(19) DANMARK

Patent- og Varemærkestyrelsen

(10) **DK/EP 3725161 T3**

(12) Oversættelse af europæisk patentskrift

(51) Int.Cl.: ***A 23 C 9/123 (2006.01)*** ***A 23 C 9/13 (2006.01)*** ***A 23 C 9/137 (2006.01)***

(45) Oversættelsen bekendtgjort den: **2024-12-09**

(80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2024-11-20**

(86) Europæisk ansøgning nr.: **20169113.6**

(86) Europæisk indleveringsdag: **2020-04-09**

(87) Den europæiske ansøgnings publiceringsdag: **2020-10-21**

(30) Prioritet: **2019-04-15 KR 20190043610**

(84) Designerede stater: **AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**

(73) Patenthaver: **Cell Biotech Co., Ltd., 50, Aegibong-ro 409 Beon-gil, Wolgot-myeon, Gyeonggi-do 10003, Sydkorea**

(72) Opfinder: **CHUNG, Myung Jun, 113-2001, 270, Sinbanpo-ro, Seocho-gu, 06544 Seoul, Sydkorea**

(74) Fuldmægtig i Danmark: **NORDIC PATENT SERVICE A/S, Bredgade 30, 1260 København K, Danmark**

(54) Benævnelse: **FREMGANGSMÅDE TIL FREMSTILLING AF MÆLKESYREBAKTERIER DOBBELTCOATET MED PROTEIN OG POLYSACCHARID VED HJÆLP AF PROTEINHYDROLYSAT**

(56) Fremdragne publikationer:
US-A1- 2014 093 613
US-A1- 2015 152 378
DOHERTY S B ET AL: "Efficacy of whey protein gel networks as potential viability-enhancing scaffolds for cell immobilization of Lactobacillus rhamnosus GG", JOURNAL OF MICROBIOLOGICAL METHODS, ELSEVIER, AMSTERDAM, NL, vol. 80, no. 3, 1 March 2010 (2010-03-01), pages 231 - 241, XP026907759, ISSN: 0167-7012, [retrieved on 20100104], DOI: 10.1016/J.MIMET.2009.12.009

DESCRIPTION

Description

BACKGROUND

1. Technical Field

[0001] The present disclosure relates to a method of producing lactic acid bacteria dual-coated with protein and polysaccharide by using a protein hydrolysate.

2. Related Art

[0002] Lactic acid bacteria, also known as lactobacillus, are important bacteria that live in mammalian intestines and are also used as probiotics that prevent abnormal fermentation caused by various bacteria. For example, *Lactobacillus bulgaricus* is a lactic acid bacterium known for the longest time and is used in the manufacture of yogurt and also used as a starter in the manufacture of cheese or fermented butter. In addition, aerobic *Lactobacillus acidophilus* is present in the intestines of humans, all mammals and other animals, and is used in the production of butter or milk or for the treatment of intestinal auto-intoxication. In addition, *Lactobacillus lactis* is the most important dairy lactic acid bacterium that produces DL-lactic acid, which is always present in milk and used in butter or cheese production.

[0003] The useful lactic acid bacteria as described above are colonize in the intestines and activate intestinal movement, thus ameliorating constipation or diarrhea and facilitating bowel movement. In addition, these lactic acid bacteria exhibit various physiological activities, including immune enhancement, anti-hyperlipidemia, anti-atopy, diet, anti-diabetic, and anticancer activities, and thus are widely used in health functional foods, probiotic drugs, skin improvement cosmetics, feed additives, and so on. However, in order to exhibit the above-described physiological effects, the lactic acid bacteria should be ingested in amounts that are much larger than the amounts of the lactic acid bacteria contained in conventional foods such as yogurt. Therefore, a method of separating only lactic acid bacteria and taking the separated lactic acid bacteria easily in the form of powder or capsules has been popularized.

[0004] Since these lactic acid bacteria are based on live bacteria themselves, unlike other industrial microorganisms that mainly based on fermentation products, maintaining the viability of these lactic acid bacteria during their distribution period and their ingestion is a very

important challenge. That is, when the lactic acid bacteria are ingested orally as probiotics themselves, problems arise in that their growth in the human body is inhibited by digestive enzymes and bile acids secreted from the stomach with a pH of 3 or less and the small intestine, and thus the number of live bacteria that reach the large intestine decreases and the intestinal colonization rate of the lactic acid bacteria also decreases.

[0005] For this reason, the lactic acid bacteria are considerably unstable and the long-term storage and use thereof is significantly limited, even though these lactic bacteria are very beneficial in terms of their usefulness and value. Fermented milk products and probiotics based on these lactic acid bacteria show a significant decrease in lactic acid bacteria viability due to oxygen or oxidative stress during the production and storage thereof, and this decrease hinders the industrial use thereof or the exhibition of the effect thereof after oral ingestion.

[0006] In an attempt to solve the above-described problems, various methods of coating lactic acid bacteria have been developed. In the initial stage, there were enteric-coating capsulation and microencapsulation with gelatin, polysaccharides, gums, etc., but the use of expensive coating agents or additional processes has been pointed out as a problem.

[0007] In an attempt to solve this problem, the following methods have been competitively developed: a method for producing a double-structured jelly containing a high concentration of live lactic acid bacteria; a method of forming a quadruple coating by adding a water-soluble polymer, hyaluronic acid, a coating agent having porous particles, and a protein (Korean Patent Application No. 10-2011-0134486); a method of forming a quadruple coating using a water-soluble polymer, a functional hydrated hyaluronic acid, porous particles, and a protein (Korean Patent Application No. 10-2015-0129986); a method of coating lactic acid bacteria with casein, a coating agent, edible oil and fat, a *Lactobacillus plantarum* extracellular polymeric substance, and alginic acid to improve the intestinal viability of the lactic acid bacteria (Korean Patent Application No. 10-2016-0172568); and a method of coating with silk fibroin to improve intestinal colonization (Korean Patent Application No. 10-2017-0045326).

[0008] However, these improved lactic acid bacteria coating technologies also problems in that they cannot completely coat the surface of lactic acid bacteria, and thus the lactic acid bacteria are still insufficient in terms of their freeze-drying viability, heat resistance, acid resistance, bile resistance, intestinal colonization ability, etc., and the viability thereof during storage and distribution is insufficient.

Prior Art Documents

Patent Documents

[0009]

Korean Patent Application No. 10-2011-0134486;

Korean Patent Application No. 10-2015-0129986;

Korean Patent Application No. 10-2016-0172568;

Korean Patent Application No. 10-2017-0045326.

United States Patent Application No. 2015/152378 A1.

SUMMARY

[0010] The present inventors have made extensive research efforts to develop a lactic acid bacteria coating method capable of improving the freeze-drying viability, storage stability, acid resistance and bile resistance of lactic acid bacteria, and as a result, have experimentally found that, when a protein is hydrolyzed and lactic acid bacteria are dual-coated with the hydrolyzed protein along with a polysaccharide and a cryoprotectant, the freeze-drying viability, acid resistance and bile resistance of the lactic acid bacteria can be greatly improved, thereby completing the present disclosure.

