornithine concentration with the elapse of time when L. lactis OLS3797 strain is cultured while intermittently adding arginine to a MRS medium 10 hr after the start of culturing.

[Fig. 5] Fig. 5 is a graph illustrating a change in pH with the elapse of time, a change in microbial cell concentration with the elapse of time, and a change in arginine concentration, citrulline concentration, and ornithine concentration with the elapse of time when L. lactis OLS3797 strain is cultured while intermittently adding arginine to a MRS medium 4 hr after the start of culturing.

#### [DESCRIPTION OF EMBODIMENTS]

##### [0024] Process for preparing citrulline

The process for preparing citrulline according to the

present invention is characterized by inoculating a microorganism having a citrulline producing ability into a medium and culturing the microorganism at a temperature above a commonly used temperature (an optimal temperature for culturing of microorganism). Specifically, the culturing temperature is 40°C to 48°C, preferably 42°C to 47°C, more preferably 44°C to 46°C. Further, in another embodiment of the present invention, the culturing temperature is 40°C to 70°C, and the upper limit of the culturing temperature is preferably 68°C, more preferably 65°C, while the lower limit of the culturing temperature is preferably 42°C, more preferably 43°C.

[0025] As described above, the culturing temperature in the present invention is a temperature above a temperature commonly used for a microorganism having a citrulline producing ability (for example, an optimal temperature). Citrulline can be efficiently produced by culturing the microorganism in the above temperature range. While the reason for this is considered to be as follows, the following description (theory) is merely hypothetical, and the present invention is not bound by this theory. As described in the column of "Background Art," it is considered that, for example, *L. lactis* having an arginine metabolizing ability has a gene encoding arginine deiminase (ADI) that is an enzyme which allows arginine to be converted into citrulline, and a gene encoding ornithine carbomyltransferase (OTC) that is an enzyme which allows citrulline to be converted into ornithine and that the expression of these two genes is regulated by an identical promotor. In the present invention, it is considered that, when the culturing temperature is high, citrulline is accumulated in a medium (a culture solution) by enhancing the transduction from arginine to citrulline by ADI, by suppressing the transduction from citrulline to ornithine by OTC, or by the action and phenomenon of both the enhancement and the suppression. That is, when the culturing temperature is high as in the present invention, arginine is efficiently converted into citrulline, leading to the realization of a process for preparing citrulline in an efficient manner.

[0026] In the present invention, the culturing time can be properly selected from conditions under which microorganisms having a citrulline producing ability can be cultured and is not particularly limited, but is preferably 2 to 48 hr, more preferably 3 to 36 hr, still more

preferably 4 to 24 hr.

[0027] In the present invention, while the microorganism having a citrulline producing ability is not particularly limited as long as the microorganism produces citrulline, the microorganism is preferably one that, when cultured preferably at 40°C to 48°C, more preferably 42°C to 47°C, still more preferably 44°C to 46°C, can efficiently produce citrulline. In another embodiment of the present invention, the temperature is 40°C to 70°C, and the upper limit of the temperature is preferably 68°C, more preferably 65°C, and the lower limit of the temperature is preferably 42°C, more preferably 43°C. In the present invention, the microorganism having a citrulline producing ability is preferably lactic acid bacterium, bifidobacteria, or propionibacteriaceae having a citrulline producing ability, more preferably lactic acid bacterium or bifidobacteria having a citrulline producing ability, still more preferably lactic acid bacterium having a citrulline producing ability.

[0028] In the present invention, lactic acid bacterium having a citrulline producing ability is preferably one or more members selected from the group consisting of *Lactococcus lactis*, *Lactobacillus fermentum*, *Lactobacillus buchneri*, *Enterococcus faecalis*, *Oenococcus oeni*, *Pediococcus pentosaceus*, *Lactobacillus amylovorus*, *Lactobacillus brevis*, and *Lactobacillus reuteri*, more preferably one or more members selected from the group consisting of *Lactococcus lactis*, *Lactobacillus fermentum*, and *Lactobacillus buchneri*, still more preferably *Lactococcus lactis*.

[0029] In the present invention, *Lactococcus lactis* having a citrulline producing ability is preferably one or more members selected from the group consisting of *Lactococcus lactis* spp. *lactis* and *Lactococcus lactis* spp. *cremoris*, more preferably *Lactococcus lactis* spp. *lactis*, still more preferably one or more members selected from the group consisting of *Lactococcus lactis* spp. *lactis* OLS3789 strain, OLS3797 strain, and OLS3818 strain. As will be described below, these strains are deposited with depository institutions.

[0030] As described above, *Lactococcus lactis* spp. *lactis* having a citrulline producing ability is *Lactococcus lactis* spp. *lactis* OLS3789 that is deposited with Patent Microorganisms Depository Center of National Institute of Technology and Evaluation (NITE) as an accession

number NITE BP-1387 (indication of identification: Lactococcus lactis spp. lactis OLS3789, date of deposit: July 18, 2012), Lactococcus lactis spp. lactis OLS3797 that is deposited with Patent Microorganisms Depository Center of National Institute of Technology and Evaluation (NITE) as an accession number NITE BP-1388 (indication of identification: Lactococcus lactis spp. lactis OLS3797, date of deposit: July 18, 2012), and Lactococcus lactis spp. lactis OLS3818 that is deposited with Patent Microorganisms Depository Center of National Institute of Technology and Evaluation (NITE) as an accession number NITE BP-1389 (indication of identification: Lactococcus lactis spp. lactis OLS3818, date of deposit: July 18, 2012).

[0031] Lactococcus lactis spp. lactis OLS3789 has the following scientific properties (such as morphological characteristics, characteristics of medium, and physiological properties).

(a) Morphological properties

Properties of colony on medium (Lactobacilli MRS Agar, DIFCO): circular, white, smooth, and flat

(b) Physiological properties

Bacterial morphology: micrococcus, Gram's staining: positive, lactic acid fermentation type: homolactic acid fermentation, aerobic growth: +

[0032]

(c) Other properties that characterize the microorganism

The 16srDNA sequence is as follows. (OLS3789)

GCTGGCGGGCGTGCCTAATACATGCAAGTTGAGCGCTGAAGGTTGGT  
 ACTTGTACCGACTGGATGAGCAGCGAACGGGTGAGTAACGCGTGGG  
 GAATCTGCCTTTGAGCGGGGGACAACATTTGGAAACGAATGCTAATAC  
 CGCATAAACTTTAAACACAAGTTTTAAGTTTGAAAGATGCAATTGCA  
 TCACTCAAAGATGATCCCGCGTTGTATTAGCTAGTTGGTGAGGTAAAG  
 GCTCACCAAGGCGATGATACATAGCCGACCTGAGAGGGTGATCGGCC  
 ACATTGGGACTGAGACACGGCCCAAACCTACGGGAGGCAGCAGT  
 AGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCG  
 TGAGTGAAGAAGGTTTTTCGGATCGTAAACTCTGTTGGTAGAGAAGA  
 ACGTTGGTGAGAGTGGAAGCTCATCAAGTGACGGTAACTACCCAGA  
 AAGGGACGGCTAACTACGT (SEQ ID No: 1)

[0033] Further, Lactococcus lactis spp. lactis OLS3797 has the

following scientific properties (such as morphological characteristics, characteristics of medium, and physiological properties).

(a) Morphological properties

Properties of colony on medium (Lactobacilli MRS Agar, DIFCO): circular, white, smooth, and flat

(b) Physiological properties

Bacterial morphology: micrococcus, Gram's staining: positive, lactic acid fermentation type: homolactic acid fermentation, aerobic growth: +

[0034] (c) Other properties that characterize the microorganism

The 16srDNA sequence is as follows. (OLS3797)

GAAGCTGGCGGCGTGCCTAATACATGCAAGTTGAGCGCTGAAGGTTG  
GTACTTGTACCGACTGGATGAGCAGCGAACGGGTGAGTAACGCGTG  
GGGAATCTGCCTTTGAGCGGGGGACAACATTTGGAAACGAATGCTAA  
TACCGCATAAAAACTTTAAACACAAGTTTTAAGTTTGAAAGATGCAATT  
GCATCACTCAAAGATGATCCCGCGTTGTATTAGCTAGTTGGTGAGGTA  
AAGGCTCACCAAGGCGATGATACATAGCCGACCTGAGAGGGTGATCG  
GCCACATTGGGACTGAGACACGGCCCAAACCTCCTACGGGAGGCAGC  
AGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCC  
GCGTGAGTGAAGAAGGTTTTTCGGATCGTAAACTCTGTTGGTAGAGA  
AGAACGTTGGTGAGAGTGAAAGCTCATCAAGTGACGGTAACTACCC  
AGAAAGGGACGGCTAACTACGT (SEQ ID No: 2)

[0035] Further, Lactococcus lactis spp. lactis OLS3818 has the following scientific properties (such as morphological characteristics, characteristics of medium, and physiological properties).

(a) Morphological properties

Properties of colony on medium (Lactobacilli MRS Agar, DIFCO): circular, white, smooth, and flat

(b) Physiological properties

Bacterial morphology: micrococcus, Gram's staining: positive, lactic acid fermentation type: homolactic acid fermentation, aerobic growth: +

[0036] (c) Other properties that characterize the microorganism

The 16srDNA sequence is as follows. (OLS3818)

GCGAAGCTGGCGGCGTGCCTAATACATGCAAGTTGAGCGCTGAAGG  
TTGGTACTTGTACCGACTGGATGAGCAGCGAACGGGTGAGTAACGCG

TGGGGAATCTGCCTTTGAGCGGGGGACAACATTTGGAAACGAATGCT  
 AATACCGCATAAAACTTTAAACACAAGTTTTAAGTTTGAAAGATGCAA  
 TTGCATCACTCAAAGATGATCCCGCGTTGTATTAGCTAGTTGGTGAGG  
 TAAAGGCTCACCAAGGCGATGATACATAGCCGACCTGAGAGGGTGAT  
 CGGCCACATTGGGACTGAGACACGGCCCAAACCTCCTACGGGAGGCA  
 GCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACG  
 CCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGGTAGA  
 GAAGAACGTTGGTGAGAGTGGAAGCTCATCAAGTGACGGTAACTAC  
 CCAGAAAGGGACGGCTAACTACGT (SEQ ID No: 3)

[0037] In the present invention, while a culture medium is not particularly limited as long as the medium is properly selected from media in which microorganisms having a citrulline producing ability can be cultured, examples of such media include: defatted milks, defatted concentrated milks, reconstituted skim milks and proteolyzates thereof; wheys, whey concentrates, reduced wheys and proteolyzates of thereof; and raw milks, whole milks (sterilized milks), whole milk concentrated milks, and reduced whole milks. Preferred media include: defatted milks, reconstituted skim milks and proteolyzates thereof; and wheys, reduced wheys, and proteolyzates thereof.

[0038] In the method for producing citrulline according to the present invention, citrulline is produced into the medium. The citrulline-containing media as such can be utilized, for example, as starting materials or materials for foods and drinks, preferably as starting materials or materials for milk products. Alternatively, citrulline can be processed by treatment such as separation (for example, isolation) from the medium or concentration before citrulline having a desired purity is prepared and utilized. Foods and drinks include, for example, functional foods and nutritional foods, and milk products include, for example, milk beverages, fermented milks (more preferably yoghurts), lactic acid bacterium beverages, cheeses, and ice creams.

