

DESCRIPTION

TITLE OF INVENTION:

YOGURT AND PRODUCTION METHOD THEREOF, PRODUCTION METHOD OF LACTIC ACID BACTERIA EXTRACELLULAR FUNCTIONAL PRODUCT AND AGENT FOR ENHANCING YIELD OF LACTIC ACID BACTERIA EXTRACELLULAR FUNCTIONAL PRODUCT

TECHNICAL FIELD

[0001] The present invention relates to a production method of yogurt containing an increased amount of a lactic acid bacteria functional product and to yogurt produced by the production method of yogurt. The present invention also relates to a production method of a lactic acid bacteria extracellular functional product, the production method allowing enhancement in the yield of the lactic acid bacteria extracellular functional product, and to an agent for enhancing the yield of the lactic acid bacteria extracellular functional product used for the production method.

BACKGROUND ART

[0002] An intake of lactic acid bacteria and functional products thereof is known to improve digestive bacterial flora and confer advantageous effects (maintenance of good health, reduction of risk of illness) to hosts. For example, *Lactobacillus delbruechii* subsp. *bulgaricus* is one lactic acid bacterium used as a starter for production of fermented milk such as yogurt. Many strains

belonging to *Lactobacillus delbruechii* subsp. *bulgaricus* produce exopolysaccharides (EPS). EPS are known to contribute not only to physical properties and stability of fermented milk products but also to a manifestation of probiotic effects. For example, it has been known that EPS produced by *Lactobacillus delbruechii* subsp. *bulgaricus* OLL1073R-1 strain (hereinafter also referred to as "OLL1073R-1 strain") may prevent autoimmune diseases. Fermented milk produced with the strain is also known to be effective in activating NK cells, reducing incidence of common colds, preventing influenza and the like (for example, see Patent documents 1 to 3). By utilizing EPS-producing lactic acid bacteria and EPS produced by lactic acid bacteria and the like, functional food and supplements with health-promoting benefits can be obtained.

[0003] In order to effectively produce such functional food and supplements, it is necessary to increase the content of EPS in the functional food.

[0004] Various methods have been previously proposed in order to increase production of EPS from lactic acid bacteria, not to mention *Lactobacillus delbruechii* subsp. *bulgaricus*. For example, Patent document 4 discloses that culturing lactic acid bacteria capable of producing kefiran such as *Lactobacillus kefiranofaciens* in a medium containing peptone, yeast extract and an unsaturated fatty acid or an ester thereof allows production of high concentration of EPS in the culture medium. Patent document 5 also discloses fermentation of milk supplemented with whey protein or soya protein with *Lactococcus lactis* subsp. *cremoris* can

significantly increase EPS from the lactic acid bacteria.

PRIOR ART

PATENT DOCUMENT

[0005] Patent document 1: Japanese Published Unexamined Patent Application No. 2000-247895

Patent document 2: Japanese Published Unexamined Patent Application No. 2005-194259

Patent document 3: WO 2011/065300

Patent document 4: Japanese Published Unexamined Patent Application No. 2011-250756

Patent document 5: Japanese Published Unexamined Patent Application No. 2011-51268

DISCLOSURE OF THE INVENTION

PROBLEM TO BE SOLVED BY THE INVENTION

[0006] An object of the present invention is to provide a novel method that allows an effective increase in the yield of functional products from lactic acid bacteria.

MEANS FOR SOLVING THE PROBLEM

[0007] A production method of yogurt according to an aspect of the present invention includes a pH buffering agent addition step and a fermentation step. In the pH buffering agent addition step, a pH buffering agent is added to a yogurt starting material. In the fermentation step, the yogurt starting material to which the pH buffering agent has been added (hereinafter also referred to as "pH buffering agent-containing yogurt starting material") is

fermented by lactic acid bacteria. The lactic acid bacteria may be added to the yogurt starting material before or after addition of the pH buffering agent.

[0008] As a result of exhaustive studies carried out by the inventors of the present invention, it is now found that, as the production method of the yogurt as described above, fermenting a yogurt starting material after addition of a pH buffering agent thereto can increase the period of time during which the yogurt starting material has pH at which lactic acid bacteria can grow, resulting in an increase in the yield of functional products from the lactic acid bacteria in yogurt. Therefore the production method of yogurt can effectively increase the yield of functional products from lactic acid bacteria.

[0009] In the production method of yogurt, the lactic acid bacteria preferably include at least lactic acid bacteria capable of producing lactic acid bacteria exopolysaccharides. The lactic acid bacteria capable of producing lactic acid bacteria exopolysaccharides preferably include at least lactic acid bacteria belonging to *Lactobacillus delbruechii* subsp. *bulgaricus*. In this case, the lactic acid bacteria capable of producing lactic acid bacteria exopolysaccharides may be solely *Lactobacillus delbruechii* subsp. *bulgaricus* or a mixture of *Lactobacillus delbruechii* subsp. *bulgaricus* and different lactic acid bacteria.

[0010] In the production method of yogurt, the pH buffering agent is preferably a phosphate salt because when the lactic acid bacteria used are a mixture of *Lactobacillus delbruechii* subsp. *bulgaricus*

and *Streptococcus thermophilus*, growth of *Streptococcus thermophilus* can be suppressed while promoting growth of *Lactobacillus delbruechii* subsp. *bulgaricus*. The phosphate salt is preferably an alkali metal salt. The phosphate salt is further preferably at least one phosphate salt selected from the group consisting of disodium hydrogen phosphate and dipotassium hydrogen phosphate.