[0011] Therefore, it is an object of the present disclosure to provide a method of effectively dual-coating lactic acid bacteria using a hydrolyzed protein so as to improve the freeze-drying viability, storage stability, acid resistance and bile resistance of the lactic acid bacteria.

[0012] Other objects and technical features of the present disclosure will be disclosed in more detail with reference to the following description of the disclosure, the appended claims and the accompanying drawings.

[0013] In accordance with one aspect of the present disclosure, there is provided a method for producing lactic acid bacteria having a dual-coating of protein and polysaccharide coatings, according to claim 1.

Preparation of Protein Hydrolysate

[0014] In the present disclosure, the protein that is used for coating of lactic acid bacteria is used after hydrolytic treatment.

[0015] According to one embodiment of the present disclosure, hydrolytic treatment of the protein may be performed by adding a protease to an aqueous protein solution containing the protein, and then subjecting the aqueous protein solution to a hydrolysis reaction.

[0016] In the present disclosure, the hydrolysis rate of the protein refers to the degree to which the protein was hydrolyzed by the enzyme. The hydrolysis rate of the protein is calculated by: i) a method of measuring the difference in optical density of the aqueous protein solution between before and after enzymatic hydrolytic treatment; ii) a method of measuring the difference in weight of a precipitate, obtained by centrifuging the aqueous protein solution, between before and after enzymatic hydrolytic treatment;

[0017] According to one embodiment of the present disclosure, the hydrolysis rate of the protein in the present disclosure is a percentage value (%) obtained by dividing a ΔOD value, which is a difference between the optical density (OD) value ("start-point OD") of the aqueous protein solution, measured before treatment with the protease, and the optical density (OD) value ("endpoint OD") of the aqueous protein solution, measured after treatment with the protease, by the start-point OD value. That is, the hydrolysis rate of the protein may be calculated by the following equation: hydrolysis rate (%) = [ΔOD]/[start-point OD] X 100.

[0018] According to one embodiment of the present disclosure, the hydrolysis rate of the protein in the present disclosure is a percentage value (%) obtained by dividing a Δppt value, which is a difference between the measured weight value ("start-point ppt") of a precipitate, obtained by centrifuging the aqueous protein solution before treatment with the protease, and the measured weight value ("endpoint ppt") of a precipitate, obtained by centrifuging the aqueous protein solution after treatment with the protease, by the start-point ppt value. That is, the hydrolysis rate of the protein may be calculated by the following equation: hydrolysis rate (%) = [Δppt]/[start-point ppt] X 100.

[0019] According to one embodiment of the present disclosure, the protein that is used for lactic acid bacteria coating is isolated soy protein (ISP).

[0020] According to one embodiment of the present disclosure, the hydrolysis rate of isolated soy protein in the present disclosure is 74%.

[0021] As used herein, the term "protease" refers to an enzyme that grades the proteins into amino acids or peptide fragments. The protease that is used in the present disclosure may be selected from among commercially available Alcalase, Flavourzyme, Neutrase, Protamax, and the like, but are not limited thereto.

[0022] It is presumed that hydrolytic treatment of the protein in the present disclosure produces a water-semisoluble peptide. The water semisoluble peptide refers to a peptide that remains without being completely hydrolyzed into amino acids upon hydrolytic treatment of the protein. In the method of the present disclosure, as the hydrolysis rate increases, the amount of the water semisoluble peptide decreases, and as the hydrolysis rate decreases, the amount of water semisoluble peptide increases. The water semisoluble peptide functions to increase freeze-drying viability by alleviating protein denaturation caused by a physical impact which is applied to the cells during freeze drying. However, if the water-semisoluble peptide is present in excessive amounts in the aqueous protein hydrolysate solution, the culture may change to a

hydrophobic state, thereby inducing agglomeration of the lactic acid bacteria and suppressing the culturability of the lactic acid bacteria.

[0023] Accordingly, if the hydrolysis rate of the protein in the present disclosure is lower problems arise in that the hydrophobicity of the culture is increased by a large amount of the non-hydrolyzed water-semisoluble peptide, and thus the lactic acid bacteria agglomerate and are not easily divided, resulting in deterioration in the culturability of the lactic acid bacteria. On the contrary, if the hydrolysis rate of the protein is higher a problem arises in that the non-hydrolyzed water-semisoluble peptide is present in insufficient amounts, and thus cannot sufficiently alleviate protein denaturation caused by a physical impact which is applied to the lactic acid bacteria during freezes drying, resulting in a decrease in freeze-drying viability.

[0024] According to one embodiment of the present disclosure, the concentration of isolated soy protein (ISP) in an aqueous isolated soy protein solution during hydrolysis of the isolated soy protein 0.45 wt%, and the concentration of the protease in the aqueous isolated soy protein solution is 0.015 wt%.

Lactic Acid Bacteria Culture and Protein Coating

[0025] Sugar and nitrogen source components for lactic acid bacteria culture are added to the prepared aqueous protein hydrolysate solution which is then sterilized. Next, lactic acid bacteria are inoculated and cultured in the sterilized aqueous solution.

[0026] In the present disclosure, the lactic acid bacteria to be coated are lactic acid bacteria that produce an acid and can proliferate even under weakly acidic conditions,

[0027] In addition, in the present disclosure, the lactic acid bacteria is *Bifidobacterium breve* CBT-BR (accession number: KCTC 12201BP),

[0028] In the present disclosure, as lactic acid bacteria to be coated, any raw material may be used without limitation on its formulation or preparation method, as long as it includes live lactic acid bacteria. However, a culture of lactic acid bacteria may preferably be used.

[0029] In the present disclosure, lactic acid bacteria culture conditions for obtaining the culture of lactic acid bacteria are also not particularly limited, and any conditions may be used without limitation as long as they are conditions that are generally used for lactic acid bacteria culture in the art. For example, the lactic acid bacteria may be inoculated in medium, such as MRS, BL, M17, NB or BHI, and cultured at a temperature of 35 to 40°C under anaerobic conditions substituted with nitrogen gas or carbon dioxide gas for 6 to 48 hours.

[0030] In the present disclosure, as the sugar component for lactic acid bacteria culture, there may be used mixed lactose, fructose, sucrose or glucose. Preferably, the sugar component may be selected depending on the characteristics of the strain and used.

[0031] In the present disclosure, as the nitrogen source component for lactic acid bacteria culture, yeast extract or soy peptone may be used, but is not limited thereto.

[0032] In addition, for lactic acid bacteria culture, trace components may further be added, such as potassium phosphate dibasic, magnesium sulfate, manganese sulfate, calcium chloride, potassium citrate, sodium acetate, L-ascorbic acid, L-glutamic acid, L-cysteine hydrochloride, polysorbate 80, etc.

Recovery and Concentration of Lactic Acid Bacteria Cells

[0033] A filtrate is removed as much as possible from a culture obtained by culturing the lactic acid bacteria, and the remaining lactic acid bacteria cell mass as a solid is concentrated to have high specific gravity. The densely concentrated lactic acid bacteria cell mass has the following advantages in the preparation of freeze-dried lactic acid bacteria: i) the amount of cell mass dispensed per tray decreases and the amount of water to be evaporated is also small; ii) the freezing rate is high due to water discharge caused by an osmotic pressure difference in a freezing process; iii) ice crystals are small and uniform and a change in cell morphology is also small, and thus damage to the lactic acid bacteria cell membrane during freezing is also small; and iv) modification of the lactic acid bacteria in a freezing process also less occurs.