[0039] Method for producing citrulline-containing composition

The method for producing a citrulline-containing composition according to the present invention is characterized by adding (or mixing) a microorganism having a citrulline producing ability into a starting material and fermenting the mixture at a temperature above a commonly used temperature (a temperature optimal for

fermentation of microorganisms). The fermentation temperature is specifically 40°C to 48°C, preferably 42°C to 47°C, more preferably 44°C to 46°C. In another embodiment of the present invention, the fermentation temperature is 40°C to 70°C, and the upper limit of the fermentation temperature is preferably 68°C, more preferably 65°C while the lower limit of the fermentation temperature is preferably 42°C, more preferably 43°C.

[0040] As described above, the fermentation temperature in the present invention is a temperature above the commonly used temperature in the fermentation (e.g., an optimal temperature) of a microorganism having a citrulline producing ability. Citrulline can be efficiently produced by culturing of microorganisms in this temperature range. The reason for this is considered to be the same as already described in the method for preparing of citrulline according to the present invention.

[0041] In the method for preparing citrulline according to the present invention, citrulline-containing medium as such may be utilized as a citrulline-containing composition without isolating citrulline from a citrulline-containing medium, or alternatively, a medium treated by a desired procedure (for example, centrifugation or membrane separation) may be utilized as a citrulline-containing composition.

[0042] In the present invention, while the fermentation time is not particularly limited as long as microorganisms having a citrulline producing ability can be fermented, it is preferably 2 to 24 hr, more preferably 3 to 16 hr, still more preferably 4 to 12 hr.

[0043] In the present invention, while the starting material is not particularly limited and can be properly selected from those that allow microorganisms having a citrulline producing ability to be fermented, examples thereof include defatted milks, defatted concentrated milks, reconstituted skim milks, and their proteolyzates, wheys, whey concentrates, reduced wheys, and their proteolyzates, raw milks, whole milks (sterilized milks), whole milk concentrated milks, and reduced whole milks. Preferred are defatted milks, reconstituted skim milks, their proteolyzates, wheys, reduced wheys, and their proteolyzates.

[0044] In a preferred embodiment of the present invention, when a raw milk is used as the starting material, for example, a milk material

or a milk product is obtained as a citrulline-containing composition. In the milk materials or milk products (citrulline-containing composition), citrulline-derived functions are added, and the nutritional value is enhanced. Further, in the milk materials, milk products and the like, the content of citrulline can be enhanced in the production process without external addition of citrulline, and, thus, for example, the production cost of final products based on the milk materials and milk products can be low.

[0045] In another preferred embodiment of the present invention, when fermented milk mixes (more preferably, yoghurt mixes) are used as a starting material, fermented milks (more preferably, yoghurts) are obtained as the citrulline-containing composition. In the fermented milks (more preferably, yoghurts) thus obtained, citrulline-derived functions are added, and the nutritional value is enhanced. Furthermore, in the fermented milks (more preferably, yoghurts), milk products and the like, the content of citrulline can be enhanced in the production process without external addition of citrulline, and, thus, for example, the production cost of final products based on the fermented milks (more preferably, yoghurts) can be low.

[0046] In the method for producing a citrulline-containing composition according to the present invention, citrulline is produced into the material. The material containing citrulline as such can be used, for example, as starting materials and materials for foods and drinks, preferably as starting materials and materials for milk products, or alternatively, citrulline having a desired purity can be prepared and used after processing such as citrulline separation (for example, isolation) and concentration. Foods and drinks include, for example, functional foods and nutritional foods, and, the milk products includes, for example, milk beverages, fermented milks (more preferably, yoghurts), lactic acid bacterium beverages, cheeses, and ice creams.

[0047] Citrulline-containing foods and drinks

As described above, the media containing citrulline obtained by the method for preparing citrulline according to the present invention, or the citrulline-containing starting materials obtained by the method for preparing a citrulline-containing composition according to the present invention, or the products processed from them can be used as

foods and drinks as such or starting materials or ingredients for foods and drinks when a culture medium or a starting material is properly selected and used. Accordingly, according to the present invention, there is provided a citrulline-containing food and drink utilizing, as foods and drinks as such or a starting material or material for foods and drinks, a citrulline-containing medium, or a citrulline-containing starting material, or a processed product thereof.

[0048] The functions derived from citrulline can be added to foods and drinks by adding (incorporating) citrulline or a citrulline-containing composition or the processed product thereof to foods and drinks (preferably, milk products, more preferably, fermented milks). Thus, according to the present invention, citrulline-containing foods and drinks can be provided by adding the citrulline or citrulline-containing composition or the processed product thereof to foods and drinks.

[0049] According to the method for producing citrulline or a citrulline-containing composition according to the present invention, citrulline or a citrulline-containing composition, or a processed product thereof can be efficiently provided with a low manufacturing cost (a processing cost) or production cost. Accordingly, when the citrulline or citrulline-containing composition according to the present invention is used, citrulline-containing foods and drinks can be efficiently provided at a low production cost or the like while enhancing the value of products, for example, by adding a citrulline-derived function, for example, because the citrulline content can be enhanced in the production process.

[0050] Citrulline-containing foods and drinks according to the present invention are not particularly limited as long as the foods and drinks may contain citrulline, and examples thereof include milk products such as milk beverages, fermented milks (more preferably, yoghurts), lactic acid bacterium beverages, cheeses, ice creams.

[0051] Method for preparing culture solution containing microorganism that has citrulline producing ability wherein a conversion efficiency from arginine into citrulline is enhanced

According to the present invention, the method for preparing a culture solution containing a microorganism that has a citrulline producing ability wherein a conversion efficiency from arginine

into citrulline is enhanced is characterized in that the microorganism is cultured in a medium to which arginine is added at a culturing temperature above the commonly used temperature (temperature optimal for fermentation of microorganism). The culturing temperature is specifically 40°C to 48°C, preferably 42°C to 47°C, more preferably 44°C to 46°C. In another embodiment of the present invention, the culturing temperature is 40°C to 70°C, and the upper limit of the culturing temperature is preferably 68°C, more preferably 65°C, while the lower limit of the culturing temperature is 42°C, more preferably 43°C.

[0052] In a preferred embodiment of the present invention, there is provided a method for improving a citrulline producing ability of a microorganism, the method comprising culturing a microorganism having a citrulline producing ability at a temperature of 40°C to 48°C, preferably 42°C to 47°C, more preferably 44°C to 46°C. In another embodiment of the present invention, the culturing temperature is 40°C to 70°C, and the upper limit of the culturing temperature is preferably 68°C, more preferably 65°C while the lower limit of the culturing temperature is 42°C, more preferably 43°C. In this context, as the microorganism having a citrulline producing ability, it is possible to use the microorganism having an enhanced efficiency of converting arginine into citrulline, which is obtained by properly treating a cultured product or composition obtained by a process for preparing citrulline or a process for producing a citrulline-containing composition according to the present invention.

[0053] As described above, the medium in a preferred embodiment of the present invention is a medium with arginine added thereto. Citrulline can be efficiently produced by culturing a microorganism in the above medium composition (blend). While the reason for this is considered to be as follows, the following description (theory) is hypothetical, and the present invention is not bound by this theory. As described in the prior art, for example, NPLs 1 and 2 report that, in the metabolic activity of arginine of *L. lactis*, the addition of arginine to the medium is expected to contribute to an increase in transcript amount of a gene coding for ADI and OCT. According to this finding, when arginine is added to a medium, an increase in the transcript amount of a gene coding for ADI is expected, and it is considered that this phenomenon is maintained even when the culturing

temperature is high, that is, 40°C to 70°C, in some cases 48°C or below. An increase in transcript amount of a gene coding for ADI by the addition of arginine to a medium, maintaining of this phenomenon or suppression of the transcript amount of a gene coding for OCT also at a high culturing temperature of 40°C to 70°C, in some cases 48°C or below, is not easily expected. Accordingly, it not reasonable to determine that the present invention is obvious over NPLs 1 and 2.

[0054] According to a preferred embodiment of the present invention, when culturing a microorganism having a citrulline producing ability, arginine is added to a medium, and/or arginine is intermittently added to a medium during culturing, or arginine is continuously added to a medium during culturing. The citrulline producing ability of the microorganism having a citrulline producing ability thus can be enhanced. In this embodiment, preferably, the time of contact between a microorganism having a citrulline producing ability and arginine having a satisfactory concentration is long. Accordingly, when culturing the microorganism having a citrulline producing ability, arginine is preferably added (blended) into a medium before culturing, more preferably, arginine is intermittently or continuously added to a medium before culturing and, at the same time, still more preferably, arginine is continuously added to a medium during culturing.

[0055] In the present invention, while amount of arginine added is not particularly limited and may be properly selected from addition amount conditions that allow citrulline or a citrulline-containing composition, or processed products thereof to be produced, the amount to the medium is preferably 0.5 to 5% by mass, more preferably 0.7 to 4% by mass, still more preferably 1 to 3% by mass.

[0056] In the present invention, while means for adding arginine is not particularly limited as long as arginine can be aseptically added (blended) into a medium. After arginine is added to the medium, the medium is heat-sterilized or filtration-sterilized, or alternatively an aqueous arginine solution is separately prepared and the solution is heat-sterilized or filtration-sterilized followed by adding the sterilized aqueous arginine solution to the medium.

[0057] According to a preferred embodiment of the present invention, when culturing a microorganism having a citrulline producing

ability, a medium having a pH range of 5 to 7, preferably 5 to 6.5, more preferably 5 to 6, still more preferably 5.5 to 6, is used. The concentration of the microorganism having a citrulline producing ability (microbial cell concentration) thus can be enhanced, and the citrulline producing ability can be enhanced. In this context, the pH of the medium can be preferably regulated by adding an alkali, and the use of arginine or an alkali metal hydroxide as an alkali is more preferred.

[0058] In an embodiment of the present invention, while the expression "culturing is carried out in a pH range of 5 to 7" means preferably that the pH of the medium is 5 to 7 from an early stage to the end of the culturing, it should be noted that this expression does not necessarily mean that pH of the medium is always in a range of 5 to 7 from an early stage to the end of culturing. That is, in such a state that pH of the medium is in a range of 5 to 7, culturing may be carried out for a period of time long enough to enhance the efficiency of transduction from arginine to citrulline, and, specifically, the culturing time is 1 to 60 hr, preferably 2 to 48 hr, more preferably 3 to 36 hr, still more preferably 4 to 24 hr.

[0059] In the present invention, the means for adding an alkali is not particularly limited and may be properly selected from alkali addition conditions that allow pH of the medium to be regulated or controlled to a predetermined range. For example, pH of the medium is preferably intermittently or continuously regulated or controlled in a predetermined range while intermittently or continuously observing pH of the medium. More preferably, pH of the medium is continuously regulated or controlled in a predetermined pH range while continuously observing pH with, for example, a pH meter and a computer.

[0060] According to a preferred embodiment of the present invention, a microorganism having a citrulline producing ability may be cultured after the microorganism is subjected to activation culturing. Conditions for activation culturing are not particularly limited and may be properly selected depending upon the type of the microorganism having a citrulline producing ability.