[0011] Further, in the production method of yogurt, the yogurt starting material preferably contains solids-not-fat at from 8% by weight to 20% by weight. The yogurt starting material in this context means the one before addition of the pH buffering agent.

[0012] In addition, in the fermentation step of the production method of yogurt, the pH buffering agent-containing yogurt starting material is preferably fermented by the lactic acid bacteria at a temperature in the range from 30°C to 40°C.

[0013] The yogurt produced by the production method of yogurt contains the lactic acid bacteria and the pH buffering agent. The yogurt contains the pH buffering agent homogeneously dissolved therein. The lactic acid bacteria and the pH buffering agent are as described above. The pH buffering agent in the yogurt can be detected by, for example, chromatographic techniques such as high performance liquid chromatography, nuclear magnetic resonance (NMR) techniques (for example, see Szlyk E, Hrynczyszyn P, "Phosphate additives determination in meat products by ³¹-phosphorus nuclear magnetic resonance using new internal reference standard: hexamethylphosphoroamide phosphate solution",

Talanta. 15 Mar 2011; 84(1): 199-203, doi:
10.1016/j.talanta.2010.12.046) and the like.

[0014] The yogurt preferably further contains a lactic acid bacteria extracellular functional product (for example, lactic acid bacteria exopolysaccharides). In this case, the content of the lactic acid bacteria extracellular functional product is preferably in the range from 40 mg/kg to 100 mg/kg relative to the total amount of the yogurt.

[0015] A production method of a lactic acid bacteria extracellular functional product according to another aspect of the present invention includes a pH buffering agent addition step and a fermentation step. In the pH buffering agent addition step, a pH buffering agent is added to milk as a raw material. In the fermentation step, the milk as a raw material to which the pH buffering agent has been added is fermented by lactic acid bacteria (hereinafter also referred to as "extracellular functional product-producing lactic acid bacteria") capable of producing the lactic acid bacteria extracellular functional product. The lactic acid bacteria may be added to the milk as a raw material before or after addition of the pH buffering agent.

[0016] As a result of exhaustive studies carried out by the inventors of the present invention, it is now found that, as the production method of the lactic acid bacteria extracellular functional product as described above, fermenting milk as a raw material after addition of a pH buffering agent thereto can increase the period of time during which the milk as a raw material has pH

at which extracellular functional product-producing lactic acid bacteria can grow, resulting in an increase in the yield of the functional product from the lactic acid bacteria. Therefore the production method of the lactic acid bacteria extracellular functional product can effectively increase the yield of functional products from lactic acid bacteria. Thus in the present aspect, the pH buffering agent serves as an active substance of an agent for enhancing the yield of the lactic acid bacteria extracellular functional product.

[0017] The production method of the lactic acid bacteria extracellular functional product described above may be described as, in different standpoints, "a method for enhancing the yield of a lactic acid bacteria extracellular functional product by adding a pH buffering agent to milk as a raw material and fermenting the milk as a raw material by lactic acid bacteria", "a method for enhancing the yield of a lactic acid bacteria extracellular functional product by adding a pH buffering agent to milk as a raw material", "a method of adding a pH buffering agent to milk as a raw material in order to enhance the yield of a lactic acid bacteria extracellular functional product", "use of a pH buffering agent to milk as a raw material for enhancing the yield of a lactic acid bacteria extracellular functional product" and the like.

EFFECT OF THE INVENTION

[0018] In the production method of yogurt according to an aspect of the present invention, fermenting a yogurt starting material after addition of a pH buffering agent thereto can increase the

period of time during which the yogurt starting material has pH at which lactic acid bacteria can grow, resulting in an increase in the yield of functional products from the lactic acid bacteria in yogurt. Therefore the production method of yogurt can effectively increase the yield of functional products from lactic acid bacteria.

[0019] In the production method of a lactic acid bacteria extracellular functional product according to another aspect of the present invention, fermenting milk as a raw material after addition of a pH buffering agent thereto can increase the period of time during which the milk as a raw material has pH at which lactic acid bacteria can grow, resulting in an increase of the yield of the functional product from the lactic acid bacteria. Therefore the production method of the lactic acid bacteria extracellular functional product can effectively increase the yield of functional products from lactic acid bacteria.

BEST MODE FOR CARRYING OUT THE INVENTION

[0020] Embodiments for carrying out the present invention are hereinafter specifically described. However, the present invention is not limited to the respective embodiments described hereinafter.

[0021] -First embodiment-

Yogurt according to the present embodiment is produced through a pH buffering agent addition step and a fermentation step. In the pH buffering agent addition step, a pH buffering agent is added

to a yogurt starting material. In the fermentation step, the yogurt starting material to which the pH buffering agent has been added (hereinafter also referred to as "pH buffering agent-containing yogurt starting material") is fermented by lactic acid bacteria.