[0034] In the present disclosure, the method of recovering and concentrating the lactic acid bacteria cells is not particularly limited, but the lactic acid bacteria cells may preferably be recovered and concentrated by a centrifugation method. For example, the lactic acid bacteria cells may be concentrated using a disc type high-speed centrifuge or tubular type high-speed centrifuge, but are not limited thereto.

Addition of Cryoprotectant

[0035] In the method of the present disclosure, the storage stability of the lactic acid bacteria may be improved by freeze-drying the lactic acid bacteria. For freeze-drying treatment, a cryoprotectant is added to the recovered and concentrated lactic acid bacteria cells, and then mixed and homogenized. Preferably, an aqueous cryoprotectant solution containing a cryoprotectant may be used.

[0036] A cryoprotectant that may be used in the method of the present disclosure may be one or a mixture of two or more selected from the group consisting of trehalose, maltodextrin, mannitol, and skim milk powder. Preferably, the cryoprotectant that is used in the method of the present disclosure may be a mixture of trehalose, maltodextrin, mannitol and skim milk powder. In the present disclosure, the cryoprotectant may be prepared as an aqueous solution containing the cryoprotectant in an amount of 1 to 90 wt%, 1 to 80 wet%, 1 to 75 wt%, 1 to 70

wt%, 1 to 65 wt%, or 1 to 60 wt%, and may be added to and used in the concentrated lactic acid bacteria cells.

[0037] The aqueous cryoprotectant solution may be added and used in an appropriate amount based on the lactic acid bacteria cells. Preferably, it may be added and used in an amount of 0.001 to 99 wt%, 0.01 to 95 wt%, or 0.1 to 95 wt%, based on the weight of the lactic acid bacteria cells.

Coating with Polysaccharide

[0038] In the present disclosure, the concentrated lactic acid bacteria is coated with a polysaccharide to improve the freeze-drying viability, acid resistance and bile resistance of the lactic acid bacteria.

[0039] For coating with a polysaccharide, a polysaccharide is added to the concentrated lactic acid bacteria cells, and then mixed and homogenized. Preferably, an aqueous polysaccharide solution containing a polysaccharide may be used.

[0040] In the present disclosure, a polysaccharide that may be used for polysaccharide coating of the lactic acid bacteria may be one or more selected from the group consisting of gums, water-soluble dietary fiber, indigestible maltodextrin, insoluble dietary fiber, starch, Levan, and resistant starch. The gums may be one or more selected from the group consisting of xanthan gum, Arabic gum, guar gum, gellan gum, Karaya gum, and locust bean gum. The water-soluble dietary fiber may be polydextrose, glucomannan, β-glucan, pectin, or alginic acid. The insoluble dietary fiber may be chicory extract powder, cellulose, hemicellulose, lignin, or inulin.

[0041] According to one embodiment of the present disclosure, the polysaccharide that is used for polysaccharide coating may be a mixture of xanthan gum and cellulose.

[0042] In the present disclosure, the polysaccharide coating may be performed by preparing an aqueous polysaccharide solution containing the polysaccharide in an amount of 1 to 80 wt%, 1 to 75 wt%, 1 to 70 wt%, 1 to 65 wt%, 1 to 60 wt%, or 1 to 50 wt%, and adding the prepared aqueous polysaccharide solution to the concentrated lactic acid bacteria cells, followed by mixing and homogenization.

[0043] The aqueous polysaccharide solution may be added and used in an appropriate amount based on the weight of the lactic acid bacteria cells. Preferably, it may be added and used in an amount of 0.001 to 99 wt%, 0.01 to 95 wt%, or 0.1 to 95 wt%, based on the weight of the lactic acid bacteria cells.

Freeze-Drying

[0044] As described above, the lactic acid bacteria cells obtained by adding the cryoprotectant and polysaccharide components to the protein-coated lactic acid bacteria cells, followed by mixing and homogenization, are freeze-dried.

BRIEF DESCRIPTION OF THE DRAWINGS

[0045]

FIG. 1 is a microscopic photograph of a culture of *Streptococcus thermophilus* CBT ST3 (6.0% skim milk powder and 0.003% protease).

FIG. 2 is a microscopic photograph of a culture of *Bifidobacterium breve* CBT BR3 (0.90% isolated soy protein and 0.015% protease).

FIG. 3 is a microscopic photograph of a culture of *Lactobacillus acidophilus* CBT LA1 (2.0% skim milk powder, 0.90% isolated soy protein, and 0.015% protease).

FIG. 4 is a SEM photograph of *Streptococcus thermophilus* CBT ST3 cells dual-coated according to a method of the present disclosure.

FIG. 5 is a SEM photograph of *Bifidobacterium breve* CBT BR3 cells dual-coated according to a method of the present disclosure.

FIG. 6 is a SEM photograph of *Lactobacillus acidophilus CBT LA1* cells dual-coated according to a method of the present disclosure.

DETAILED DESCRIPTION

[0046] Specific examples described in the present specification are intended to represent exemplary embodiments or examples of the present disclosure, and the scope of the present disclosure is not limited thereby

Examples

Example 1: Measurement of Protein Hydrolysis Rate

<u>1-1. Method for Measurement of Hydrolysis Rate</u>

[0047] As protein sources that are used for dual coating of lactic acid bacteria, skim milk powder, isolated soy protein (ISP) and a mixture of skim milk powder and isolated soy protein were measured for their rate of hydrolysis induced by protease. Aqueous protein solutions containing different concentrations of each of skim milk powder and isolated soy protein were prepared. Each of the prepared aqueous protein solutions containing different concentrations of each of skim milk powder and isolated soy protein was placed in a reactor equipped with a stirrer, and then suspended and homogenized at 100 RPM at 60°C. Next, the prepared suspensions were adjusted to a pH of 8.2±0.2 by adding 1N NaOH thereto. Tables 1, 2 and 3 below show examples in which suspensions containing different concentrations of each of skim milk powder, isolated soy protein, and skim milk powder + isolated soy protein mixture were prepared.