[0061] According to one embodiment of the present invention, a culture solution containing a microorganism that has a citrulline producing ability wherein a conversion efficiency from arginine into

citrulline is enhanced may be subjected to centrifugation or membrane separation to obtain microbial cells of the microorganism before the microbial cell is used in the process for preparing citrulline or the process for producing citrulline or the process for producing a citrulline-containing composition.

[0062] Method for producing composition containing microorganism having a citrulline producing ability that has enhanced efficiency of converting arginine into citrulline

The process for producing a composition containing a microorganism that has a citrulline producing ability wherein a conversion efficiency from arginine into citrulline is enhanced according to the present invention is characterized in that the microorganism is cultured using a starting material with arginine added thereto at a culturing temperature above a commonly used temperature (temperature optimal for culturing microorganism). The culturing temperature is specifically 40°C to 48°C, preferably 42°C to 47°C, more preferably 44°C to 46°C. In another embodiment of the present invention, the culturing temperature is 40°C to 70°C, and the upper limit of the culturing temperature is 68°C, more preferably 65°C, while the lower limit of the culturing temperature is 42°C, more preferably 43°C.

[0063] In a preferred embodiment of the present invention, there is provided a method for improving a citrulline producing ability of the microorganism, the method comprising culturing a microorganism having a citrulline producing ability of the present invention at a temperature of 40°C to 48°C, preferably 42°C to 47°C, more preferably 44°C to 46°C. Further, in another embodiment of the present invention, the culturing temperature is 40°C to 70°C, and the upper limit of the culturing temperature is preferably 68°C, more preferably 65°C, and the lower limit of the culturing temperature is preferably 42°C, more preferably 43°C. In this case, a microorganism that has an enhanced efficiency of conversion from arginine into citrulline and is obtained by properly treating a cultured product or a composition obtained by the process for preparing citrulline or the process for producing a citrulline-containing composition can be used in the microorganism having a citrulline producing ability according to the present invention.

[0064] As described above, the starting material in a preferred

embodiment of the present invention is a material to which arginine is added. Citrulline can be efficiently produced by culturing the microorganism in a composition (blend) of the starting material. The reason for this is considered to be the same as that described above in connection with the process for preparing a culture solution containing a microorganism that has a citrulline producing ability wherein a conversion efficiency from arginine into citrulline is enhanced according to the present invention.

[0065] In the process for preparing a culture solution containing a microorganism that has a citrulline producing ability and has an enhanced efficiency of transduction from arginine to citrulline according to the present invention, the medium as such can be utilized as a citrulline-containing composition without isolating citrulline from the citrulline-containing medium, or alternatively a medium treated by a desired procedure (for example, centrifugation or membrane separation) can be used as a citrulline-containing composition.

[0066] In the present invention, while the amount of arginine added is not particularly limited and may be properly selected from addition amount conditions that allow citrulline or a citrulline-containing composition, or a processed product thereof to be prepared or produced, the amount of arginine added to the starting material is preferably 0.5 to 5% by mass, more preferably 0.7 to 4% by mass, still more preferably 1 to 3% by mass.

[0067] In the present invention, means for adding arginine is not particularly limited as long as arginine can be aseptically added (blended) into the medium. After the addition of arginine to the material, the material is heat-sterilized or filtration-sterilized, or alternatively an aqueous arginine solution is separately prepared and the aqueous arginine solution is heat-sterilized or filtration-sterilized followed by the addition of the sterilized aqueous arginine solution to the medium.

[0068] In a preferred embodiment of the present invention, arginine is added to the medium before the culturing, and/or is intermittently added in the process of the culturing, or is continuously added in the process of the culturing. According to this embodiment, the citrulline producing ability of the microorganism having a citrulline producing ability can be enhanced. In this case, preferably, the time of

contact between a microorganism having a citrulline producing ability and arginine having a satisfactory concentration is long. Accordingly, in culturing the microorganism having a citrulline producing ability, preferably, arginine is added (blended) into a medium before culturing, more preferably, arginine is added to a medium before culturing and, at the same time, arginine is added (blended) intermittently (for example, in a predetermined amount at a time) in the process of culturing or continuously (for example, little by little) into the medium, still more preferably, arginine is continuously added to a medium in the process of culturing. According to this method, when the microorganism having a citrulline producing ability is cultured, the efficiency of transduction from arginine to citrulline can be enhanced.

[0069] According to a preferred embodiment of the present invention, fermentation is carried out using a microorganism having a citrulline producing ability after the microorganism is subjected to activation culturing. Conditions for activation culturing are not particularly limited and may be properly selected depending upon the type of the microorganism having a citrulline producing ability.

[0070] According to one embodiment of the present invention, the composition containing a microorganism that has a citrulline producing ability wherein a conversion efficiency from arginine to citrulline is enhanced can be subjected to centrifugation or membrane separation to separate microbial cells before the microbial cells are used in the process for preparing citrulline or the process for producing a citrulline-containing composition according to the present invention.

[0071] Method for preparing microorganism that has citrulline producing ability and has enhanced microorganism concentration (microbial cell concentration)

The process for preparing a culture solution containing a microorganism that has a citrulline producing ability and an enhanced microorganism concentration (a microbial cell concentration) is characterized in that the microorganism is cultured in a medium having a pH value of 5 to 7 at a commonly used temperature (a temperature optimal for culturing microorganism) and is characterized in that the microorganism is cultured in a medium with arginine added thereto in a pH range of 5 to 7, preferably 5 to 6.5, more preferably 5 to 6, still more

preferably 5.5 to 6 at a culturing temperature of 35°C to 48°C, preferably 38°C to 48°C, more preferably 40°C to 48°C, still more preferably 42°C to 47°C, particularly preferably 44°C to 46°C. That is, the microorganism may be cultured at a temperature suitable for culturing of a microorganism having a citrulline producing ability (for example, an optimal temperature). Another embodiment of the present invention is characterized in that the microorganism is cultured in a medium with arginine added thereto in a pH range of 5 to 7, preferably 5 to 6.5, more preferably 5 to 6, still more preferably 5.5 to 6 at a culturing temperature of 35°C to 70°C (the lower limit culturing temperature being preferably 38°C, more preferably 40°C, still more preferably 42°C, particularly preferably 44°C, and the upper limit culturing temperature being preferably 68°C, more preferably 65°C).

[0072] According to a preferred embodiment of the present invention, there is provided a method for improving the concentration of the microorganism (microbial cell concentration), the method comprising culturing the microorganism having a citrulline producing ability in the present invention at a temperature of 35°C to 48°C, preferably 38°C to 48°C, more preferably 40°C to 48°C, still more preferably 42°C to 47°C, particularly preferably 44°C to 46°C. According to another embodiment of the present invention, there is provided a method for improving the concentration of the microorganism (microbial cell concentration), the method comprising culturing the microorganism at a culturing temperature of 35°C to 70°C (the lower limit culturing temperature being preferably 38°C, more preferably 40°C, still more preferably 42°C, particularly preferably 44°C, and the upper limit culturing temperature being preferably 68°C, more preferably 65°C). In this case, a microorganism that has an enhanced efficiency of converting arginine into citrulline and is obtained by properly treating a cultured product or a composition obtained by a process for preparing citrulline or a process for producing a citrulline-containing composition according to the present invention can be used in the microorganism having a citrulline producing ability according to the present invention.

[0073] According to a preferred embodiment of the present invention, there is provided a method for improving the concentration of the microorganism (microbial cell concentration), the method comprising

using a medium having a pH value in the range of 5 to 7, preferably 5 to 6.5, more preferably 5 to 6, still more preferably 5.5 to 6, in culturing the microorganism having a citrulline producing ability. According to this method, the concentration of the microorganism having a citrulline producing ability (microbial cell concentration) can be enhanced, and the citrulline producing ability can be enhanced. The pH value of the medium is preferably regulated by the addition of an alkali. More preferably, arginine or an alkali metal hydroxide is used as the alkali.

[0074] In the present invention, while the amount of arginine added is not particularly limited and may be properly selected from addition amount conditions that allow citrulline or citrulline-containing composition or processed products thereof to be prepared and produced, the amount of addition to the medium is preferably 0.5 to 5% by mass, more preferably 0.7 to 4% by mass, still more preferably 1 to 3% by mass.

[0075] In one embodiment of the present invention, the expression "cultured in a medium having a pH range of 5 to 7" means that, preferably, the pH range of the medium is in a pH range of 5 to 7 in a period from an early stage to the end of culturing. In this context, it should be noted that this expression does not mean that the pH range of the medium is always in a pH range of 5 to 7 in a period from an early stage to the end of culturing. In such a state that the pH value of the medium is in a range of 5 to 7, culturing for a period of time enough to satisfactorily enhance the concentration of the microbial cells is sufficient for contemplated results, and, specifically, the culturing time is 1 to 60 hr, preferably 2 to 48 hr, more preferably 3 to 36 hr, still more preferably 4 to 24 hr.

[0076] The means for adding an alkali is not particularly limited and may be properly selected from alkali addition conditions that allow pH of the medium to be regulated or controlled in a predetermined pH range. For example, preferably, while intermittently or continuously observing pH of the medium, for example, with a pH meter and a computer, the pH value of the medium is regulated or controlled in a predetermined pH range. More preferably, while continuously observing pH of the medium, the pH value of the medium is regulated or controlled in a predetermined pH range.

[0077] In a preferred embodiment of the present invention, a microorganism having a citrulline producing ability may be cultured after the microorganism is subjected to activation culturing. Conditions for activation culturing are not particularly limited and may be properly selected depending upon the type of the microorganism having a citrulline producing ability.

[0078] In one embodiment of the present invention, a culture solution that contains a microorganism having a citrulline producing ability and has a microbial cell concentration may be subjected to centrifugation or membrane separation to obtain microbial cells of the microorganism before the microbial cell is used in the process for preparing citrulline or the process for producing a citrulline-containing composition in the present invention.

[0079] In one embodiment of the present invention, when arginine is used as the alkali, the arginine is common to those added to the medium in the process for preparing citrulline in the present invention and the process for preparing a culture solution containing a microorganism having a citrulline producing ability in the present invention. Accordingly, when arginine is added in an excessive amount to the medium, for example, in the preparation processes, in some cases, pH of the medium is change to the alkaline side and, consequently, pH of the medium is significantly outside the pH range of 5 to 7. Thus, when arginine is added in an excessive amount to the medium, the citrulline producing ability is somewhat enhanced but the microbial cell concentration cannot be sometimes satisfactorily enhanced. Therefore, this is satisfactory when the purpose is only to enhance the citrulline producing ability. Accordingly, in this case, the production amount of citrulline per microorganism having a citrulline producing ability (microbial cells) (relative amount of citrulline) can be enhanced. However, care should be taken because there is a possibility that the production amount of citrulline (absolute amount of citrulline) per culture solution (medium) containing the microorganism.