[0022] Yogurt in the present embodiment refers to "fermented milk" and "fermented milk drink" defined in Ministerial Ordinance Concerning Compositional Standards, etc. for Milk and Milk Products. It is defined in Ministerial Ordinance Concerning Compositional Standards, etc. for Milk and Milk Products that "fermented milk" is "the products which are obtained by fermenting milk, or milk, etc. containing an equal or greater amount of milk solids-not-fat with lactic acid bacteria or yeast and then forming a paste or liquid" or such milk and milk products that are frozen. Fermented milk is broadly classified into "firm yogurt (solid fermented milk, set-type yogurt) obtained by fermenting and solidifying after filling into a container", "stirred yogurt (paste-like fermented milk) which is fermented curd crushed and filled into a container" and "liquid yogurt (liquid fermented milk) obtained by further crushing stirred yogurt in a homogenizer in order to increase the fluidity". It is defined in Ministerial Ordinance Concerning Compositional Standards, etc. for Milk and Milk Products that "fermented milk drink" are "the drinks which are obtained by fermenting milk, etc. with lactic acid bacteria or yeast and then processing it or using it as the principal ingredient (excluding fermented milk)".

[0023] The yogurt starting material contains at least one of milk,

a milk product and a milk protein. Examples of the yogurt starting material include animal milk such as cow, sheep or goat milk and processed products thereof; milk as raw materials such as pasteurised/sterilised milk, skim milk, whole powdered milk, partly skimmed milk, powdered skim milk, whole fat concentrated milk, concentrated skim milk, cream, butter, butter milk, whey, whey protein concentrate (WPC), whey protein isolate (WPI), α -lactalbumin (α -La), β -lactoglobulin (β -Lg), α -casein, β -casein, κ -casein and non-protein nitrogen; sugar, saccharides, processed starch (dextrin, soluble starch, British starch, oxidized starch, starch esters, starch ethers and the like), dietary fibre, sweeteners, organic acids (malic acid, citric acid, lactic acid, tartaric acid and the like), flavours, water and the like. The yogurt starting material is obtained by mixing conventional raw materials as above and dissolving them while heating. The yogurt starting material may contain a gelling agent, a thickening agent, a stabilizer such as gelatin, agar, pectin and carboxymethylcellulose (CMC) and the like. In this case, the yogurt starting material is prepared by dissolving a stabilizer and the like such as gelatin in a solvent such as water and then mixing the aqueous solution of the stabilizer with other components.

[0024] It is preferable that the yogurt starting material contains solids-not-fat (hereinafter referred to as "SNF") at from 8% by weight to 20% by weight. This is because when the lactic acid

bacteria employed are EPS-producing *Lactobacillus delbruechii* subsp. *bulgaricus* and *Streptococcus thermophilus* and the pH buffering agent employed is a phosphate salt in the production method of yogurt according to the embodiment, the flavour of yogurt can be suitably maintained while increasing the content of exopolysaccharides (hereinafter referred to as "EPS") in the yogurt. More preferably, the yogurt starting material contains SNF at from 8.5% by weight to 18.5% by weight, still more preferably from 9% by weight to 15% by weight and particularly preferably from 9.5% by weight to 14% by weight.

[0025] In the present embodiment, the pH buffering agent is not particularly limited; however it is particularly preferably a phosphate salt. It should be noted that the phosphate salt as in the embodiment encompasses hydrates thereof. Examples of the phosphate salt include monosodium dihydrogen phosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), monopotassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4) and the like. These phosphate salts may be used independently, or two or more kinds may be used in combination. In the production method of yogurt according to the embodiment, it is preferable that disodium hydrogen phosphate (Na_2HPO_4) and dipotassium hydrogen phosphate (K_2HPO_4) are used independently or in combination and it is particularly preferable that disodium hydrogen phosphate (Na_2HPO_4) is solely used.

[0026] In the pH buffering agent addition step of the production method of yogurt according to the embodiment, it is preferable that

the amount of the pH buffering agent to be added is in the range from 0.05% by weight to 0.55% by weight relative to the yogurt starting material. This is because when the lactic acid bacteria employed are EPS-producing *Lactobacillus delbruechii* subsp. *bulgaricus* and *Streptococcus thermophilus* and the pH buffering agent employed is a phosphate salt in the production method of yogurt according to the embodiment, the pH buffering agent added within the above range can maintain preferable curd strength (curd tension) and flavour of yogurt while promoting growth of *Lactobacillus delbruechii* subsp. *bulgaricus* as well as enhancing the yield of EPS from *Lactobacillus delbruechii* subsp. *bulgaricus*. The amount of the pH buffering agent added is more preferably in the range from 0.08% by weight to 0.45% by weight and particularly preferably in the range from 0.1% by weight to 0.35% by weight.

[0027] In the present embodiment, the lactic acid bacteria are lactic acid bacteria capable of producing an extracellular functional product e.g. EPS (hereinafter referred to as "extracellular functional product-producing lactic acid bacteria") and are, for example, lactic acid bacteria belonging to *Lactobacillus delbruechii* subsp. *bulgaricus*. In the present embodiment, the lactic acid bacteria may be any strain of extracellular functional product-producing lactic acid bacteria; however it is suitably *Lactobacillus delbruechii* subsp. *bulgaricus* OLL1073R-1 strain (FERM BP-10741) which produces EPS. In the present embodiment, it is preferable that *Lactobacillus delbruechii* subsp. *bulgaricus* is predominant; however

Lactobacillus delbruechii subsp. *bulgaricus* may be used in combination with different lactic acid bacteria producing EPS (hereinafter also referred to as "EPS-producing lactic acid bacteria") such as *Lactobacillus bulgaricus* and *Lactococcus lactis* ssp. *cremoris*.