[Table 1]

Skim milk powder suspension		
Concentration (%)	Substrate amount (kg)	Purified water (ℓ)
1.0%	6	594
2.0%	12	588
3.0%	18	582
4.0%	24	576
5.0%	30	570
6.0%	36	564

[Table 2]

Isolated soy protein (ISP) suspension		
Concentration (%)	Substrate amount (kg)	Purified water (ℓ)
0.15%	0.9	599.1
0.30%	1.8	598.2
0.45%	2.7	597.3
0.60%	3.6	596.4
0.75%	4.5	595.5
0.90%	5.4	594.6

[Table 3]

<table>
<tr><td colspan="5">Skim milk powder + isolated soy protein (ISP) mixture suspension</td></tr>
<tr><td colspan="2">Skim milk powder</td><td colspan="2">Isolated soy protein (ISP)</td><td rowspan="2">Purified water (ℓ)</td></tr>
<tr><td>Concentration</td><td>Substrate</td><td>Concentration</td><td>Substrate</td></tr>
<tr><td>(%)</td><td>amount (kg)</td><td>(%)</td><td>amount (kg)</td><td rowspan="3">Purified water added to 600 f of aqueous solution</td></tr>
<tr><td>0.5%</td><td>3.0</td><td>0.10%</td><td>0.6</td></tr>
<tr><td>1.0%</td><td>6.0</td><td>0.20%</td><td>1.2</td></tr>
</table>

Skim milk powder + isolated soy protein (ISP) mixture suspension				
Skim milk powder		Isolated soy protein (ISP)		Purified water (ℓ)
Concentration	Substrate	Concentration	Substrate	
1.5%	9.0	0.30%	1.8	
2.0%	12.0	0.40%	2.4	

[0048] Different concentrations of protease (Alcalase) were added to each of the pH-adjusted suspensions which were then subjected to a hydrolysis reaction at a pH of 6.8 or less for 2 hours. Using "before pH adjustment of each suspension" as a start point (before enzymatic treatment) and using "after 2 hours of the enzymatic reaction" as an endpoint, the hydrolysis rates of the protein sources were measured.

[0049] For measurement of the hydrolysis rate of skim milk power, the optical density at 610 nm was measured using a spectrophotometer (SHIMADZU, UV-1280).

Hydrolysis rate S (%) = [ΔOD]/[start-point OD] x 100%

[ΔOD] = start point OD – endpoint OD

[0050] For measurement of the hydrolysis rate of isolated soy protein (ISP), first-step centrifugation was performed using a microcentrifuge (Hanil, micro-12) at 13,000 rpm for 15 minutes, the supernatant was discarded, second-step centrifugation for 15 minutes was performed, and then the weight of the precipitate (ppt) was measured.

Hydrolysis rate I (%) =[Δppt]/[start-point ppt] x 100%

[Δ ppt] = start point ppt – endpoint ppt

[0051] For measurement of the hydrolysis rate of a mixture of skim milk powder and isolated soy protein, the two characteristics (optical density and ppt) were measured and the average value of the measured values was calculated.

Hydrolysis rate S & I (%) =[hydrolysis rate S + hydrolysis rate I]/2

1-2. Results of Hydrolysis Rate Measurement

[0052] Table 4 below shows the results of measuring the hydrolysis rate of the skim milk powder depending on the concentration of the skim milk powder and the concentration of the enzyme.

[Table 4]

Results of measuring the hydrolysis rate of skim milk powder depending on the concentrations of skim milk powder and protease						
Enzyme/Skim	**1.0%**	**2.0%**	**3.0%**	**4.0%**	**5.0%**	**6.0%**
0.001 %	90.9%	75.4%	73.2%	68.9%	65.4%	59.4%
0.002 %	91.9%	78.7%	75.6%	72.1%	69.7%	66.7%
0.003%	92.1%	85.0%	80.6%	76.2%	73.9%	71.6%
0.004%	92.7%	88.2%	86.1%	85.3%	80.4%	76.4%
0.005%	93.4%	91.0%	87.9%	86.9%	84.2%	81.9%

[0053] Concentration: based on skim milk powder solution, w/w%.

[0054] Enzyme used: protease (product name: Alcalase 2.4L FG, manufactured by Novozymes A/S Denmark).

[0055] From the experimental results, it was confirmed that the hydrolysis rate of the skim milk powder at the same enzyme concentration decreased as the concentration of the skim milk powder increased, and the hydrolysis rate of the skim milk powder increased as the concentration of the enzyme increased.

[0056] Table 5 below shows the results of measuring the hydrolysis rate of the isolated soy protein depending on the concentration of the isolated soy protein and the concentration of the enzyme.

[Table 5]

Results of measuring the hydrolysis rate of isolated soy protein depending on the concentrations of isolated soy protein and protease						
Enzyme/ISP	**0.15%**	**0.30%**	**0.45%**	**0.60%**	**0.75%**	**0.90%**
0.005%	81.6%	75.9%	68.1%	62.7%	53.3%	46.8%
0.010%	82.4%	77.3%	71.1%	65.4%	58.6%	50.3%
0.015%	83.7%	78.9%	74.0%	68.6%	62.7%	55.5%
0.020%	84.1%	80.0%	77.9%	72.6%	66.3%	60.7%
0.025%	85.6%	81.9%	79.1%	74.9%	69.9%	66.3%

[0057] Concentration: based on isolated soy protein (ISP) solution, w/w%

[0058] Enzyme used: protease (product name: Alcalase 2.4L FG, manufactured by Novozymes A/S Denmark).

[0059] It was confirmed that the hydrolysis rate of the isolated soy protein at the same enzyme concentration decreased as the concentration of the isolated soy protein increased, and the

hydrolysis rate of the isolated soy protein increased as the concentration of the enzyme increased.

[0060] Table 6 below shows the results of measuring the hydrolysis rate depending on the concentration of the mixture of skim milk powder and isolated soy protein.

[Table 6]

Results of measuring the hydrolysis rate depending on the concentration of the mixture of skim milk powder and isolated soy protein and the concentration of the enzyme				
Skim/ISP	**0.10%**	**0.20%**	**0.30%**	**0.40%**
0.5%	92.7%	91.8%	90.6%	88.8%
1.0%	88.4%	87.2%	86.3%	85.1%
1.5%	84.6%	83.7%	82.6%	80.4%
2.0%	80.1%	78.9%	77.6%	76.8%

[0061] Concentration: based on the solution of the mixture of skim milk powder and isolated soy protein, w/w%;

Enzyme used: 0.015% protease (product name: Alcalase 2.4L FG, manufactured by Novozymes A/S Denmark).

[0062] It was confirmed that the hydrolysis rate at the same enzyme concentration decreased as the concentration of the mixture of skim milk powder and isolated soy protein increased. In addition, at the same enzyme concentration, the hydrolysis rate of the isolated soy protein in the mixture was higher than when the isolated soy protein was used alone.

Example 2: Measurement of Culturability depending on Hydrolysis Rate of Protein

2-1: Experiment on Culturability depending on Concentration-Dependent Hydrolysis Rate of Skim Milk Powder (comparative)

[0063] In the aqueous protein hydrolysate solution prepared in Example 1, 30 kg of mixed lactose, 6 kg of soy peptone, 12 kg of yeast extract, 1.2 kg of potassium phosphate dibasic, 120 g of magnesium sulfate, 600 g of L-ascorbic acid, 240 g of L-glutamic acid and 600 g of polysorbate-80 were dissolved to a final volume of 1,200 L. The resulting solution was sterilized using a heat exchanger (Alfalaval, Sweden) at a temperature of 130°C and a flow rate of 1,850 ℓ/hr and transferred to a 1.2-KL-volume anaerobic fermentation tube. Next, 5 L of *Streptococcus thermophilus* CBT ST3 as a starter was inoculated into the solution and then fermented for 13 hours while maintaining the pH at 6.0 with ammonia. After fermentation, the culturability of the lactic acid bacteria depending on the concentration-dependent hydrolysis

rate of the skim milk powder and on the concentration of protease was measured. For measurement of the culturability of the lactic acid bacteria, 1 ml of the culture was taken in 9 ml of dilution water and vortexed, and then the number of viable cells was analyzed by the decimal dilution method. The results of the measurement are shown in Table 7 below.