[0080] Method for preparing culture solution that contains microorganism having citrulline producing ability, has enhanced efficiency of converting arginine into citrulline and has enhanced concentration of microorganism (microbial cell concentration)

The method for preparing culture solution that contains microorganism having citrulline producing ability, has enhanced efficiency of converting arginine into citrulline and has enhanced concentration of microorganism (microbial cell concentration) in the present invention is characterized in that the microorganism is cultured in a medium at a culturing temperature above a commonly used temperature (temperature optimal for culturing microorganism) and having pH value of 5 to 7 and that the microorganism is cultured in a medium with arginine added thereto at a culturing temperature of 40°C to 48°C, preferably 42°C to 47°C, more preferably 44°C to 46°C, the medium having a pH value in a range of 5 to 7, preferably 5 to 6.5, more preferably 5 to 6, still more preferably 5.5 to 6. According to another aspect of the present invention, the preparation process in the present invention is characterized in that the microorganism is cultured in a medium with arginine added thereto at a culturing temperature of 40°C to 70°C (here the upper limit culturing temperature is 68°C, more preferably 65°C, and the lower limit culturing temperature is 42°C, more preferably 43°C), the medium having a pH range of 5 to 7, preferably 5 to 6.5, more preferably 5 to 6, still more preferably 5.5 to 6.

[0081] That is, a culture solution that contains a microorganism having a citrulline producing ability and has enhanced efficiency of converting arginine into citrulline and enhanced microorganism concentration (microbial cell concentration) can be prepared by the combined use of conditions for the preparation of a culture solution that contains a microorganism having a citrulline producing ability wherein a conversion efficiency from arginine into citrulline is enhanced according to the present invention, and conditions for the preparation of a culture solution that contains a microorganism having a citrulline producing ability and has an enhanced microorganism concentration (microbial cell concentration) according to the present invention.

[0082] In one aspect of the present invention, the above process can be applied to means for adding arginine to a medium (a culture solution) and a method for the regulation (control) of pH of the medium (culture solution) as long as the efficiency of transduction from arginine to citrulline can be enhanced and the concentration of microorganism (microbial cell concentration) can be enhanced.

[0083] According to a preferred embodiment of the present invention, when culturing a microorganism having a citrulline producing ability, arginine is added to the medium before culturing, and/or arginine is intermittently added to the medium during culturing, or arginine is continuously added to the medium during culturing. They can enhance the citrulline producing ability of a microorganism having a citrulline producing ability. At that time, for the microorganism having a citrulline producing ability, the pH value of the medium is preferably approximately neutral at the time of start of the culturing. Accordingly, preferably, arginine is not added (blended) to the medium before the culturing. The addition of arginine to the medium in an intermittent manner (for example, the addition of a predetermined amount at a time) or in a continuous manner (for example, gradually little by little) in the process of the culturing without the addition of arginine to the medium before the culturing is more preferred. The addition of arginine to the medium in the process of the culturing in a continuous manner is still more preferred. This can enhance the efficiency of converting arginine into citrulline and can enhance the concentration of microorganism (microbial cell concentration) in culturing or fermenting microorganism having a citrulline producing ability.

[0084] In the present invention, while the amount of arginine added is not particularly limited and may be properly selected from conditions for the preparation or production of citrulline or citrulline-containing composition, or processed products thereof, the amount of arginine to the medium is preferably 0.5 to 5% by mass, more preferably 0.7 to 4% by mass, still more preferably 1 to 3% by mass, to the medium.

#### [EXAMPLES]

[0085] The present invention is further illustrated by the following Examples that are not intended as a limitation of the scope of the present invention.

[0086] In the Examples of the present invention, the microbial cell concentration was measured by a measuring method for the number of lactic acid bacterium cells specified in "the Ministerial Ordinance concerning Compositional Standards, Etc. for Milk and Milk Products (Ministerial Ordinance concerning Milk, Japan). The culturing

temperature of a BCP medium was 30°C. The concentration of arginine, the concentration of citrulline, and the concentration of ornithine were measured by a HPLC method.

[0087] Example 1: Arginine metabolizing ability for lactic acid bacterium and bifidobacteria

For lactic acid bacterium and bifidobacteria specified in Table 1, the arginine metabolizing ability was examined. At the outset, a MRS medium or a GAM medium was sterilized at 121°C for 15 min. Thereafter, an aqueous arginine solution was filtered and sterilized. The sterilized aqueous arginine solution was added to these media, followed by adjustment to provide an arginine concentration of 27 mM. The adjusted media were inoculated with an activating culture solution of a MRS medium of lactic acid bacterium and bifidobacteria specified in Table 1 below at a concentration of 1% by weight. For lactococcus, aerobic culturing was carried out in a stationary state under conditions of a culturing temperature of 30°C and a culturing time of 16 hr, and, for other microbial cells, aerobic culturing was carried out in a stationary state under conditions of a culturing temperature of 37°C and a culturing time of 16 hr.

[0088] The results were as shown in Table 1 below. The arginine metabolizing ability was confirmed for Lactobacillus fermentum JCM1173T, Lactobacillus buchneri NCIMB8007T, Lactococcus lactis ssp.lactis JCM5805T, and IFO12007.

[0089]

Table 1: Comparison of arginine metabolizing ability between lactic acid bacterium and bifidobacteria

| Strain                                  | Type Strain NO. | Media | Temp. (°C) | Arg (mM) | Cit (mM) | Orn (mM) | OD (600nm) |
|-----------------------------------------|-----------------|-------|------------|----------|----------|----------|------------|
| <i>Bif breve</i>                        | JCM 1192T       | MRS   | 37         | 25.0     | 0.1      | 1.8      | 2.20       |
|                                         |                 | GAM   |            | 25.7     | 0        | 6.9      | 2.69       |
| <i>Bif. Longum</i>                      | JCM 1217T       | MRS   |            | 26.5     | 0.1      | 1.8      | 3.12       |
|                                         |                 | GAM   |            | 25.2     | 0        | 6.6      | 1.46       |
| <i>Bif. Bifidum</i>                     | JCM 1255T       | MRS   |            | 25.2     | 0        | 1.9      | 1.24       |
|                                         |                 | GAM   |            | 24.3     | 0        | 6.4      | 0.73       |
| <i>S. thermophilus</i>                  | JCM 17834T      | MRS   |            | 27.2     | 0        | 2.7      | 4.42       |
| <i>Pediococcus pentosus</i>             | JCM 5890T       | MRS   |            | 25.9     | 0        | 1.3      | 1.99       |
| <i>L. gasseri</i>                       | JCM 1131T       | MRS   |            | 27.7     | 0        | 1.6      | 5.80       |
| <i>L. rhamnosus</i>                     | JCM 1136T       | MRS   |            | 27.7     | 0        | 1.1      | 5.82       |
| <i>L. fermentum</i>                     | JCM 1173T       | MRS   |            | 0        | 1.3      | 24.9     | 5.26       |
| <i>L. buchneri</i>                      | NCIMB 8007T     | MRS   |            | 1.8      | 3.1      | 22.3     | 3.39       |
| <i>L. plamtarum</i>                     | NCIMB 11974T    | MRS   |            | 25.9     | 0        | 1.2      | 9.01       |
| <i>L. bulgaricus</i>                    | JCM 1002T       | MRS   |            | 25.9     | 0        | 1.5      | 3.02       |
| <i>L. casei</i>                         | JCM 1134T       | MRS   |            | 25.5     | 0        | 1.4      | 4.91       |
| <i>Lac. Lactis</i> ssp. <i>Lactis</i>   | JCM 5805T       | MRS   | 30         | 0.8      | 2.2      | 24.6     | 2.48       |
| <i>Lac. Lactis</i> ssp. <i>Lactis</i>   | IFO 12007       | MRS   |            | 0        | 0.9      | 25.4     | 3.62       |
| <i>Lac. Lactis</i> ssp. <i>cremoris</i> | JCM 16167T      | MRS   |            | 25.2     | 0        | 1.5      | 1.70       |

In the table, Arg: arginine, Cit: citrulline, and Orn: ornithine

[0090] Example 2: Arginine metabolizing ability for Lactococcus lactis ssp.lactis and Lactococcus lactis ssp.diacetylactis

The results of Example 1 demonstrate that, for Lactococcus lactis ssp. lactis, both two strains indicated the arginine metabolizing ability. In these two strains, arginine was entirely converted into citrulline that was then mostly converted to ornithine.

[0091] Accordingly, for 29 strains of Lactococcus lactis ssp.lactis and one strain of Lactococcus lactis ssp.diacetylactis that is allied species of Lactococcus lactis ssp.lactis specified in Table 2 below, search was carried out for strains that do not convert citrulline into ornithine. Nos. 1 to 29 in Table 2 are Lactococcus lactis ssp.lactis, while No. 30 is Lactococcus lactis ssp.diacetylactis.

[0092] The MRS media were sterilized at 121°C for 15 min. Thereafter, an aqueous arginine solution was filtered and sterilized, the sterilized solution was added to the media, and the media were adjusted to an arginine concentration of 27 mM. These adjusted media were inoculated with 1% by weight of an activating culture solution of strains specified in Table 2 below in a MRS medium. For these strains, aerobic culturing was carried out in a stationary state under conditions of a culturing temperature of 30°C and a culturing time of 16 hr.

[0093] The results were as shown in Table 2 below. In all of the 29 strains of Lactococcus lactis ssp.lactis, an arginine metabolizing ability was identified. The arginine was largely converted into ornithine, and the production ratio of citrulline to ornithine ("Cit/Orn") was low and 0.02 to 0.13. Further, in one strain of Lactococcus lactis ssp.diacetylactis, an arginine metabolizing ability was not identified.

[0094]

Table 2: Comparison of arginine metabolizing ability with lactis

| NO. | Strain         | Arg<br>(mM) | Cit<br>(mM) | Orn<br>(mM) | Cit/Orn | OD<br>(600nm) |
|-----|----------------|-------------|-------------|-------------|---------|---------------|
| 1   | MEP12<br>03401 | 0.4         | 0.6         | 20.6        | 0.03    | 4.64          |
| 2   | MEP12<br>03402 | 0           | 0.5         | 21.5        | 0.02    | 4.33          |
| 3   | MEP12<br>03403 | 0           | 2.0         | 20.7        | 0.09    | 3.91          |
| 4   | OLS<br>3789    | 0           | 1.1         | 21.4        | 0.05    | 4.56          |
| 5   | MEP12<br>03404 | 0           | 0.6         | 21.2        | 0.03    | 4.51          |
| 6   | MEP12<br>03405 | 0           | 1.0         | 20.8        | 0.05    | 4.08          |
| 7   | MEP12<br>03406 | 0           | 0.6         | 21.0        | 0.03    | 3.97          |
| 8   | MEP12<br>03407 | 0           | 0.7         | 21.1        | 0.03    | 4.05          |
| 9   | MEP12<br>03408 | 0           | 0.9         | 23.7        | 0.04    | 5.05          |
| 10  | MEP12<br>03409 | 0           | 0.7         | 21.4        | 0.03    | 4.11          |
| 11  | MEP12<br>03410 | 0           | 0.7         | 20.9        | 0.03    | 4.37          |
| 12  | OLS<br>3797    | 0           | 1.8         | 20.2        | 0.09    | 5.00          |
| 13  | MEP12<br>03411 | 0           | 2.5         | 19.8        | 0.13    | 3.76          |
| 14  | MEP12<br>03412 | 0           | 2.5         | 20.9        | 0.12    | 3.73          |
| 15  | MEP12<br>03413 | 0           | 1.0         | 20.7        | 0.05    | 3.80          |
| 16  | MEP12<br>03414 | 0           | 0.9         | 21.0        | 0.04    | 3.91          |
| 17  | MEP12<br>03415 | 0           | 0.9         | 21.7        | 0.04    | 4.29          |
| 18  | MEP12<br>03416 | 0           | 0.6         | 21.9        | 0.03    | 4.38          |
| 19  | MEP12<br>03417 | 0           | 0.6         | 21.4        | 0.03    | 4.04          |
| 20  | MEP12<br>03418 | 0           | 0.8         | 22.5        | 0.03    | 4.09          |
| 21  | MEP12<br>03419 | 0           | 0.6         | 21.6        | 0.03    | 4.33          |
| 22  | MEP12<br>03420 | 0           | 0.6         | 21.8        | 0.03    | 4.07          |
| 23  | MEP12<br>03421 | 0           | 0.7         | 21.4        | 0.03    | 3.98          |
| 24  | MEP12<br>03422 | 0           | 0.5         | 21.4        | 0.02    | 4.46          |

Table 2 (Continued)

|    |                |      |     |      |      |      |
|----|----------------|------|-----|------|------|------|
| 25 | MEP12<br>03423 | 0    | 0.6 | 21.2 | 0.03 | 4.01 |
| 26 | MEP12<br>03424 | 0    | 0.9 | 21.1 | 0.04 | 3.95 |
| 27 | OLS<br>3818    | 0    | 0.7 | 20.1 | 0.04 | 4.34 |
| 28 | MEP12<br>03425 | 0    | 0.4 | 21.1 | 0.02 | 4.48 |
| 29 | MEP12<br>03426 | 0    | 0.4 | 21.2 | 0.02 | 3.84 |
| 30 | MEP12<br>03427 | 24.0 | 0.2 | 2.4  | 0.07 | 2.81 |

[0095] Example 3: Influence of culturing temperature (37°C, 40°C) on arginine metabolism with Lactococcus lactis ssp.lactis.

