

PATENT SPECIFICATION

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(19)



(54) A METHOD FOR MANUFACTURING LACTIC ACID FERMENTED MILK BEVERAGES

(71) We, KABUSHIKI KAISHA YAKULT HONSHA, a Japanese Body Corporate, of 1-19, Higashi Shinbashi 1-chome, Minato-ku, Tokyo, Japan, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention relates to a method for manufacturing lactic acid fermented milk beverages.

Lactic acid fermented milk beverages (or, more simply lactic acid beverages) are now widely manufactured by fermenting with lactic acid bacteria a medium comprising mainly milk which is cow's milk or the milk of other animals, skim milk, powdered milk, or other milk products; sweetening agents, flavoring materials, fruit juices and the like additive; and by diluting and sterilizing the resultant products.

Lactic acid beverages can be divided into two classes: (i) beverages contained viable lactic acid bacteria from the fermentation and (ii) heat-sterilized beverages. The former, which hereinafter will be referred to as beverages containing viable bacteria, have recently attracted notice because of their intestinal controlling activity, and their production has been increased remarkably.

While strains like *Lactobacillus casei*, and *L. acidophilus* are generally used for the manufacture of lactic acid beverages, lactic acid production is slow as compared to their bacterial growth. Since lactic acid beverages are characterized by moderate acidity with a fresh taste, lactic acid fermentation should be continued until a sufficient amount of lactic acid is produced; that is a titratable acidity (volume in ml of 0.1N sodium hydroxide required to neutralize a sample of 1 ml) of from 2.8 to 3.0, in the product to be consumed and after any necessary dilution. Generally, at least 5 to 7 days' incubation is required for this purpose.

To reduce this long incubation time, the use is widely accepted of mixed strain cultures with bacteria, which rapidly produce lactic

acid, as also is the use of lactic acid bacteria growth-promoting substances such as yeast extract, chlorella, or a chlorella extract. The use of a mixed strain culture, however, has in no instance been satisfactory because the bacteria which rapidly produce lactic acid also generally produce diacetyl and the other characteristic by-products which result in deterioration of the final product and often also result in mutual growth inhibition between the strains used. Moreover, a diacetyl odour noticeably spoils the taste in a liquid-type lactic acid beverage while it is not noticeable in a solid-type fermented milk such as yoghurt.

The invention thus provides a method for producing lactic acid beverages which comprises lactic acid fermentation of a medium comprising milk using *Lactobacillus helveticus* YIT-0100 (FERM — P No. 2946) or *L. Helveticus* YIT-0019 (FERM — P No. 3083). Preferably the fermentation uses a mixed strain culture of the present strains with complementary lactic acid bacillus strains. The complementary strain will typically be of a low acid-producing, acid-resistant bacillus, preferably that which we call PS — 1, i.e. *L. casei* PS — 1 (FERM — P 1746).

The above mentioned two strains of *L. helveticus* used in the method of this invention were so identified according to the classification standard in Bergey's Manual Volume 8 (1974) and have features commonly found in strains of *L. helveticus* such as: (1) No gas is formed from glucose, and (2) Acid producing activity is high — an acidity of 2.8 to 3.0 is reached in 40 to 48 hours' incubation at a temperature of 37°C. The two strains also exhibit specific desirable features when used in a mixed strain culture with various strains of *L. casei* especially with PS — 1 such as (A) Diacetyl production is negligible and other flavor components produced do not spoil the otherwise pleasant flavor of the products, and (b) Inhibition of mutual bacterial growth does not take place. Other biological properties are shown in Table 1.

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TABLE 1.

Microscopic appearance	Gram-positive, medium-size rods
Growth at 15°C	—
Growth at 45°C	+
Skim milk coagulability	+
Carbohydrate reactions	
Arabinose	—
Xylose	—
Rhamnose	—
Glucose	+
Mannose	+
Galactose	+
Sucrose	+
Maltose	+
Lactose	+
Cellobiose	—
Trehalose	—
Melibiose	—
Raffinose	—
Melezitose	—
Mannitol	—
Sorbitol	—
Salicin	—
Amygdalin	—
Optimum growth conditions	pH 6.6 to 7.0 Temperature: 37°C to 40°C
Growth temperature range	YIT-0100: 24°C to 46°C YIT-0019: 25°C to 48°C
Presence of serological group A (according to Rogosa and Sharp)	+
Methyl red test	—
Voges-Proskauer reaction	—
Indole test	—
Hydrogen sulfide formation	—
Ammonia production	—
Nitrate reduction	—
Catalase production	—
Liquifaction of gelatin and casein	—
Citrate utilization	—
Milk coagulability	+
Litmus milk reduction	+
Ammonium salt and urea utilization	—

Note: No difference between YIT-0100 and YIT-0019 was observed except the growth temperatures.

The strain PS — 1 has already disclosed in a Japanese Patent, Petition No. SHO-45 — 71777, (Patent Publication No. SHO-49 — 38824), as an excellent lactobacillus strain for manufacture of lactic acid beverages providing fast growth rate in a single strain

culture and a pleasant flavor in final products.