[Table 7]

Experimental results for the culturability of *Streptococcus thermophilus* CBT ST3 (KCTC 11870BP) depending on the concentration-dependent hydrolysis rate of skim milk powder and on the concentration of protease						
Enzyme/Skim	**1.0%**	**2.0%**	**3.0%**	**4.0%**	**5.0%**	**6.0%**
0.001%	4.9E+09	7.1E+09	7.4E+09	7.3E+09	7.0E+09	6.7E+09
0.002%	5.0E+09	7.4E+09	7.6E+09	7.5E+09	7.2E+09	6.9E+09
0.003%	5.2E+09	8.3E+09	8.2E+09	8.3E+09	7.7E+09	7.1E+09
0.004%	5.2E+09	8.2E+09	8.3E+09	8.2E+09	8.2E+09	7.4E+09
0.005%	5.3E+09	8.3E+09	8.4E+09	8.3E+09	8.3E+09	7.6E+09

[0064] From the experimental results shown in Table 7 above, it was confirmed that when the skim milk powder and the protease were used at concentrations of 2% or higher and 0.003% or higher, respectively, excellent culturability could be ensured. At the skim milk powder concentration that was increased to exceed a certain level, the culturability tended to decrease rather than increase, as the proportion of the skim milk powder not degraded by the enzyme increased. As can be seen in a microscopic photograph of FIG. 1, these results were presumed to be because the hydrophobicity of the culture was increased due to an increase in the amount of the non-degraded, non-covalently bonded hydrophobic water-semisoluble peptide, and thus the lactic acid bacteria agglomerated and were not easily divided.

2-2: Experiment on Culturability depending on Concentration-Dependent Hydrolysis Rate of Isolated Soy Protein

[0065] Like the case of the skim milk powder, in the hydrolysate solution obtained in Example 1, 24 kg of glucose, 6 kg of soy peptone, 18 kg of yeast extract, 1.2 kg of sodium acetate, 1.2 kg of potassium citrate, 120 g of magnesium sulfate, 1.8 kg of L-cysteine hydrochloride, 600 g of L-ascorbic acid and 1.2 kg of polysorbate-80 were dissolved to a final volume of 1,200 L. The resulting solution was sterilized using a heat exchanger (Alfalaval, Sweden) at a temperature of 130°C and a flow rate of 1,850 ℓ/hr and transferred to a 1.2-KL-volume anaerobic fermentation tube. Next, 5 L of *Bifidobacterium breve* CBT BR3 as a starter was inoculated into the solution, and then fermented for 14 hours while maintaining the pH at 6.5 with ammonia. After fermentation, the culturability of the lactic acid bacteria depending on the concentration-dependent hydrolysis rate of the isolated soy protein and on the concentration of protease was measured. For measurement of the culturability of the lactic acid bacteria, 1 ml of the culture was taken in 9 ml of dilution water and vortexed, and then the number of viable

cells was analyzed by the decimal dilution method. The results of the measurement are shown in Table 8 below.

[Table 8]

Results of measuring the culturability of *Bifidobacterium breve* CBT BR3 (KCTC 12201BP) depending on the concentration-dependent hydrolysis rate of isolated soy protein and on the concentration of protease						
Enzyme/ISP	**0.15%**	**0.30%**	**0.45%**	**0.60%**	**0.75%**	**0.90%**
0.005%	6.0E+09	8.0E+09	8.9E+09	9.3E+09	7.8E+09	6.9E+09
0.010%	6.1E+09	8.1E+09	9.0E+09	9.4E+09	8.3E+09	7.4E+09
0.015%	6.3E+09	8.2E+09	9.3E+09	9.4E+09	9.3E+09	8.2E+09
0.020%	6.3E+09	8.2E+09	9.3E+09	9.4E+09	9.1E+09	8.0E+09
0.025%	6.4E+09	8.4E+09	9.4E+09	9.5E+09	9.5E+09	7.9E+09

[0066] From the experimental results shown in Table 8 above, it was confirmed that when the isolated soy protein and the protease were used at concentrations of 0.45% or higher and 0.015% or higher, respectively, excellent culturability could be ensured. At the isolated soy protein concentration that was increased to exceed a certain level, the culturability tended to slightly decrease rather than increase, as the proportion of the isolated soy protein not degraded by the enzyme increased. As can be seen in a microscopic photograph of FIG. 2, these results were presumed to be because the hydrophobicity of the culture was increased due to an increase in the amount of the non-degraded water-semisoluble peptide, and thus the lactic acid bacteria agglomerated and were not easily divided.

2-3: Experiment on Culturability depending on Concentration-Dependent Hydrolysis Rate of Mixture of Skim Milk Powder and Isolated Soy Protein (comparative)

[0067] In the hydrolysate solution of the mixture of skim milk powder and isolated soy protein, prepared in Example 1, 36 kg of crystalline fructose, 36 kg of yeast extract, 2.4 kg of potassium phosphate dibasic, 6 kg of sodium acetate, 1.2 kg of magnesium sulfate, 6 g of manganese sulfate, 1.2 kg of L-cysteine hydrochloride, 1.2 kg of L-ascorbic acid, 2.4 kg of polysorbate-80 and 7.2 kg of refined salt were dissolved to a final volume of 1,200 L. The resulting solution was sterilized using a heat exchanger (Alfalaval, Sweden) at a temperature of 130°C and a flow rate of 1,850 ℓ/hr and transferred to a 1.2-KL-volume anaerobic fermentation tube. Next, 5 L of *Lactobacillus acidophilus* CBT LA1 as a starter was inoculated into the solution, and then fermented for 20 hours while maintaining the pH at 5.5 with ammonia. After fermentation, the culturability of the lactic acid bacteria depending on the concentration-dependent hydrolysis rate of the mixture of skim milk powder and isolated soy protein was measured. For measurement of the culturability of the lactic acid bacteria, 1 ml of the culture was taken in 9 ml of dilution water and vortexed, and then the number of viable cells was analyzed by the decimal dilution method. The results of the measurement are shown in Table 9 below.

[Table 9]

Results of measuring the culturability of *Lactobacillus acidophilus* CBT LA1(KCTC 11906BP) depending on the concentration-dependent hydrolysis rate of the mixture of skim milk powder and isolated soy protein (ISP)				
Skim/ISP	**0.10%**	**0.20%**	**0.30%**	**0.40%**
0.5%	5.9E+09	6.1E+09	6.4E+09	6.3E+09
1.0%	6.2E+09	6.3E+09	7.2E+09	6.8E+09
1.5%	6.4E+09	7.0E+09	8.2E+09	7.2E+09
2.0%	6.8E+10	7.1E+09	8.1E+09	6.8E+09

[0068] From the experimental results shown in Table 9 above, it was confirmed that when the skim milk powder and the isolated soy protein were used as a mixture at concentrations of 1.5% or higher and 0.3% or higher, respectively, excellent culturability could be ensured. At the mixture concentration that was increased to exceed a certain level, the culturability tended to decrease rather than increase. As can be seen in a microscopic photograph of FIG. 3, these results were presumed to be because the hydrophobicity of the culture was increased due to an increase in the amount of the non-degraded water-semisoluble peptide, and thus the lactic acid bacteria agglomerated and were not easily divided.