[0028] The EPS-producing lactic acid bacteria may also be used in combination with lactic acid bacteria other than the EPS-producing lactic acid bacteria, for example, other lactic acid bacteria that are generally used for production of fermented milk such as *Streptococcus thermophilus*, *Lactobacillus gasseri*, Bifidobacteria and Propionibacteria. The lactic acid bacteria may be used together with yeast. In the production method of yogurt according to the present embodiment, the lactic acid bacteria are particularly preferably EPS-producing *Lactobacillus delbruechii* subsp. *bulgaricus* in combination with *Streptococcus thermophilus*.

[0029] In the fermentation step of the production method of yogurt according to the present embodiment, it is preferable that the fermentation temperature is in the range from 30°C to 40°C. This is because when the lactic acid bacteria employed are EPS-producing *Lactobacillus delbruechii* subsp. *bulgaricus* and *Streptococcus thermophilus* and the pH buffering agent employed is a phosphate salt, EPS content of yogurt can be increased. The fermentation temperature is more preferably in the range from 32°C to 39°C and still more preferably in the range from 34°C to 38°C.

[0030] The extracellular functional product-producing lactic

acid bacteria according to the present embodiment preferably produce the extracellular functional product at from 40 mg/kg to 100 mg/kg relative to the total amount. If the amount of EPS produced is within the above range when the lactic acid bacteria employed are EPS-producing *Lactobacillus delbruechii* subsp. *bulgaricus* and *Streptococcus thermophilus* and the pH buffering agent employed is a phosphate salt, it is observed that the ratio of the yield of EPS with addition of the pH buffering agent to the yield of EPS without addition of the pH buffering agent is increased, namely the effect of addition of the pH buffering agent is enhanced. It is more preferably that the extracellular functional product-producing lactic acid bacteria produce the extracellular functional product at from 42 mg/kg to 100 mg/kg, still more preferably at from 44 mg/kg to 100 mg/kg, even more preferably at from 48 mg/kg to 100 mg/kg and particularly preferably at from 50 mg/kg to 100 mg/kg, relative to the total amount.

[0031] In the production method of yogurt according to the present embodiment, the yield of the extracellular functional product is preferably 1.05 times or more higher, more preferably 1.1 times or more higher, still more preferably 1.2 times or more higher and particularly preferably 1.25 times or more higher than the yield thereof without addition of the pH buffering agent. When the lactic acid bacteria employed are EPS-producing *Lactobacillus delbruechii* subsp. *bulgaricus* and *Streptococcus thermophilus* and the pH buffering agent employed is a phosphate salt, the yield of EPS with addition of the pH buffering agent may be 1.25 times or

more higher than the yield of EPS without addition of the pH buffering agent.

[0032] -Second embodiment-

A lactic acid bacteria extracellular functional product according to the present embodiment is produced through a pH buffering agent addition step and a fermentation step. In the pH buffering agent addition step, a pH buffering agent is added to milk as a raw material. In the fermentation step, the milk as a raw material to which the pH buffering agent has been added is fermented by lactic acid bacteria capable of producing the lactic acid bacteria extracellular functional product.

[0033] The production method of lactic acid bacteria extracellular functional product according to the present embodiment is the same as the production method of yogurt according to the first embodiment except that the yogurt starting material is replaced by the milk as a raw material. The "lactic acid bacteria extracellular functional product" as used in the present embodiment is identical to the "extracellular functional product" as used in the first embodiment. The "milk as a raw material" in the present embodiment is animal milk or a processed product thereof such as cow, sheep or goat milk; pasteurised/sterilised milk, skim milk, whole powdered milk, partly skimmed milk, powdered skim milk, whole fat concentrated milk, concentrated skim milk, cream, butter, butter milk, whey, whey protein concentrate (WPC), whey protein isolate (WPI), α -lactalbumin (α -La), β -lactoglobulin (β -Lg), α -casein,

β -casein, κ -casein, non-protein nitrogen or the like. The milk as a raw material preferably contains solids-not-fat (hereinafter referred to as "SNF") at from 8% by weight to 20% by weight, more preferably at from 8.5% by weight to 18.5% by weight, still more preferably at from 9% by weight to 15% by weight and particularly preferably at from 9.5% by weight to 14% by weight.

[0034] The yield of the lactic acid bacteria extracellular functional product according to the present embodiment is preferably 1.05 times or more higher, more preferably 1.1 times or more higher, still more preferably 1.2 times or more higher and particularly preferably 1.25 times or more higher than the yield thereof without addition of the pH buffering agent.