The strains, "*Lactobacillus helveticus* YIT-0019" "*Lactobacillus helveticus* YIT-0100" and "*Lactobacillus casei* (PS — 1)" used in the present invention were respectively de-

posited at the FERMENTATION RESEARCH INSTITUTE, Agency of Industrial Science & Technology, No. 8 — 1 Inage Higashi - 5 - chome, Chiba-shi, Chiba-ken, Japan, and the deposit numbers are respectively FERM — P No. 3083, FERM — P No. 2946 and FERM — P No. 1766.

Due to their high acid and low diacetyl producing activities the strains YIT - 0100 and YIT 0019 can be to advantage in single strain cultures for manufacture of lactic acid beverages, more particularly of beverages wherein viable cell numbers and viability thereof in the human intestine are not a primary concern; however, these strains can be more effectively utilized in a combination with acid-tolerant strains of *L. casei*, *L. acidophilus* and the like known bacteria which as mentioned above require a long incubation time due to their low acid producing activity. The above mentioned *L. casei* strain PS — 1 is particularly useful in mixed strain cultures wherein fast growth of PS — 1 and high acid productivity of *L. helveticus* symbiotically cooperate to give in 40—48 hours' incubation viable cell numbers of 1.0×10^9 /ml and a titratable acidity of above 2.8. Besides, the pleasant flavor characteristic in a single strain PS — 1 culture is not spoiled at all.

The following is a detailed description for preparing lactic acid beverages in accordance with this invention and is mainly directed to preparing lactic acid beverages containing viable bacteria by use of a mixed strain culture; however, this is intended to illustrate the invention and not to limit it.

Two single strains of lactic acid bacteria are individually precultured for preparation of starter cultures. Preculturing is effected at 37°C for 24 hours in a sterile medium containing 10% nonfat milk solids and 3% glucose to obtain viable cell numbers of 3 to 4×10^9 /ml for PS — 1 and 1.0×10^9 /ml for YIT -0100 (or YIT -0019). The starter cultures thus prepared are inoculated into a sterile medium containing 8 to 16% nonfat milk solids and about 3% glucose. The amounts in the inoculum are typically 0.2 to 2.0% (by weight on the weight of the medium; the same basis is used hereinafter for all inocula percentages) for PS — 1 and 0.0005% to 0.005% for YIT -0100 (or YIT -0019), the weight ratio of the two

usually being adjusted to be in the range from 40:1 to 4,000:1 according to the incubation conditions. The medium is thoroughly mixed and incubated at a temperature of $37 \pm 2^\circ\text{C}$ for 40 to 48 hours to reach an acidity of 2.75. The resultant culture is cooled and aged at 20°C , giving a final acidity of 2.8 to 3.0. Flavoring materials, fruit juices, water and the like additives are usually added when manufactured for beverages.

Figure 1 illustrates change in the titratable acidity in mixed strain cultures of PS — 1 with YIT strain prepared as mentioned above; curve a is for a mixed strain culture whereas b is for PS — 1 in a single strain culture. Figure 2 illustrates the change in numbers of viable lactic acid bacteria in mixed strain culture; c is for PS — 1 in a mixed strain culture whereas d for YIT -0100 is a single strain culture. The incubation conditions for the plots in Figures 1 and 2 are the same as in Example 1 hereinafter. Figure 3 illustrates the change in titratable acidity in the case of a single YIT -0100 strain culture; curve e is for an inoculum of 0.0005% and f for that of 0.05%. The arrows on each curve in Figure 3 indicate the scales which apply to the particular curve. It is obvious from the Figures that the mixed strain cultures provide sufficiently high acidity and viable cell number in only half the period required in the case of a single strain culture. Shortening of the incubation time has further advantageously affects by reducing the number of bacteria introduced and other unexpected problems due to the prolonged incubation.

Moreover, since PS — 1 are still in their highly biologically active growing phase at 40 to 48 hours' incubation time, products containing the bacteria of long-lasting stable viability can be obtained.

Table 2 illustrates the results of sensory tests of product "A" from a single PS — 1 strain culture and product "B" from a mixed strain PS — 1/YIT -0100 culture, and Table 3 illustrates the results during storage stability tests. From these results it is obvious that the products obtained by the mixed strain culture using a short incubation period were comparable, in respect of sensory test and storage stability, to those obtained by a single strain PS — 1 culture.

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TABLE 2

(1) Triangle test *

Number of panel members	Number of members who differentiated	Significance in difference
Male 164	55	None
Female 127	49	None
Total 291	104	None

*A differentiation test for choosing the correct sample from three samples, see Krum, J.K.; Truest evaluation in sensory panel testing, Food Engineering, 27, 1955 No. F.74.