Example 3: Measurement of Freeze-Drying Viability depending on Protein Hydrolysis Rate

3-1: <u>Experiment on Freeze-Drying Viability depending on Concentration-Dependent Hydrolysis Rate of Skim Milk Powder</u> (comparative)

[0069] The fermentation broth obtained in Example 2-1 above was centrifuged using a tubular type high-speed centrifuge (RPM: 15,000 or more, and G-force: 13,200 or more) at a flow rate of 4.0 ℓ/min, and the cells coated with the remaining protein component were recovered by precipitation. 10 L of an aqueous cryoprotectant solution including 3 kg of trehalose, 1 kg of maltodextrin, 1 kg of mannitol and 1 kg of skim milk powder was sterilized by autoclaving and prepared, and 10 L of an aqueous polysaccharide solution obtained by dissolving 20 g of xanthan gum and 20 g of cellulose was sterilized by autoclaving and prepared. Then, the recovered cells and the prepared aqueous cryoprotectant solution and aqueous polysaccharide solution were homogenized by stirring at 200 RPM in a vertical mixer equipped with a whipper. Next, the homogenized mixture was frozen rapidly in a pre-freezer at -40°C, and then the freeze dryer shelf temperature was elevated stepwise from 0°C by a rate of 10°C/2 hour, and then the mixture was finally freeze-dried at 37°C. In the process of hydrolysis with the protease, the remaining water-semisoluble protein component formed a protein coating on the cells, and the xanthan gum and cellulose polysaccharide components formed a

cluster having a very dense structure by binding between the cells. As this time, as the amount of the water-semisoluble peptide was higher than a certain amount, the culturability of the lactic acid bacteria was reduced while the lactic acid bacteria tended to agglomerate. In addition, a suitable amount of the water-semisoluble peptide exhibited excellent freeze-drying viability, accelerated stability, acid resistance and bile resistance by protecting the cells from heat applied during the freeze-drying process.

[0070] The results of the experiment are shown in Table 10 below.

[Table 10]

Experimental results for the freeze-drying viability of *Streptococcus thermophilus* CBT ST3 (KCTC 11870BP) depending on the concentration-dependent hydrolysis rate of skim milk powder and on the concentration of protease						
Enzyme/Skim	**1.0%**	**2.0%**	**3.0%**	**4.0%**	**5.0%**	**6.0%**
0.001%	57.7%	72.9%	63.8%	60.3%	59.0%	58.1%
0.002%	56.2%	73.4%	65.6%	62.9%	59.6%	58.8%
0.003%	55.9%	81.5%	67.1%	64.4%	60.7%	59.6%
0.004%	54.5%	71.5%	68.6%	67.0%	64.3%	61.1%
0.005%	53.1%	69.0%	70.3%	67.7%	65.2%	63.9%

[0071] Freeze-drying viability (%): the viability after the freeze-drying process was determined in view of the viable cell number and weight before and after freeze-drying.

[0072] As shown in Table 10 above, a skim milk powder concentration of 2% and a protease concentration of 0.003% showed the best freeze-drying viability (81.5%). As the skim milk powder concentration increased, the hydrolysis rate tended to decrease and the freeze-drying viability tended to decrease. It was confirmed that a certain amount of the non-degraded water-semisoluble peptide reduced protein denaturation caused by a physical factor applied to the cells and improved the freeze-drying viability. The results of measurement of the culturability and the freeze-drying viability indicated that both the culturability and the freeze-drying viability were excellent when treated with 2.0% skim milk powder and 0.003% enzyme.

3-2: <u>Experiment on Freeze-Drying Viability depending on Concentration-Dependent Hydrolysis Rate of Isolated Soy Protein</u>

[0073] According to the experimental method described in Example 3-1, an experiment on freeze-drying viability depending on the concentration-dependent hydrolysis rate of isolated soy protein was performed using the fermentation broth obtained in Example 2-2. The results of the experiment are shown in Table 11 below.

[Table 11]

Experimental results for the freeze-drying viability of *Bifidobacterium breve* CBT BR3(KCTC 12201BP) depending on the concentration-dependent hydrolysis rate of isolated soy protein and on the concentration of protease						
Enzyme/ISP	**0.15%**	**0.30%**	**0.45%**	**0.60%**	**0.75%**	**0.90%**
0.005%	55.3%	64.9%	77.1%	63.2%	60.4%	58.6%
0.010%	57.9%	67.1%	78.9%	69.7%	63.2%	61.1%
0.015%	62.3%	68.1%	85.2%	70.4%	67.6%	64.8%
0.020%	65.9%	69.6%	76.1%	69.6%	65.8%	63.1%
0.025%	69.4%	70.1%	75.4%	68.4%	64.1%	61.1%

[0074] Freeze-drying viability (%): the viability after the freeze-drying process was determined in view of the viable cell number and weight before and after freeze-drying.

[0075] As shown in Table 11 above, an isolated soy protein concentration of 0.45% and a protease concentration of 0.015% showed the best freeze-drying viability (85.2%). As the isolated soy protein concentration increased, the hydrolysis rate tended to decrease and the freeze-drying viability tended to decrease. It was confirmed that a certain amount of the non-degraded water-semisoluble peptide reduced protein denaturation caused by a physical factor applied to the cells and improved the freeze-drying viability. The results of measurement of the culturability and the freeze-drying viability indicated that both the culturability and the freeze-drying viability were excellent when treated with 0.45% isolated soy protein and 0.015% enzyme.

3-3: <u>Experiment on Freeze-Drying Viability depending on Concentration-Dependent Hydrolysis Rates of Skim Milk Powder and Isolated Soy Protein</u> (comparative)

[0076] According to the experimental method described in Example 3-1, an experiment on freeze-drying viability depending on the concentration-dependent hydrolysis rates of skim milk powder and isolated soy protein was performed using the fermentation broth obtained in Example 2-3. The results of the experiment are shown in Table 12 below.

[Table 12]

Freeze-drying viability of *Lactobacillus acidophilus* CBT LA1 (KCTC 11906BP) depending on the concentration-dependent hydrolysis rates of skim milk powder and isolated soy protein				
Skim/ISP	**0.10%**	**0.20%**	**0.30%**	**0.40%**
0.5%	57.7%	59.9%	66.2%	62.9%
1.0%	63.8%	65.3%	71.6%	67.6%
1.5%	70.1%	73.0%	***83.1%***	70.3%
2.0%	68.8%	70.1%	74.2%	68.6%

* Freeze-drying viability (%): the viability after the freeze-drying process was determined in view of the viable cell number and weight before and after freeze-drying.