[0035] <Examples>

Preferable Examples of the present invention are hereinafter described. However, it is to be understood that the present invention is not limited to the above-mentioned embodiments or the following Examples and may be variously modified within the scope of the claims. *Lactobacillus delbruechii* subsp. *bulgaricus* OLL1073R-1 strain (hereinafter referred to as OLL1073R-1 strain) used in Examples is a lactic acid bacterial strain deposited in accordance with the Budapest Treaty on 29 November 2006 (date of deposit) with the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan) under Accession Number FERM BP-10741. *Streptococcus thermophilus* OLS3059 strain is a lactic acid bacterial strain deposited in

accordance with the Budapest Treaty on 15 December 2006 (date of deposit) with the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan) under Accession Number FERM BP-10740. The National Institute of Technology and Evaluation has succeeded the deposit of patent microorganisms from the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology as of 1 April 2012, and the National Institute of Technology and Evaluation Patent Microorganisms Depositary has moved to National Institute of Technology and Evaluation Biological Resource Center, 2-5-8, Kazusakamatari, Kisarazu-shi, Chiba, Japan as of 1 April 2013.

[0036] (Production Example)

(1) Preparation of yogurt starting material mix A (SNF = 9.4% by weight)

50 kg of raw material milk, 5.49 kg of powdered skim milk, 1.5 kg of unsalted butter and 43.01 kg of water were mixed. The mixture was heat-sterilised at 95°C for 5 minutes followed by cooling to around 37°C to prepare yogurt starting material mix A.

[0037] (2) Preparation of yogurt starting material mix B (SNF = 10.2% by weight)

25 kg of raw material milk, 18.4 kg of concentrated skim milk, 0.96 kg of powdered skim milk, 1.31 kg of unsalted butter and 45.75 kg of water were mixed. The mixture was heat-sterilised at 95°C

for 5 minutes followed by cooling to around 37°C to prepare yogurt starting material mix B having a higher solids-not-fat (SNF) content than yogurt starting material mix A.

Example 1

[0038] -Evaluation of effects of amount of phosphate salt on EPS yield-

A lactic acid bacteria starter (yogurt starter) which was a mixed culture of *Streptococcus thermophilus* OLS3059 strain (hereinafter also referred to as OLS3059 strain) and a strain of lactic acid bacteria producing polysaccharides, *Lactobacillus delbruechii* subsp. *bulgaricus* 1073R-1 strain, was inoculated into yogurt starting material mix A so that the lactic acid bacteria starter accounted for 2% by weight of the total amount. Thereafter the yogurt starting material mix A containing the lactic acid bacteria starter was dispensed at 20 mL each in 5 test tubes to prepare 5 samples 1-1, 1-2, 1-3, 1-4 and 1-5. To sample 1-1 was added Na_2HPO_4 so that Na_2HPO_4 accounted for 0.5% by weight of the total amount. To sample 1-2 were added Na_2HPO_4 and NaH_2PO_4 so that Na_2HPO_4 and NaH_2PO_4 accounted for 0.4% by weight and 0.1% by weight, respectively, of the total amount. To sample 1-3 were added Na_2HPO_4 and NaH_2PO_4 so that Na_2HPO_4 and NaH_2PO_4 accounted for 0.25% by weight, respectively, of the total amount. To sample 1-4 were added Na_2HPO_4 and NaH_2PO_4 so that Na_2HPO_4 and NaH_2PO_4 accounted for 0.5% by weight, respectively, of the total amount. No phosphate salt was added to sample 1-5 (control). The samples were immersed in a thermostat

bath at 43°C for fermentation. When pH of a sample reached 4.4 to 4.5, the sample was cooled to 10°C or lower to terminate fermentation. Each sample was measured for the time it took to complete fermentation (fermentation time), acidity, EPS content, the number of *Lactobacillus delbruechii* subsp. *bulgaricus* cells and the number of *Streptococcus thermophilus* cells (see Table 1).

[0039] pH was measured using a pH meter with a glass electrode (TOA-HM50V, available from DKK-TOA Corporation). Acidity was measured as follows: 500 µL of phenolphthalein was added to 9.00 g of each sample. The sample was then titrated with 0.1 N sodium hydroxide with the end point of titration being the point when pale red colour sustained over 30 seconds.

[0040] EPS content of yogurt was measured as follows: each sample (10 g) was weighed and placed in a 50-mL tube to which 1 mL of 100% trichloroacetic acid was added. The content of the tube was stirred and then left to stand at about 4°C for about 10 minutes. The content in the tube was then centrifuged at 4°C for 20 minutes with relative centrifuge force of $12000 \times g$ and the supernatant was transferred to a new 50-mL tube. While stirring the supernatant, cold ethanol in twice the volume of the supernatant was gradually added to the supernatant and the supernatant was thoroughly mixed with the cold ethanol. The mixture was then left to stand overnight at about 4°C followed by centrifugation under the same conditions as above. The supernatant from the mixture was then discarded and 10 mL of purified water was added to the precipitate in order to completely dissolve

the precipitate in the purified water. The aqueous solution (150 µL) thus obtained was injected into HPLC with a syringe attached with a filter having a pore diameter of 0.45 µm. The EPS content was calculated as the ratio of "the peak area of a single peak detected on an RI detector at around 16 minutes after injection of the sample" to "the total peak area". Analytical conditions of the HPLC are as follows:

HPLC system: Aquity H-class (Waters)

Column: OHpak 806HQ (Shodex) + SB-G (Shodex)

Column temperature: 40°C

Solvent: 0.2 M NaCl aqueous solution

Flow rate: 0.5 mL/min

Detector: RI detector 2414 (Waters), detection temperature: 40°C

Sample injection: 150 µL

Duration of analysis: 50 min

[0041] Effects of different amounts of addition of phosphate salts on the EPS content are shown in Table 1. Samples 1-1, 1-2, 1-3 and 1-4 containing the phosphate salt had longer fermentation time than the control sample 1-5 and also had the acidity at the end of fermentation of 1 or higher. Thus, addition of the phosphate salt resulted in an increase of the EPS yield in yogurt. The EPS content was highest when only Na₂HPO₄ was added as the phosphate salt (sample 1-1). The number of *Lactobacillus delbruechii* subsp. *bulgaricus* cells was increased by addition of the phosphate salt while the number of *Streptococcus thermophilus* cells was decreased

with an increase of the concentration of Na_2HPO_4 .