Microbial cells were cultured and the arginine metabolizing ability was examined in the same manner as in Example 2, except that the culturing temperature was changed to 37°C or 40°C. The results were as shown in Table 3 below. Here search for strains that provide a relatively high Cit/Orn value was carried out, and 6 strains were selected.

[0096]

Table 3: Relationship between arginine metabolizing ability with lactis and culturing temperature (MRS medium)

| NO. | Strain      | 37 °C    |          |          |         |            | 40 °C    |          |          |         |            |
|-----|-------------|----------|----------|----------|---------|------------|----------|----------|----------|---------|------------|
|     |             | Arg (mM) | Cit (mM) | Orn (mM) | Cit/Orn | OD (600nm) | Arg (mM) | Cit (mM) | Orn (mM) | Cit/Orn | OD (600nm) |
| 1   | MEP12 03401 | 2.3      | 0.8      | 21.9     | 0.03    | 4.49       | 0        | 3.2      | 18.8     | 0.17    | 2.89       |
| 2   | MEP12 03402 | 0        | 0.8      | 22.2     | 0.03    | 4.51       | 0        | 0.8      | 21.4     | 0.04    | 4.00       |
| 3   | MEP12 03403 | 0.7      | 4.8      | 18.0     | 0.27    | 2.19       | 16.6     | 1.2      | 2.5      | 0.47    | 0.48       |
| 4   | OLS 3789    | 0        | 4.3      | 19.3     | 0.22    | 4.05       | 1.2      | 11.1     | 9.2      | 1.21    | 2.95       |
| 5   | MEP12 03404 | 0        | 1.4      | 21.9     | 0.06    | 4.22       | 0        | 4.0      | 16.8     | 0.24    | 2.97       |
| 6   | MEP12 03405 | 0        | 2.1      | 21.4     | 0.10    | 3.88       | 7.5      | 3.7      | 10.6     | 0.35    | 3.25       |
| 7   | MEP12 03406 | 0        | 2.3      | 21.2     | 0.11    | 3.17       | 7.6      | 2.5      | 11.2     | 0.22    | 2.02       |
| 8   | MEP12 03407 | 0.2      | 2.0      | 22.4     | 0.09    | 3.29       | 6.0      | 2.1      | 13.6     | 0.15    | 2.21       |
| 9   | MEP12 03408 | 0.1      | 1.7      | 21.8     | 0.08    | 4.28       | 0        | 3.0      | 19.2     | 0.16    | 3.47       |
| 10  | MEP12 03409 | 0.1      | 2.2      | 21.8     | 0.10    | 3.15       | 8.0      | 2.0      | 11.6     | 0.18    | 2.13       |
| 11  | MEP12 03410 | 0        | 1.8      | 21.5     | 0.08    | 3.89       | 3.1      | 3.5      | 14.9     | 0.23    | 2.47       |
| 12  | OLS 3797    | 0        | 3.3      | 20.3     | 0.16    | 4.43       | 0        | 11.6     | 9.6      | 1.20    | 2.90       |
| 13  | MEP12 03411 | 0.3      | 4.8      | 18.9     | 0.25    | 2.94       | 3.5      | 5.0      | 12.2     | 0.41    | 1.95       |
| 14  | MEP12 03412 | 0.2      | 4.7      | 19.6     | 0.24    | 2.98       | 3.1      | 4.9      | 12.7     | 0.39    | 2.02       |
| 15  | MEP12 03413 | 1.0      | 3.3      | 19.1     | 0.17    | 3.32       | 5.0      | 3.5      | 13.2     | 0.26    | 2.55       |
| 16  | MEP12 03414 | 0        | 2.2      | 21.3     | 0.10    | 3.25       | 2.8      | 3.3      | 15.7     | 0.21    | 2.42       |
| 17  | MEP12 03415 | 0        | 1.8      | 20.9     | 0.09    | 3.53       | 1.8      | 6.8      | 12.8     | 0.53    | 2.20       |
| 18  | MEP12 03416 | 0        | 4.1      | 19.9     | 0.21    | 3.19       | 1.0      | 13.2     | 7.4      | 1.79    | 1.40       |

Table 3 (Continued)

|    |                |     |      |      |      |      |     |      |      |      |      |
|----|----------------|-----|------|------|------|------|-----|------|------|------|------|
| 19 | MEP12<br>03417 | 0   | 1.6  | 21.1 | 0.08 | 3.84 | 3.2 | 3.2  | 15.0 | 0.21 | 2.82 |
| 20 | MEP12<br>03418 | 0   | 2.1  | 21.0 | 0.10 | 3.25 | 4.5 | 1.9  | 14.5 | 0.13 | 2.27 |
| 21 | MEP12<br>03419 | 0   | 2.0  | 21.1 | 0.10 | 3.33 | 2.7 | 3.2  | 15.1 | 0.21 | 2.04 |
| 22 | MEP12<br>03420 | 0.6 | 2.4  | 20.1 | 0.12 | 3.21 | 6.9 | 8.0  | 6.0  | 1.33 | 1.59 |
| 23 | MEP12<br>03421 | 0   | 1.9  | 21.1 | 0.09 | 3.32 | 4.4 | 2.0  | 14.0 | 0.14 | 2.71 |
| 24 | MEP12<br>03422 | 0   | 4.5  | 18.1 | 0.25 | 3.06 | 0   | 13.5 | 5.7  | 2.36 | 1.42 |
| 25 | MEP12<br>03423 | 0   | 1.8  | 21.2 | 0.09 | 3.29 | 3.6 | 3.6  | 14.2 | 0.25 | 2.49 |
| 26 | MEP12<br>03424 | 0   | 1.2  | 20.8 | 0.06 | 3.87 | 6.7 | 2.0  | 13.5 | 0.15 | 2.22 |
| 27 | OLS<br>3818    | 0   | 10.5 | 11.5 | 0.91 | 3.94 | 0   | 15.4 | 4.2  | 3.68 | 3.04 |
| 28 | MEP12<br>03425 | 0   | 2.2  | 20.2 | 0.11 | 3.90 | 5.5 | 2.5  | 13.5 | 0.18 | 2.79 |
| 29 | MEP12<br>03426 | 0   | 2.8  | 18.8 | 0.15 | 3.56 | 3.6 | 3.6  | 13.8 | 0.26 | 2.45 |

[0097] Example 4: Verification of arginine metabolizing ability with Lactococcus lactis ssp. lactis in reconstituted skim milk (1)

Based on the results of Example 3, search for strains that provide a relatively high Cit/Orn value was carried out, and 5 strains were selected. Specifically, for 5 strains described in Table 4 below, the arginine metabolizing ability was examined. A reconstituted skim milk with 1% by weight of arginine added thereto (concentration of skimmed milk powder: 10% by weight) (hereinafter referred to as "1% Arg + 10% reconstituted skim milk") was used as the medium.

[0098] Activating culture solutions (20 mL) of 5 strains described in Table 4 below in a MRS medium were centrifuged (8000 rpm, 10 min) to collect microbial cells of the 5 strains. Thereafter, 40 mL of "1% Arg + 10% reconstituted skim milk" was sterilized at 95°C for 3 min. As a result, as described above, the collected microbial cells of the 5 strains were inoculated. In the addition of arginine to the reconstituted skim milk, at the outset, an aqueous solution of arginine was prepared, and pH of the aqueous solution was adjusted by the addition of hydrochloric

acid, followed by mixing with the reconstituted skim milk. These 5 strains were aerobically cultured in a stationary state under conditions of culturing temperatures of 40°C and 43°C and culturing times of 4 hr and 8 hr.

[0099] The results were as shown in Table 4 below. Here search for strains that provide a relatively high Cit/Orn value and for a culturing temperature and a culturing time that provide a high Cit/Orn value. For these strains, as a whole, there was a tendency that the Cit/Orn value increased with raising the culturing temperature and increasing the culturing time.

[0100]

Table 4: Relationship between arginine metabolizing ability with *Lactis* and culturing temperature and time (skimmed milk powder medium)

(1)

| Strain     | Temp. (°C) | Time (H) | Arg (mM) | Cit (mM) | Orn (mM) | Cit/Orn |
|------------|------------|----------|----------|----------|----------|---------|
| OLS3789    | 40         | 4        | 28.5     | 2.2      | 13.1     | 0.17    |
|            |            | 8        | 17.1     | 4.8      | 20.8     | 0.23    |
|            | 43         | 4        | 30.3     | 3.9      | 9.9      | 0.40    |
|            |            | 8        | 17.8     | 14.2     | 12.3     | 1.16    |
| OLS3797    | 40         | 4        | 31.8     | 6.8      | 4.4      | 1.54    |
|            |            | 8        | 22.2     | 16.6     | 10.8     | 1.54    |
|            | 43         | 4        | 29.7     | 11.7     | 4.6      | 2.52    |
|            |            | 8        | 7.8      | 31.1     | 5.3      | 5.91    |
| MEP1203416 | 40         | 4        | 33.6     | 1.6      | 7.0      | 0.23    |
|            |            | 8        | 27.8     | 4.2      | 14.9     | 0.28    |
|            | 43         | 4        | 32.2     | 2.3      | 9.7      | 0.23    |
|            |            | 8        | 23.3     | 5.4      | 18.7     | 0.29    |
| MEP1203422 | 40         | 4        | 31.7     | 1.6      | 8.2      | 0.20    |
|            |            | 8        | 23.3     | 4.0      | 17.4     | 0.23    |
|            | 43         | 4        | 30.6     | 2.1      | 10.4     | 0.20    |
|            |            | 8        | 19.2     | 4.4      | 19.9     | 0.22    |
| OLS3818    | 40         | 4        | 33.8     | 4.6      | 5.4      | 0.86    |
|            |            | 8        | 24.2     | 9.1      | 12.9     | 0.70    |
|            | 43         | 4        | 27.6     | 10.9     | 5.9      | 1.84    |
|            |            | 8        | 5.3      | 30.4     | 6.9      | 4.41    |

[0101] Example 5: Verification of arginine metabolizing ability with *Lactococcus lactis* ssp. *lactis* in reconstituted skim milk (2)

Microbial cells were cultured and the arginine metabolizing ability was examined in the same manner as in Example 4,

except that the culturing temperature was 44.5°C, 46°C, and temperatures increased from 50°C to 70°C by an increment of 5°C. The results were as shown in Table 5 below. Based on the results of Example 4, search for strains that provide a relatively high Cit/Orn value was carried out, and 3 strains were selected and used.