[0077] As shown in Table 12 above, when a mixture of 1.5% skim milk powder and 0.3% isolated soy protein was used, the best freeze-drying viability (83.1%) could be obtained. As the mixture concentration increased, the hydrolysis rate tended to decrease and the freeze-drying viability tended to decrease. It was confirmed that a certain amount of the non-degraded water-semisoluble peptide reduced protein denaturation caused by a physical factor applied to the cells and improved the freeze-drying viability. The results of measurement of the culturability and the freeze-drying viability indicated that both the culturability and the freeze-drying viability were excellent when treated with a mixture of 1.5% skim milk powder and 0.3% isolated soy protein.

Example 4: Experiment on Accelerated Stability, Acid Resistance and Bile Resistance by Dual Coating

[0078] An experiment was performed on the accelerated stability, acid resistance and bile resistance of non-coated lactic acid bacteria, lactic acid bacteria coated with protein alone, and dual-coated lactic acid bacteria. The results of the experiment are shown in Table 13 below.

[Table 13]

Experimental results for accelerated stability, acid resistance and bile resistance depending on non-coating, protein coating and polysaccharide coating								
Strain	CBT ST3				CBT BR3			
Protein hydrolysis conditions	Not used		2% skim milk powder + 0.003% enzyme		Not used		0.45% ISP + 0.015% enzyme	
Protein coating	X	X	O	O	X	X	O	O
Polysaccharide coating (dual coating)	X	CMC-Na, XG	X	CMC-Na, XG	X	CMC-Na, XG	X	CMC-Na, XG
Accelerated stability	19.2%	38.3%	54.2%	85.2%	12.5%	33.2%	44.1%	65.2%
Acid resistance	35.2%	52.8%	70.0%	90.7%	23.6%	51.2%	69.5%	88.6%
Bile resistance	30.7%	50.5%	70.7%	91.6%	26.6%	51.8%	56.8%	83.1%
CMC-Na: cellulose; XG: xanthan gum;								

[0079] Accelerated stability: 1 g of each sample was taken in 9 ml of dilution water and

vortexed, and then the initial number of viable cells was analyzed by the decimal dilution method. Additionally, after each sample was incubated at 40°C, the number of viable cells was analyzed once a week for 4 weeks, and the viability relative to the initial number of viable cells was examined.

[0080] Acid resistance: 0.1 g of each sample was dissolved in 9.9 ml of MRS broth solution (pH 2.1) corrected with 1M HCl solution, and then the number of viable cells at 0 hour and 2 hours was analyzed while maintaining a temperature of 37°C, and the viability relative to the viability at 0 hour was examined.

[0081] Bile resistance: 0.1 g of each sample was dissolved in 9.9 ml of 0.5% oxgall-containing MRS broth solution, and then the number of viable cells at 0 hour and 2 hours was analyzed while maintaining a temperature of 37°C, and the viability relative to the viability at 0 hour was examined.

[0082] The results of comparative analysis of non-coated lactic acid bacteria, protein-coated lactic acid bacteria and dual-coated lactic acid bacteria indicated that the dual-coated lactic acid bacteria could exhibit better accelerated stability, acid resistance and bile resistance than the non-coated or single (protein)-coated lactic acid bacteria. Since the rate of death caused by a physical factor applied to the cells is low and the physiologically active function of the lactic acid bacteria cannot be lost, the dual-coated lactic acid bacteria exhibit improved accelerated stability, acid resistance and bile resistance.

[0083] As described above, the present disclosure relates to a method of producing lactic acid bacteria dual-coated with protein and polysaccharide by using a protein hydrolysate, and lactic acid bacteria having a dual coating, produced by the method. The lactic acid bacteria having a dual coating of protein and polysaccharide, produced according to the present disclosure, have very excellent dry-freezing viability, acid resistance and bile resistance. Accordingly, the lactic acid bacteria having a dual coating of protein and polysaccharide produced according to the present disclosure will be very useful for the production of fermented milk, processed milk, fermented soy products, processed foods, functional beverages, functional foods, common foods, etc.

REFERENCES CITED IN THE DESCRIPTION

Cited references

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all

liability in this regard.

Patent documents cited in the description

- KR1020110134486 [0007] [0009]
- KR1020150129986 [0007] [0009]
- KR1020160172568 [0007] [0009]
- KR1020170045326 [0007] [0009]
- US2015152378A1 [0009]

PATENTKRAV

1. Fremgangsmåde til fremstilling af mælkesyrebakterier med en dobbelt coating af protein og polysaccharidcoatinger, hvilken fremgangsmåde omfatter følgende trin:
5 (a) behandling af en vandig proteinopløsning med en protease, hvorved der fremstilles en vandig proteinhydrolysatopløsning;
(b) tilsætning af en sukkerbestanddel og en nitrogenkildebestanddel til mælkesyrebakteriekultur til den fremstillede vandige, proteinhydrolysatopløsning, efterfulgt af sterilisering, og derefter inokulering og dyrkning af mælkesyrebakterierne i den steriliserede, vandige opløsning;
10 (c) genvinding af mælkesyrebakterieceller fra en fermenteret mælkesyrebakteriekultur opnået ved dyrkningen;
(d) tilsætning af en vandig kryoprotektantopløsning og en vandig polysaccharidopløsning til de genvundne mælkesyrebakterieceller, efterfulgt af blanding og homogenisering; og
(e) frysetørring af den homogeniserede, vandige mælkesyrebakteriecelleopløsning,
15 hvor proteinets hydrolyserate i trin (a) er
(i) en procentværdi (%) opnået ved division af en ΔOD-værdi, der er en forskel mellem den vandige proteinopløsnings optiske densitets- (OD) værdi ("startpunkts-OD"), målt før behandling med proteasen, og den vandige proteinopløsnings optiske densitets- (OD) værdi ("slutpunkts-OD"), målt efter behandling med proteasen, med startpunkt-OD-værdien; eller
20 (ii) en procentværdi (%) opnået ved division af en Δppt-værdi, der er en forskel mellem den målte vægtværdi ("startpunkts-ppt") af et præcipitat, opnået ved centrifugering af den vandige proteinopløsning før behandling med proteasen, og en målt vægtværdi ("slutpunkts-ppt") af et præcipitat, opnået ved centrifugering af den vandige proteinopløsning efter behandling med proteasen, med startpunkts-ppt-værdien,
25 hvor proteinet i trin (a) er isoleret sojaprotein (ISP),
hvor koncentrationen af det isolerede sojaprotein (ISP) er 0,45 % og koncentrationen af proteasen er 0,015 %,
hvor det isolerede sojaproteins hydrolyserate er 74 %,
hvor mælkesyrebakterierne er *Bifidobacterium breve* CBT BR3 (KCTC 12201BP).
30

2. Fremgangsmåde ifølge krav 1, hvor nitrogenkildebestanddelen til mælkesyrebakteriekulturen i trin (b) er gærekstrakt eller sojapepton.