[0042] It is believed from these results that addition of the phosphate salt could extend the duration of fermentation in pH at which the lactic acid bacteria could proliferate, resulting in an increase in the number of *Lactobacillus delbruechii* subsp. *bulgaricus* cells. Further, growth of *Streptococcus thermophilus* was observed to decrease by addition of the phosphate salt, particularly Na_2HPO_4 , and thus it is believed that the decrease of growth of *Streptococcus thermophilus* also contributed to an increase of EPS from *Lactobacillus delbruechii* subsp. *bulgaricus*.

[Table 1]

Sample No.	Na ₂ HPO ₄ (wt%)	NaH ₂ PO ₄ (wt%)	Fermentation time	Acidity	pH	EPS content		Number of <i>L.</i> <i>delbruechii</i> ssp. <i>Bulgaricus</i> cells		Number of <i>S.</i> <i>thermophiles</i> cells	
						mg/kg	Relative to Control	cfu/mL	Relative to Control	cfu/mL	Relative to Control
1-1	0.5	0	4 h 15 min	1.04	4.49	47.7	1.3	4.1×10^8	9.5	3.6×10^8	0.4
1-2	0.4	0.1	3 h 50 min	1.09	4.47	42.2	1.1	2.8×10^8	6.5	5.9×10^8	0.6
1-3	0.25	0.25	3 h 30 min	1.09	4.48	42.7	1.1	3.0×10^8	7.0	1.1×10^9	1.1
1-4	0.5	0.5	4 h 50 min	1.3	4.51	41.2	1.1	3.5×10^8	8.1	1.4×10^8	0.1
1-5 (Control)	0	0	3 h 00 min	0.81	4.38	37.6	1.0	4.3×10^7	1.0	9.9×10^8	1.0

Example 2

[0043] -Evaluation of effects of the type of phosphate salt on EPS yield-

A lactic acid bacteria starter (yogurt starter) which was a mixed culture of OLS3059 strain and 1073R-1 strain was inoculated into 500 g of yogurt starting material mix A so that the lactic acid bacteria starter accounted for 2% by weight of the total amount. Thereafter the yogurt starting material mix A containing the lactic acid bacteria starter was dispensed at 20 mL each in 6 test tubes to prepare 6 samples 2-1, 2-2, 2-3, 2-4, 2-5 and 2-6. To sample 2-1 was added Na_2HPO_4 so that Na_2HPO_4 accounted for 0.5% by weight of the total amount. To sample 2-2 was added K_2HPO_4 so that K_2HPO_4 accounted for 0.61% by weight of the total amount. The molar concentration of K_2HPO_4 in sample 2-2 is the same molar concentration of Na_2HPO_4 in sample 2-1. To sample 2-3 was added NaCl so that NaCl accounted for 0.21% by weight of the total amount. The molar concentration of NaCl in sample 2-3 is the same molar concentration of Na_2HPO_4 in sample 2-1. To sample 2-4 was added Na_2HPO_4 so that Na_2HPO_4 accounted for 0.3% by weight of the total amount. To sample 2-5 was added Na_2HPO_4 so that Na_2HPO_4 accounted for 0.1% by weight of the total amount. No phosphate salt was added to sample 2-6 (control). The samples were immersed in a thermostat bath at 43°C for fermentation. When pH of a sample reached 4.4 to 4.5, the sample was cooled to 10°C or lower to terminate fermentation. Each sample was measured for the duration until fermentation was terminated

(fermentation time), acidity, EPS content, the number of *Lactobacillus delbruechii* subsp. *bulgaricus* cells and the number of *Streptococcus thermophilus* cells (see Table 2). These physical values were measured in the same manners as in Example 1.

[0044] Effects of the type of phosphate salt on the EPS content are shown in Table 2. Samples 2-1, 2-4 and 2-5 to which only Na_2HPO_4 was added showed an increase in fermentation time and an increase in the EPS content with an increase in the amount of Na_2HPO_4 . Further, an increase in the amount of Na_2HPO_4 increased the number of *Lactobacillus delbruechii* subsp. *bulgaricus* cells and decreased the number of *Streptococcus thermophilus* cells. The factor which may affect the decrease in the number of *Streptococcus thermophilus* cells was thus investigated. Specifically the number of *Streptococcus thermophilus* cells and the like were compared between samples 2-1, 2-2 and 2-3. Sample 2-2 showed almost similar number of *Streptococcus thermophilus* cells and EPS content as those of sample 2-1. Sample 2-3 did not show an increase in the EPS content or a decrease in the number of *Streptococcus thermophilus* cells, thus those values were similar to those of sample 2-6 (control). It is considered that this is because NaCl did not exert pH buffering action. Therefore, it was suggested that addition of the phosphate salt may suppress growth of *Streptococcus thermophilus*.