[0102]

Table 5: Relationship between arginine metabolizing ability with lactis and culturing temperature and time (skimmed milk powder medium)

(2)

| Strain      | Temp.<br>(°C) | Time<br>(H) | Arg<br>(mM) | Cit<br>(mM) | Orn<br>(mM) | Cit/Orn |
|-------------|---------------|-------------|-------------|-------------|-------------|---------|
| OLS<br>3789 | 44.5          | 4           | 28.1        | 4.3         | 8.3         | 0.51    |
|             |               | 8           | 18.6        | 13.3        | 10.9        | 1.22    |
|             | 46            | 4           | 27.4        | 7.4         | 7.1         | 1.04    |
|             |               | 8           | 17.3        | 18.0        | 8.4         | 2.14    |
|             | 50            | 4           | 28.51       | 22.53       | 2.56        | 8.79    |
|             |               | 8           | 8.90        | 44.05       | 2.72        | 16.17   |
|             | 55            | 4           | 43.19       | 7.28        | 2.97        | 2.45    |
|             |               | 8           | 38.70       | 15.24       | 3.89        | 3.92    |
|             | 60            | 4           | 34.43       | 21.77       | 0.67        | 32.39   |
|             |               | 8           | 23.28       | 34.58       | 0.84        | 41.16   |
|             | 65            | 4           | 42.13       | 13.65       | 0.54        | 25.47   |
|             |               | 8           | 36.82       | 12.95       | 0.67        | 19.35   |
|             | 70            | 4           | 52.05       | 2.48        | 0.40        | 6.25    |
|             |               | 8           | 53.60       | 2.26        | 0.50        | 4.57    |

Table 5 (Continued)

| Strain      | Temp.<br>(°C) | Time<br>(H) | Arg<br>(mM) | Cit<br>(mM) | Orn<br>(mM) | Cit/Orn |
|-------------|---------------|-------------|-------------|-------------|-------------|---------|
| OLS<br>3797 | 44.5          | 4           | 27.0        | 8.4         | 5.6         | 1.51    |
|             |               | 8           | 8.5         | 27.7        | 6.3         | 4.43    |
|             | 46            | 4           | 25.3        | 13.8        | 3.1         | 4.42    |
|             |               | 8           | 9.3         | 28.9        | 3.1         | 9.41    |
|             | 50            | 4           | 28.52       | 23.55       | 1.88        | 12.56   |
|             |               | 8           | 9.17        | 40.65       | 1.93        | 21.09   |
|             | 55            | 4           | 24.59       | 27.86       | 1.22        | 22.76   |
|             |               | 8           | 4.05        | 50.45       | 1.52        | 33.25   |
|             | 60            | 4           | 20.32       | 36.10       | 1.00        | 36.10   |
|             |               | 8           | 1.93        | 54.84       | 1.65        | 33.24   |
|             | 65            | 4           | 35.22       | 19.91       | 0.96        | 20.85   |
|             |               | 8           | 31.83       | 22.94       | 1.46        | 15.71   |
|             | 70            | 4           | 50.83       | 2.49        | 0.48        | 5.19    |
|             |               | 8           | 52.73       | 2.17        | 0.58        | 3.77    |

Table 5 (Continued)

| Strain      | Temp.<br>(°C) | Time<br>(H) | Arg<br>(mM) | Cit<br>(mM) | Orn<br>(mM) | Cit/Orn |
|-------------|---------------|-------------|-------------|-------------|-------------|---------|
| OLS<br>3818 | 44.5          | 4           | 27.3        | 10.2        | 5.4         | 1.89    |
|             |               | 8           | 9.3         | 26.2        | 6.3         | 4.14    |
|             | 46            | 4           | 25.1        | 14.5        | 2.5         | 5.83    |
|             |               | 8           | 11.4        | 28.8        | 2.6         | 11.25   |
|             | 50            | 4           | 35.23       | 19.12       | 2.22        | 8.62    |
|             |               | 8           | 34.55       | 19.13       | 2.20        | 8.71    |
|             | 55            | 4           | 35.60       | 16.52       | 2.29        | 7.23    |
|             |               | 8           | 29.38       | 26.70       | 1.96        | 13.63   |
|             | 60            | 4           | 37.19       | 18.64       | 0.62        | 30.07   |
|             |               | 8           | 28.48       | 28.24       | 0.82        | 34.64   |
|             | 65            | 4           | 43.40       | 8.71        | 0.48        | 18.22   |
|             |               | 8           | 31.04       | 7.73        | 0.52        | 14.90   |
|             | 70            | 4           | 54.33       | 1.59        | 0.47        | 3.42    |
|             |               | 8           | 54.49       | 1.30        | 0.54        | 2.40    |

[0103] Example 6: Production of citrulline-containing yoghurt using Lactococcus lactis ssp.lactis OLS3797 strain or OLS3818 strain

A citrulline-containing fermented milk (yoghurt) was produced using Lactococcus lactis ssp.lactis OLS3797 strain or OLS3818 strain. Specifically, a raw milk (a yoghurt base) described in Table 6 below was prepared by blending, and the blend was sterilized at 95°C for 3 min. A mixed starter (2% by weight) of Lactobacillus bulgaricus and Streptococcus thermophilus was added as a yoghurt starter to the raw milk. Further, Lactococcus lactis ssp.lactis OLS3797 strain or OLS3818 strain was added, and the mixture was fermented at 45°C to produce yoghurt. Here a culture solution in a MRS medium was centrifuged (8000 rpm, 10 min), and only microbial cells were collected and used as Lactococcus lactis ssp.lactis OLS3797 strain and OLS3818 strain. In this case, the amount (weight) of the MRS medium used was the same as the raw milk described in Table 6 below.

[0104]

Table 6: Composition of raw milk (yoghurt base) (% by mass)

|                                    |      |
|------------------------------------|------|
| Milk                               | 78.2 |
| Skimmed milk powder                | 2.5  |
| Whey protein isolate (WPI)         | 0.2  |
| Arginine solution (10% by weight)* | 10.0 |
| Water                              | 9.1  |

\* Arginine was dissolved in a hydrochloric acid solution, and pH of the solution after dissolution was adjusted to about 5.

[0105] When Lactococcus lactis ssp.lactis OLS3797 strain was used, the acidity was reached 0.80% in 5 hr 30 min. On the other hand, when Lactococcus lactis ssp.lactis OLS3818 strain was used, the acidity was reached 0.83% in 4 hr 30 min. When these 2 strains were used, it was confirmed that the fermentation proceeded smoothly. Further, there was no significant difference in flavor and physical properties between these fermented milks (yoghurts) and common products and conventional products.

[0106] The concentration of each of arginine, citrulline, and ornithine in these fermented milks (yoghurts) was measured. The results were as shown in Table 7 below. It was confirmed that arginine

was largely metabolized, and a large portion of the metabolite was converted into citrulline. That is, it was demonstrated that fermented milks (yoghurts) containing citrulline at a high concentration could be produced using Lactococcus lactis ssp.lactis OLS3797 strain or OLS3818 strain.

[0107]

Table 7: Arginine metabolizing ability of lactis in yoghurt

| Strain   | Fermentation time | Arg (mM) | Cit (mM) | Orn (mM) |
|----------|-------------------|----------|----------|----------|
| OLS 3797 | 5.5               | 0        | 46       | 10.6     |
| OLS 3818 | 4.5               | 11.6     | 34.8     | 13.1     |

[0108] Example 7: Study on process for preparing culture solution of a strain (a Lactococcus lactis ssp.lactis OLS3797 strain) having a high efficiency of transduction from arginine to citrulline (1)

A study was made on a process for preparing a culture solution having a high efficiency of transduction (activity) from arginine to citrulline. In the above Examples, a culture solution was prepared by subjecting L. lactis in a MRS medium to stationary culturing. As a result, it was observed that, when L. lactis was subjected to stationary culturing, a lowering in pH of the culture solution led to termination of the growth of L. lactis and made it impossible to enhance the concentration of microbial cells. A study on an influence on microbial cell concentration was made by subjecting L. lactis to stirring culturing in a MRS medium while regulating pH through the addition of an alkali to a culture solution to inhibit a lowering in pH of the culture solution (culturing with pH regulation). That is, a study on the possibility of an enhancement in efficiency of transduction from arginine to citrulline per unit volume in a culture solution of L. lactis by culturing L. lactis in a MRS medium while regulating pH. The MRS medium was sterilized at 121°C for 15 min.

[0109]

An aqueous sodium hydroxide solution (NaOH: 10% by weight) was added to the culture solution, L. lactis OLS3797 strain was subjected to stirring culturing while regulating pH of the culture solution at 5.5 (culturing while regulating pH), and the concentration of microbial cells was measured with the elapse of time. Specifically, a MRS medium was inoculated with the activating culture solution (culturing

temperature: 30°C, culturing time: 16 hr) of L. lactis OLS3797 strain in a MRS medium at a concentration of 1% by weight, followed by stirring culturing (culturing temperature: 30°C, stirring speed: 200 rpm). A change in microbial cell concentration with the elapse of time was as shown in Fig. 1.

[0110] The culture solution being cultured while regulating pH was collected 10, 11, and 12 hr after the start of culturing, and these culture solutions were stored with cooling in ice water. "1% Arg + 10% reconstituted skim milk" was inoculated with 5% by weight of the culture solution, and the mixture was subjected to stationary culturing (culturing temperature: 44.5°C, culturing time: 8 hr) to examine the efficiency of transduction from arginine to citrulline. For comparison, a MRS medium was inoculated with 5% by weight of the culture solution, and the mixture was subjected to stationary culturing (culturing temperature: 30°C, culturing time: 16 hr) to examine the efficiency of transduction from arginine to citrulline. The results were as shown in Fig. 2 and Table 8 below.