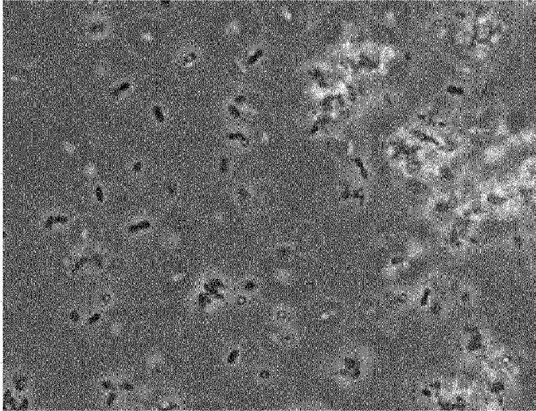
3. Fremgangsmåde ifølge krav 1, hvor kryoprotektanten i trin (d) er én eller en blanding af to eller flere valgt fra gruppen bestående af trehalose, maltodextrin, mannitol og skummetmælkspulver.

5 **4.** Fremgangsmåde ifølge krav 1, hvor polysaccharidet i trin (d) er én eller flere valgt fra gruppen bestående af gummier, vandopløselige kostfibre, ufordøjeligt maltodextrin, uopløselige kostfibre, stivelse, Levan og resistent stivelse.

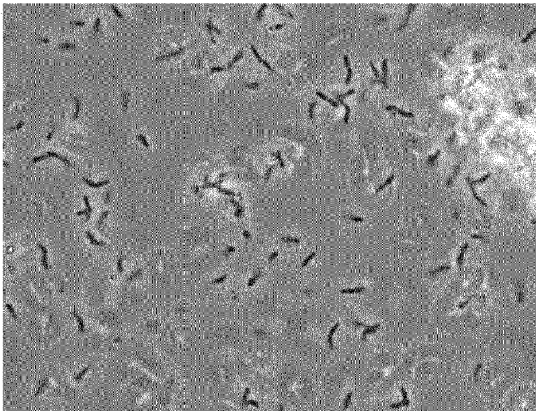
DRAWINGS

Drawing

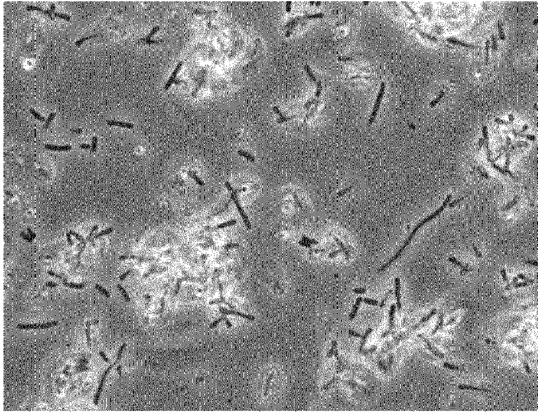
[FIG. 1]



[FIG. 2]

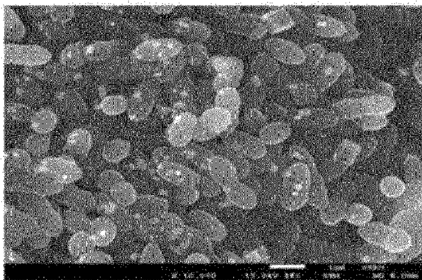


[FIG. 3]

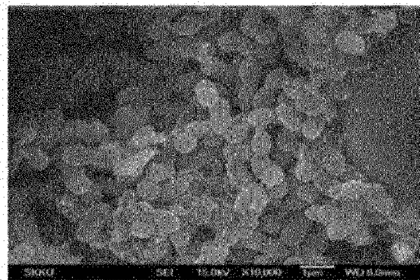


[FIG. 4]

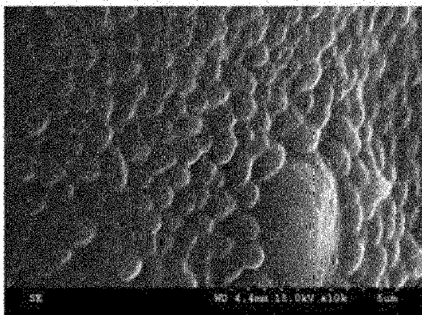
Inside and outside photographs of dual-coated *Streptococcus thermophilus* CBT ST3



10,000 X inside photograph



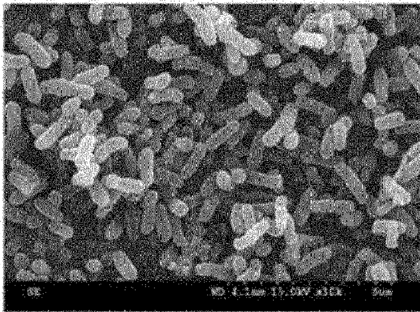
10,000 X inside photograph



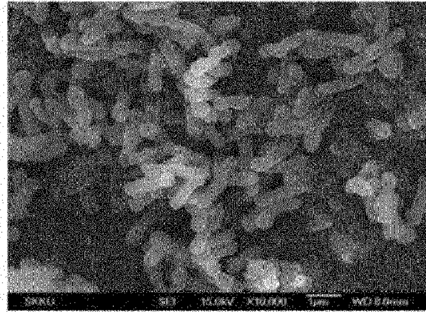
10,000 X outside photograph

[FIG. 5]

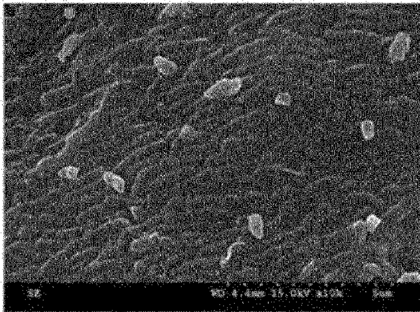
Inside and outside photographs of dual-coated *Bifidobacterium breve* CBT BR3



10,000 X inside photograph



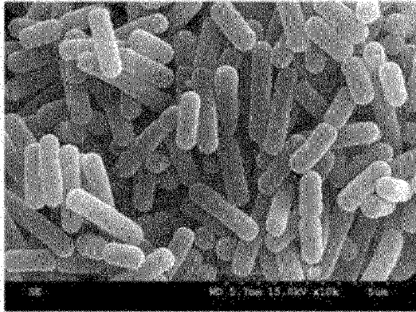
10,000 X inside photograph



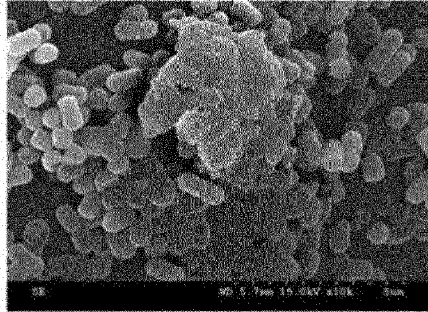
10,000 X outside photograph

[FIG. 6]

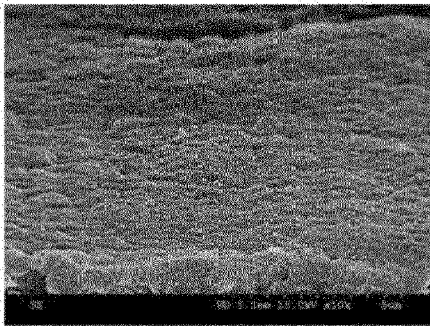
Photographs of non-coated and dual-coated *Lactobacillus acidophilus* CBT LA1



10,000 X (non-coated)



10,000 X (coated, inside)



10,000 X (coated, outside)