[Table 2]

Sample No.	Na ₂ HPO ₄ (wt%)	K ₂ HPO ₄ (wt%)	NaCl (wt%)	Fermentation time	Acidity	pH	EPS content		Number of <i>L. delbrueckii</i> ssp. <i>bulgaricus</i>		Number of <i>S. thermophilus</i>	
							mg/kg	Relative to Control	cfu/mL	Relative to Control	cfu/mL	Relative to Control
2-1	0.5	0	0	4 h 35 min	1.08	4.43	51.5	1.53	4.0×10^8	12.0	4.0×10^8	0.3
2-2	0	0.61	0	5 h 32 min	1.11	4.43	49.4	1.47	3.2×10^8	10.3	2.5×10^8	0.2
2-3	0	0	0.21	3 h 13 min	0.81	4.42	32.7	0.97	1.8×10^7	0.6	1.3×10^9	1.1
2-4	0.3	0	0	4 h 06 min	1.01	4.42	41.9	1.25	1.3×10^8	4.2	6.2×10^8	0.5
2-5	0.1	0	0	3 h 28 min	0.89	4.42	38.4	1.14	5.8×10^7	1.9	1.3×10^9	1.1
2-6 (Control)	0	0	0	3 h 24 min	0.80	4.42	33.6	1.00	3.1×10^7	1.0	1.2×10^9	1.0

Example 3

[0045] -Evaluation of effects of amount of phosphate salt on EPS yield and curd tension-

100 kg of yogurt starting material mix A was divided into 20 kg portions to prepare 5 samples 3-1, 3-2, 3-3, 3-4 and 3-5. No phosphate salt was added to sample 3-1 (control). To sample 3-2 was added Na_2HPO_4 so that Na_2HPO_4 accounted for 0.1% by weight. To sample 3-3 was added Na_2HPO_4 so that Na_2HPO_4 accounted for 0.3% by weight. To sample 3-4 was added Na_2HPO_4 so that Na_2HPO_4 accounted for 0.5% by weight. To sample 3-5 was added Na_2HPO_4 so that Na_2HPO_4 accounted for 1.0% by weight. A lactic acid bacteria starter which was a mixed culture of *Streptococcus thermophilus* generally used for production of yogurt and 1073R-1 strain was then inoculated into each sample so that the lactic acid bacteria starter accounted for 2% by weight of the total amount. Each sample containing the lactic acid bacteria starter was dispensed at 85 g in a container with 85 g capacity and fermented in a thermostat chamber at 43°C. When pH of a sample reached 4.4, the sample was cooled to 10°C or lower to terminate fermentation.

[0046] Each sample was measured for the duration until fermentation was terminated (fermentation time), EPS content and curd tension (CT) (see Table 3). EPS content of the respective samples was measured in the same manner as in Example 1. CT was measured as follows: a weight (100 g) was attached to a sample and the elasticity at breakage of the sample was measured on the

Curdmeter Max (ME-500, available from Asuka Kiki). The elasticity was regarded as an index of CT.

[0047] The resulting fermentation time, EPS content and CT of the samples are shown in Table 3. With an increase in the amount of Na_2HPO_4 , the EPS content in samples was increased and the CT value was decreased (namely, the sample had increased softness).

[Table 3]

Sample No.	Na_2HPO_4 (wt%)	Fermentation time (fermentation was terminated at pH 4.4)	EPS content		CT (g)
			mg/kg	Relative to sample No. 3-1	
3-1	0.0	4 h 00 min	40.8	1.00	51.6
3-2	0.1	4 h 25 min	42.8	1.05	46.1
3-3	0.3	5 h 00 min	45.9	1.13	48.4
3-4	0.5	5 h 25 min	49.1	1.20	43
3-5	1.0	6 h 00 min	51.8	1.27	33.2

Example 4

[0048] -Evaluation of effects of SNF content in yogurt starting material mix on EPS yield-

A lactic acid bacteria starter (yogurt starter) which was a mixed culture of OLS-3059 strain and 1073R-1 strain was inoculated into each of yogurt starting material mix A and yogurt starting material mix B so that the lactic acid bacteria starter accounted for 2% by weight of the total amount. Thereafter each of yogurt starting material mixes A and B respectively containing the lactic acid bacteria starter was dispensed at 85 g in a container with

85 g capacity to prepare 2 samples 4-1 (SNF = 9.4% by weight) and 4-2 (SNF = 10.2% by weight). Samples 4-1 and 4-2 were fermented in a thermostat chamber at 43°C. When acidity of a sample reached 0.8, the sample was cooled to 10°C or lower to terminate fermentation. Each sample was measured for EPS content. EPS content was measured in the same manner as in Example 1.

[0049] Sample 4-1 had an EPS content of 46.0 mg/kg, while sample 4-2 had an EPS content of 48.6 mg/kg. Thus the EPS content of sample 4-2 was 1.1 times of that of sample 4-1. Therefore it was revealed that using a yogurt starting material having high SNF content, yogurt having an increased EPS content could be obtained.