[0111]

Table 8: Relationship between efficiency of transduction to citrulline in lactis and culturing conditions

| Medium               | Cit Production (mM) |     | Initial Cell (cfu/mL) | Cit Production ( $\times 10^9$ mM)<br>Initial Cell · H (cfu · H /mL) |     |
|----------------------|---------------------|-----|-----------------------|----------------------------------------------------------------------|-----|
|                      | 4H                  | 8H  |                       | 4H                                                                   | 8H  |
| Stationary           | 1.3                 | 2.0 | $1.0 \times 10^8$     | 3.3                                                                  | 2.7 |
| pH regulation<br>10H | 3.1                 | 5.4 | $2.3 \times 10^8$     | 3.4                                                                  | 3.0 |
| pH regulation<br>11H | 3.3                 | 5.5 | $2.3 \times 10^8$     | 3.6                                                                  | 3.0 |
| pH regulation<br>12H | 3.4                 | 5.9 | $2.5 \times 10^8$     | 3.5                                                                  | 3.0 |

[0112] As described above, it was demonstrated that, as compared with stationary culturing in the MRS medium, culturing while regulating pH in a MRS medium exhibited an enhancement in efficiency of transduction from arginine to citrulline per unit volume in the culture solution of L. lactis. On the other hand, there was no difference in efficiency of transduction from arginine to citrulline per unit time and unit

microbial cell in the culture solution of *L. lactis* between stationary culturing in the MRS medium and culturing while regulating pH in the MRS medium. That is, it could be determined that, as compared with the stationary culturing, in the culturing while regulating pH, the microbial cell concentration is enhanced while there is no change in the efficiency of transduction from arginine to citrulline per unit microbial cell. The efficiency of transduction from arginine to citrulline per unit time and unit microbial cell was calculated as a numeric value obtained by dividing the concentration of citrulline 4 hr and 8 hr after the start of culturing by an incipient viable cell count and culturing times (4 hr and 8 hr).

[0113] For an experiment where a culture solution 12 hr after the start of culturing while regulating pH, the number of cells were counted 4 hr and 8 hr after the start of culturing in "1% Arg + 10% reconstituted skim milk." As a result, the number of cells 4 hr after the start of culturing and the number of cells 8 hr after the start of culturing were  $1 \times 10^6$  cfu/mL and  $1 \times 10^4$  cfu/mL (cfu: colony forming unit), respectively, demonstrating that the number of cells decreased with the elapse of time from the start of culturing. Accordingly, it could be estimated that there is a low possibility of the production of ADI during the measurement of the efficiency of transduction from arginine to citrulline and arginine is converted into citrulline through ADI possessed by microbial cells themselves. There is a possibility of some lowering in ADI activity with the elapse of time from the start of culturing. However, there was no significant difference in transduction efficiency between 4 hr after the start of culturing and 8 hr after the start of culturing.

[0114] Example 8: Study on process for preparing culture solution of strain (*Lactococcus lactis* ssp. *lactis* 3797 strain) having a high efficiency of transduction from arginine to citrulline (2)

In Example 7, when *L. lactis* was cultured while regulating pH, the microbial cell concentration (number of cells) increased, but on the other hand, the efficiency of transduction from arginine to citrulline per unit time and unit microbial cell remained unchanged. Accordingly, a study was made on a possibility of an enhancement in efficiency of transduction from arginine to citrulline per unit volume in a culture solution of *L. lactis* by stationary culturing after the addition of arginine to a MRS medium before the start of culturing of *L. lactis*. Specifically, *L.*

lactis OLS3797 strain was subjected to stationary culturing in a conventional MRS medium and a MRS medium containing 0.5% by weight of arginine, and the efficiency of transduction from arginine to citrulline in "1% Arg + 10% reconstituted skim milk" using the culture solution was measured. Here the MRS medium was sterilized at 121°C for 15 min.

[0115] Specifically, a MRS medium was inoculated with an activating culture solution of L. lactis 3797 strain (culturing temperature 30°C, culturing time 16 hr) at a concentration of 1% by weight, and the mixture was subjected to stationary culturing (culturing temperature: 30°C, culturing time: 16 hr). The culture solution (20 mL) was centrifuged (8000 rpm, 10 min), only microbial cells were collected, and 40 mL of "1% Arg + 10% reconstituted skim milk" was inoculated with the cells, followed by stirring culturing (culturing temperature: 44.5°C, culturing time: 4 hr) to examine the efficiency of transduction from arginine to citrulline. The results were as shown in Table 9 below.

[0116] On the other hand, a MRS medium containing 0.5% by weight of arginine was inoculated with the activating culture solution at a concentration of 1% by weight, followed by stationary culturing (culturing temperature: 30°C, culturing time: 16 hr). The culture solution (20 mL) was centrifuged (8000 rpm, 10 min), only microbial cells were collected, and 40 mL of "1% Arg + 10% reconstituted skim milk" was inoculated with the cells, followed by stirring culturing (culturing temperature: 44.5°C, culturing time: 4 hr) to examine the efficiency of transduction from arginine to citrulline. The results were as shown in Table 9 below.

[0117]

Table 9: Relationship between efficiency of transduction to citrulline in lactis and composition of medium

| Media             | Cit Production (mM) | Initial Cell (cfu/mL) | Cit Production ( $\times 10^9$ mM)<br>Initial Cell $\cdot$ H (cfu $\cdot$ H /mL) |
|-------------------|---------------------|-----------------------|----------------------------------------------------------------------------------|
| MRS               | 8.4                 | $6.7 \times 10^8$     | 3.14                                                                             |
| MRS<br>+ 0.5% Arg | 27.9                | $1.1 \times 10^9$     | 6.41                                                                             |

[0118] As described above, it was demonstrated that, as compared with stationary culturing in ordinary (conventional) MRS media,

stationary culturing in an arginine-containing MRS medium results in an enhancement in efficiency of transduction from arginine to citrulline per unit time and unit microbial cell in a culture solution of L. lactis by a factor of 2 or more.

[0119] Example 9: Study on process for preparing culture solution of strain (Lactococcus lactis ssp. lactis OLS3797 strain) having a high efficiency of transduction from arginine to citrulline (3)

In Example 8, the addition of arginine to media resulted in an enhancement in efficiency of transduction with L. lactis from arginine to citrulline. A study was made on a possibility of an enhancement in efficiency of transduction from arginine to citrulline per unit volume in a culture solution of L. lactis by adding arginine to a MRS medium and performing culturing while regulating pH before the start of culturing of L. lactis. Specifically, L. lactis OLS3797 strain was cultured while regulating pH in a MRS medium containing 0.5% by weight of arginine, and the efficiency of transduction from arginine to citrulline in "1% Arg + 10% reconstituted skim milk" using the culture solution was measured. Here the MRS medium was sterilized at 121°C for 15 min. Thereafter, the aqueous arginine solution was filtered and sterilized, and the sterilized solution was added to the medium to adjust the concentration of arginine to 0.5% by weight.

[0120] An aqueous sodium hydroxide solution (NaOH: 10% by weight) was added to the culture solution, L. lactis OLS3797 strain was subjected to stirring culturing (culturing while regulating pH) while regulating pH of the culture solution at 5.5, and the microbial cell concentration and the like were measured with the elapse of time. Specifically, a MRS medium containing 0.5% by weight of arginine was inoculated with an activating culture solution of L. lactis OLS3797 strain in a MRS medium (culturing temperature: 30°C, culturing time: 16 hr) at a concentration of 1% by weight, NaOH was added, and stirring culturing (culturing temperature: 30°C, stirring speed: 200 rpm) was carried out while regulating pH of the culture solution at 5.5. A change in microbial cell concentration, pH arginine concentration, citrulline concentration, and ornithine concentration with the elapse of time were as shown in Fig. 3.

[0121] As described above, arginine was added to the medium

before the start of culturing of L. lactis, and, as a result, pH of the medium at the start of culturing was increased to 8.3. It is believed that an increase in pH of the medium at the start of culturing (in an early stage of culturing) led to delay of the growth of the strain. At a later stage (a second half) of the culturing, lactic acid bacterium (microbial cells) were smoothly grown, the consumption of arginine was started after the elapse of 10 hr or more from the start of the culturing, and the concentration of arginine became zero (0) 13 hr after the start of the culturing. In the culturing, the highest attainable microbial cell concentration was  $2.7 \times 10^9$  cfu/mL, and the microbial cell concentration was lowered to a value that was half of that in Example 7.

[0122] After the elapse of 8 hr, 9 hr, 10 hr, 11 hr, 12 hr, and 13 hr from the start of culturing, the culture solution was collected and was cooled and stored in ice water. "1% Arg + 10% reconstituted skim milk" was inoculated with the culture solution at a concentration of 5% by weight, and stationary culturing (culturing temperature: 44.5°C, culturing time: 8 hr) was carried out to examine the efficiency of transduction from arginine to citrulline. The results were as shown in Table 10 below.

[0123]

Table 10: Relationship between efficiency of transduction to citrulline and culturing while regulating pH in *lactis*

| Cultivation Time (H) | Cit Production (mM) | Initial Cell (cfu/mL) | Cit Production ( $\times 10^9$ mM) / Initial Cell $\cdot$ H (cfu $\cdot$ H / mL) |
|----------------------|---------------------|-----------------------|----------------------------------------------------------------------------------|
| 8                    | 0.0                 | $6.3 \times 10^6$     | 0.0                                                                              |
| 9                    | 0.7                 | $3.4 \times 10^7$     | 4.2                                                                              |
| 10                   | 1.1                 | $3.4 \times 10^7$     | 4.0                                                                              |
| 11                   | 2.9                 | $8.6 \times 10^7$     | 4.3                                                                              |
| 12                   | 13.5                | $1.3 \times 10^8$     | 13.0                                                                             |
| 13                   | 21.5                | $1.3 \times 10^8$     | 20.3                                                                             |

As described above, the consumption of arginine increased rapidly from after the elapse of 11 hr from the start of culturing. It was confirmed that, when the culture solution that allows arginine to be consumed fast is used, the efficiency of transduction from arginine to citrulline is enhanced.

[0124] Example 10: Study on process for preparing culture solution of strain (Lactococcus lactis ssp.lactis OLS3797 strain) having a high

efficiency of transduction from arginine to citrulline (4)

In Example 9, the addition of arginine to the medium before the start of culturing of lactic acid bacterium (microbial cell) significantly enhances the efficiency of transduction from arginine to citrulline, but on the other hand, the highest attainable microbial cell concentration was lower than that when arginine was not added. It was considered that, when pH of the medium at the time of start of culturing is high, the highest attainable microbial cell concentration is likely to be lowered. Accordingly, a study was made on the possibility of an enhancement in efficiency of transduction with *L. lactis* from arginine to citrulline in a culture solution by culturing while regulating pH through the addition of arginine to the medium (culture solution) at a time (intermittently) in the process of culturing of *L. lactis*. The MRS medium was sterilized at 121°C for 15 min.

[0125] An aqueous sodium hydroxide solution (NaOH: 10% by weight) was added to the culture solution, *L. lactis* OLS3797 strain was subjected to stirring culturing (culturing while regulating pH) while regulating pH of the culture solution at 5.5, and the microbial cell concentration and the like were measured with the elapse of time. At that time: 10 hr after the start from culturing, an aqueous arginine solution (arginine: 10% by weight) was filtered and sterilized before the addition to the medium (culture solution) to regulate the arginine concentration to 0.5% by weight. Specifically, a MRS medium was inoculated with an activating culture solution (culturing temperature: 30°C, culturing time: 16 hr) of *L. lactis* OLS3797 strain in a MRS medium, NaOH was added thereto, and the mixture was subjected to stirring culturing (culturing temperature: 30°C, stirring speed: 200 rpm) while adjusting pH of the culture solution to 5.5. Further, 10 hr after the start of culturing, an aqueous arginine solution was filtered and sterilized before the addition to the medium to regulate the arginine concentration to 0.5% by weight. A change in microbial cell concentration, pH, arginine concentration, citrulline concentration, and ornithine concentration with the elapse of time was as shown in Fig. 4.