Example 5

[0050] -Evaluation of effects of fermentation temperature on EPS yield-

A lactic acid bacteria starter (yogurt starter) which was a mixed culture of OLS-3059 strain and 1073R-1 strain was inoculated into yogurt starting material mix A so that the lactic acid bacteria starter accounted for 2% by weight of the total amount. Thereafter yogurt starting material mix A containing the lactic acid bacteria starter was dispensed at 85 g in containers with 85 g capacity to prepare 2 samples 5-1 and 5-2. Sample 5-1 was then fermented at 43°C while sample 5-2 was fermented at 37°C. When acidity of a sample reached 0.8, the sample was cooled to 10°C or lower to terminate fermentation. Each sample was measured for EPS content. EPS content was measured in the same manner as in Example 1.

[0051] Sample 5-1 had an EPS content of 43.4 mg/kg, while sample 5-2 had an EPS content of 51.1 mg/kg. Thus the EPS content of sample 5-2 was 1.2 times of that of sample 5.1. Therefore it was revealed that decreased fermentation temperature could increase EPS content of yogurt.

Example 6

[0052] Sample 6-1 (control) first prepared was yogurt starting material mix A. Three samples 6-2, 6-3 and 6-4 were prepared by dividing yogurt starting material mix B into three. A lactic acid bacteria starter which was a mixed culture of *Streptococcus thermophilus* generally used for production of yogurt and 1073R-1 strain was then inoculated into each sample so that the lactic acid bacteria starter accounted for 2% by weight of the total amount. To sample 6-4 was further added Na_2HPO_4 so that Na_2HPO_4 accounted for 0.3% by weight of the total amount. Samples 6-1 and 6-2 were fermented at 43°C while samples 6-3 and 6-4 were fermented at 37°C. When acidity of a sample reached 0.8, the sample was cooled to 10°C or lower to terminate fermentation. Each sample was measured for EPS content. EPS content was measured in the same manner as in Example 1.

[0053] SNF, fermentation temperature, amount of phosphate salt (Na_2HPO_4) added and EPS content in yogurt for each sample are shown in Table 4. Sample 6-2 (high SNF content) had an EPS content that was 1.15 times higher than that of the control sample 6-1, and sample 6-3 (high SNF content, low fermentation temperature) had an EPS

content that was 1.19 times higher than that of the control sample 6-1. Further, sample 6-4 (high SNF content, low fermentation temperature, phosphate salt added) had an EPS content in yogurt that was as high as 1.37 times of that of the control sample 6-1.

[Table 4]

Sample No.	SNF (wt%)	Na ₂ HPO ₄ (wt%)	Fermentation temperature (°C)	EPS content	
				mg/kg	Relative to sample 6-1
6-1	9.4	Not added	43.0	43.6	1.00
6-2	10.2	Not added	43.0	50.1	1.15
6-3	10.2	Not added	37.0	51.7	1.19
6-4	10.2	0.3	37.0	59.7	1.37

INDUSTRIAL APPLICABILITY

[0054] The production method of yogurt according to the present invention can effectively increase the content of functional products from lactic acid bacteria. Therefore it allows effective production of yogurt with enhanced health-promoting benefits thanks to the functional products (polysaccharides and the like) from lactic acid bacteria and small-volume yogurt sufficiently containing an effective amount of functional products from lactic acid bacteria.

CLAIMS

What is claimed is:

1. A production method of yogurt including exopolysaccharides, comprising:
 - a phosphate salt addition step for adding a phosphate salt to a yogurt starting material; and
 - a fermentation step for fermenting the yogurt starting material to which the phosphate salt has been added by lactic acid bacteria including at least lactic acid bacteria capable of producing exopolysaccharides, wherein the lactic acid bacteria includes mixture of *Lactobacillus delbruechii* subsp. *bulgaricus* OLL1073R-1 (FERM BP-10741) and *Streptococcus thermophilus* OLS3059 (FERM BP-10740), and the growth of *Streptococcus thermophilus* OLS3059 is suppressed while the growth of *Lactobacillus delbruechii* subsp. *bulgaricus* OLL1073R-1 is promoted during the fermentation step so as to increase a yield of the exopolysaccharides.
2. The production method of yogurt according to claim 1, wherein the phosphate salt is at least one phosphate salt selected from the group consisting of disodium hydrogen phosphate and dipotassium hydrogen phosphate.
3. Yogurt obtained by the production method of yogurt according to claim 1 or 2.
4. A production method of a lactic acid bacteria extracellular functional product,
 - the production method comprising:
 - a phosphate salt addition step for adding a phosphate salt to milk as a raw material; and
 - a fermentation step for fermenting the milk as a raw material, to which the phosphate salt has been added, by lactic acid bacteria capable of producing a lactic acid bacteria extracellular functional product, wherein the lactic acid bacteria includes mixture of *Lactobacillus delbruechii* subsp. *bulgaricus* OLL1073R-1 (FERM BP-10741) and *Streptococcus thermophilus* OLS3059 (FERM BP-10740), and the growth of *Streptococcus thermophilus* OLS3059 is suppressed while the growth of *Lactobacillus delbruechii* subsp. *bulgaricus* OLL1073R-1 is promoted during the fermentation step so as to increase a yield of the lactic acid bacteria extracellular functional product.