[0126] After the elapse of 8hr, 10hr, 12hr, and 13 hr after the start of culturing, the culture solution was collected and was cooled and stored in ice water. "1% Arg + 10% reconstituted skim milk" was

inoculated with the culture solution at a concentration of 5% by weight, and stationary culturing (culturing temperature: 44.5°C, culturing time: 4.5 hr) was carried out to examine the efficiency of transduction from arginine to citrulline. The results were as shown in Table 11 below.

[0127]

Table 11: Relationship between efficiency of transduction to citrulline and timing of addition of arginine in lactis

| Cultivation Time (H) | Cit Production (mM) | Initial Cell (cfu/mL) | Cit Production ( $\times 10^9$ mM) / Initial Cell $\cdot$ H (cfu $\cdot$ H /mL) |
|----------------------|---------------------|-----------------------|---------------------------------------------------------------------------------|
| 8                    | 1.7                 | $1.4 \times 10^8$     | 4.2                                                                             |
| 10                   | 2.4                 | $1.4 \times 10^8$     | 8.0                                                                             |
| 12                   | 7.4                 | $2.5 \times 10^8$     | 7.5                                                                             |
| 13                   | 9.1                 | $3.6 \times 10^8$     | 6.4                                                                             |

As described above, the highest attainable microbial cell concentration was  $7.1 \times 10^9$  cfu/mL, and the microbial cell concentration was the same as that in Example 7. On the other hand, the efficiency of transduction from arginine to citrulline was lower than that in Example 9.

[0128] Example 11: Study on process for preparing culture solution of strain (Lactococcus lactis ssp.lactis OLS3797 strain) having a high efficiency of transduction from arginine to citrulline (5)

Culturing while regulating pH was carried out in the same manner as in Example 10, except that arginine was added to the medium (culture solution) at a time (intermittently) in the process of culturing of lactic acid bacterium (microbial cells). As a result, the highest attainable microbial cell concentration was the same as that in ordinary (conventional) culturing while regulating pH, but on the other hand, the efficiency of transduction from arginine to citrulline was lower than that when arginine was added to the medium before the start of culturing of lactic acid bacterium (microbial cells). Accordingly, a study was made on a possibility of an enhancement in efficiency of transduction from arginine to citrulline per unit volume in a culture solution of L. lactis by culturing while regulating pH and while adding arginine to a medium (culture solution) little by little (intermittently) in the process of culturing of L. lactis. Here the MRS medium was sterilized at 121°C for 15 min.

[0129] An aqueous arginine solution (arginine: 10% by weight) was added to the culture solution, and L. lactis OLS3797 strain was subjected to stirring culturing (culturing while regulating pH) while regulating pH of the culture solution to 5.5 to measure the microbial cell concentration and the like with the elapse of time. At that time, 4 hr after the start of culturing, an aqueous arginine solution (arginine: 10% by weight) was filtered and sterilized, and the sterilized solution was added to the medium (culture solution) to adjust pH of the medium to 5.5. Specifically, a MRS medium was inoculated with an activating culture solution of L. lactis OLS3797 strain in a MRS medium (culturing temperature: 30°C, culturing time: 16 hr) at a concentration of 1% by weight, and arginine was added thereto, the mixture was subjected to stirring culturing (culturing temperature: 30°C, stirring speed: 200rpm) while regulating pH of the culture solution to 5.5. After the elapse of 4 hr from the start of culturing, an aqueous arginine solution was filtered and sterilized, and the sterilized solution was added to the medium to adjust pH of the medium to 5.5. A change in microbial cell concentration, pH, arginine concentration, citrulline concentration, and ornithine concentration with the elapse of time was as shown in Fig. 5.

[0130] After the elapse of 8 hr, 10 hr, 12 hr, and 13 hr from the start of culturing, the culture solution was collected and was cooled and stored in ice water. "1% Arg + 10% reconstituted skim milk" was inoculated with the culture solution at a concentration of 5% by weight, and stationary culturing (culturing temperature: 44.5°C, culturing time: 4.5 hr) was carried out to examine the efficiency of transduction from arginine to citrulline. The results were as shown in Table 12 below. Here, in the table, "culture" refers to a solution obtained by inoculating "1% Arg + 10% reconstituted skim milk" with the culture solution per se at a concentration of 5% by weight. Further, "supernatant" refers to a solution obtained by inoculating "1% Arg + 10% reconstituted skim milk" with a supernatant obtained by centrifuging a culture solution at a concentration of 5% by weight. "Cell" refers to a product obtained by inoculating "1% Arg + 10% reconstituted skim milk" with a product obtained by cryopreserving at -80°C microbial cells obtained by centrifuging the culture solution and removing the supernatant and then thawing the cryopreserved product at a concentration of 5% by weight.

[0131]

Table 12: Relationship between efficiency of transduction to citrulline in lactis and timing of addition of arginine/culturing while regulating pH

| Cultivation | Form of cell for test | Cit (mM) | Bacterial Count (cfu/mL) | Cit Production ( $\times 10^9$ mM)<br>Initial Cell·H (cfu·H /mL) |
|-------------|-----------------------|----------|--------------------------|------------------------------------------------------------------|
| 8           | Culture               | 11.8     | $2.5 \times 10^8$        | 12.1                                                             |
| 10          | Culture               | 23.0     | $2.4 \times 10^8$        | 23.7                                                             |
| 12          | Culture               | 25.6     | $3.1 \times 10^8$        | 20.6                                                             |
| 13          | Culture               | 24.9     | $3.2 \times 10^8$        | 19.3                                                             |
| 10          | Cell                  | 19.3     | $2.4 \times 10^8$        | 19.9                                                             |
| 10          | Supernatant           | 0.9      |                          |                                                                  |

[0132] Here the addition of arginine (neutralizing agent, substrate) to the medium (culture solution) was started 4 hr after the start of culturing and was continued until the end of culturing. At that time, arginine was immediately converted into ornithine for a while from the start of addition of arginine to the medium. At a second half of culturing, however, some of arginine remained as it is without transduction to ornithine.

[0133] As described above, the highest attainable cell concentration was  $6.5 \times 10^9$  cfu/mL, and the microbial cell concentration was substantially the same as those in Example 7 and Example 10. The efficiency of transduction from arginine to citrulline was substantially the same as that in Example 9. That is, here the efficiency of transduction from arginine to citrulline was enhanced with an enhancement in microbial cell concentration.

[0134] Further, in the experiment where only microbial cells were collected and cryopreserved, the efficiency of transduction from arginine to citrulline was also substantially the same as the experiment where the culture solution of the microbial cell as such was used. The arginine was wholly metabolized by the elapse of 4 to 8 hr from the start of culturing, and most of arginine was converted into citrulline.

[0135] In the experiment where only microbial cells were collected before use, the concentration of addition (inoculated dose) to the medium (culture solution) is about 1/20 to 1/50 of that in the experiment where the culture solution of the microbial cell as such was

used. Here since the concentration of addition to the medium is 5% by weight, when only microbial cells are collected before use, the concentration of addition to the medium can be estimated to be 0.25 to 0.1% by weight. That is, when the culture solution was prepared by culturing while regulating pH and while adding arginine (as a neutralizing agent) continuously in the process of culturing (for example, to the end of culturing), the collection and use of only microbial cells can realize the production of milk products containing citrulline at a high concentration in a highly efficient manner.

[Sequence listing]

## [CLAIMS]

1. A method for preparing citrulline, comprising the steps of inoculating a microorganism having a citrulline producing ability into a culture medium and culturing the microorganism at a temperature from 40°C to 70°C.
2. The method for preparing citrulline according to claim 1, wherein the microorganism is lactic acid bacterium, bifidobacteria, or propionibacteriaceae.
3. The method for preparing citrulline according to claim 2, wherein the lactic acid bacterium is one or more members selected from the group consisting of *Lactococcus lactis*, *Lactobacillus fermentum*, and *Lactobacillus buchneri*.
4. The method for preparing citrulline according to claim 2, wherein the lactic acid bacterium is one or more members selected from the group consisting of *Lactococcus lactis* spp. *lactis* strain OLS3789, strain OLS3797, and strain OLS3818.
5. A method for preparing a citrulline-containing composition, comprising the steps of adding a microorganism having a citrulline producing ability to a starting material and culturing the microorganism at a temperature from 40°C to 70°C.
6. The method for preparing a citrulline-containing composition according to claim 5, wherein the microorganism having a citrulline producing ability is lactic acid bacterium, bifidobacteria, or propionibacteriaceae.
7. The method for preparing a citrulline-containing composition according to claim 6, wherein the lactic acid bacterium is one or more members selected from *Lactococcus lactis*, *Lactobacillus fermentum*, and *Lactobacillus buchneri*.
8. The method for preparing a citrulline-containing composition according to claim 6, wherein the lactic acid bacterium is one or more members selected from the group consisting of *Lactococcus lactis* spp. *lactis* strain OLS3789, strain OLS3797, and strain OLS3818.
9. The method for preparing a citrulline-containing composition according to any one of claims 5 to 8, wherein the starting material is a raw milk.

10. The method for preparing a citrulline-containing composition according to any one of claims 5 to 9, wherein the citrulline-containing composition is food and drink.

5 11. A method for preparing a culture solution containing a microorganism that has a citrulline producing ability wherein a conversion efficiency from arginine into citrulline at a temperature from 40°C to 70°C, the method comprising the step of culturing a microorganism having a citrulline producing ability in a culture medium to which arginine is added.

10 12. The method for preparing a culture solution according to claim 11, wherein arginine is added to the medium before the culturing and/or is intermittently added during culturing, or is continuously added during culturing.

15 13. A method for preparing a culture solution containing a microorganism having a citrulline producing ability and having an increased concentration of the microorganism contained, the method comprising the step of culturing a microorganism having a citrulline producing ability in a culture medium of which pH is adjusted between 5 to 7 thereby increasing a concentration of the microorganism.

20 14. The method for preparing a culture solution according to claim 13, wherein pH of the medium is adjusted by the addition of an alkali.

25 15. A method for preparing a culture solution containing a microorganism having a citrulline producing ability wherein a conversion efficiency from arginine into citrulline at a temperature from 35°C to 70°C and a concentration of the microorganism contained therein being increased, the method comprising the step of culturing a microorganism having a citrulline producing ability in a culture medium to which arginine is added and of which pH is set between 5 to 7 thereby increasing a concentration of the microorganism.

30 16. The method for preparing a culture solution according to claim 15, wherein arginine is added to the medium before the culturing and/or is intermittently added during culturing, or is continuously added during culturing.

35 17. A method for preparing a microorganism having a citrulline producing ability wherein conversion efficiency from arginine into citrulline, the method comprising the step of subjecting a culture solution

obtained by a method according to any one of claims 11 to 16 to centrifugation and/or membrane separation.

18. A method for preparing citrulline according to any one of claims 1 to 4, comprising the steps of inoculating into a medium a culture solution  
5 obtained by a method for preparing a culture solution according to any one of claims 11 to 16 or a microorganism obtained by a method for preparing a microorganism according to claim 17, and culturing the culture solution or the microorganism, or a method for preparing a citrulline-containing composition according to any one of claims 5 to 10,  
10 the method comprising adding the culture solution or the microorganism to a starting material and fermenting the mixture.