5 Furthermore advantageous features in the use of either strain of YIT -0100 or YIT -0019 as a complement strain for a mixed strain culture with PS — 1 are demonstrated in Table 4 in comparison with other strains of lactic acid bacteria. A medium containing 14% powdered skim milk and 3% glucose was inoculated with 0.5% PS — 1 and 10 0.005% of a complement strain, and incubated at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$. The results in Table 4 represent summarized data for about 20 strains, except for YIT -0100 and YIT -0019, of each complement species. Sensory tests for

significance in difference were conducted by 20 trained panel members repeatedly at regular intervals: — represents non-significant, and + significant in difference.

It is clearly understood that strains YIT -0100 and YIT -0019 have characteristics such as not to interfere with the fermentation by PS — 1 without spoiling flavors of the final products, while other strains tested have adverse effects. (The effect on flavor was quantitatively confirmed by remarked difference in diacetyl productivity.)

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(2) Sensory test

Number of panel members	No. of members who preferred "A"	No. of members who preferred "B"	No Special Preference	Significance in difference
Male 91	38	30	23	None
Female 69	22	29	18	None
Total 160	60	59	41	None

TABLE 3

Measurement	Storage temperature C	Product	Storage in days		
			0	3	7
Acidity	5	A	0.69	0.71	0.72
		B	0.69	0.69	0.70
	24	A	—	0.87	1.0
		B	—	0.83	0.99
pH	5	A	3.69	3.68	3.55
		B	3.68	3.60	3.56
	24	A	—	3.53	3.37
		B	—	3.83	3.32
Viable cells/ml (P8-1)	5	A	1.2×10^9	9.7×10^8	5.3×10^8
		B	1.1×10^9	9.8×10^8	5.1×10^8
	24	A	—	7.6×10^8	3.4×10^8
		B	—	7.8×10^8	3.0×10^8
Viable cells/ml (YIT-0100)	5	B	6.8×10^7	5.9×10^7	3.5×10^7
	24	B	—	4.5×10^7	2.8×10^7

TABLE 4

	Complementary strain				
	YIT-0100	YIT-0019	Other strains of <i>L. helveticus</i>	<i>Streptococcus thermophilus</i>	<i>L. jugurti</i> fast acid producing
Difference in fermentative odour from that in single-strain culture	—	—	+	+	+
Diacetyl production (ppm)	<0.3	~0.5	>0.5	>1	>1
Sensory test for diacetyl odour	—	—	+	+	+
Time required to reach an acidity of above 2.75 (hours)	40 ± 3	40 ± 3	40 ± 3	40 ± 3	40 ± 3
Viable PS-1 cells/ml at 40 hours' incubation	3.5×10^9	3.0×10^9	3.5×10^9	$<10^7$	$<10^9$

In order to illustrate the present invention, the following examples are given.

Example 1.

5 A milk medium containing 14% skim milk powder and 3% glucose was heat-sterilized, cooled to 37°C and simultaneously inoculated with PS — 1 and YIT -0100 starter cultures which had been individually precultured in a 10% skim milk medium at 37°C for 24 hours. 10 The amounts of inoculum were 0.5% for PS — 1 and 0.005% for YIT -0100 weight. After being stirred for 30 minutes, the medium was incubated at 37±2°C until the acidity

reached above 2.75. The culture was then cooled to 20°C by stirring and allowed to stand for 1 hour for ageing. It required 48 hours' incubation to obtain viable cell numbers of 2.0×10^9 /ml for PS — 1 and 3.0×10^9 /ml for YIT -0100. The acidity increased to 2.8 during ageing. The resultant milk culture was homogenized with a homogenizer, and sugar syrup and flavoring materials were added to produce a stock liquid for lactic acid beverages containing viable bacteria. The stock liquid is diluted with 1.5 times its volume of sterile water to manufacture beverages for drinking.

Example 2.

A mixed strain culture of PS — 1 and YIT -0019 was prepared as in Example 1. The amounts of inoculum were 0.5% for PS — 1 and 0.005% for YIT -0019. The incubation was continued for 40 hours to give an acidity of 2.75 and viable cell numbers of 3.0×10^8 /ml for PS — 1 and 5.0×10^8 /ml for YIT -0019. The acidity increased to 2.8 during ageing. The culture was homogenized and sugar syrup, flavoring materials and fruit juices were added to prepare a stock liquid for lactic acid beverages containing viable bacteria. The stock liquid was diluted with 1.5 times its volume of sterile water to manufacture a beverage for drinking.

Example 3.

A starter culture was previously prepared by incubating a 10% nonfat milk solids medium inoculated with YIT -0100 at 37°C for 24 hours, and said starter culture (0.05%) was inoculated in to the same medium as in Example 1. After being stirred for 30 min the medium was incubated at $37 \pm 2^\circ\text{C}$ for about 40 hours, when the acidity thereof had reached 2.5. The culture was cooled to 20°C, allowed to stand for 1 hour at the same temperature, heat-sterilized at 100°C for 2 seconds and homogenized. A stock liquid for a lactic acid beverage was prepared by adding sweetenings and flavoring materials to said culture. The product when diluted 2.5 times was sensory-evaluated by 20 trained panel members. No diacetyl odor and no astringency was reported.

Example 4.

Starter cultures of *L. acidophilus* and YIT -0100 were individually prepared as in Example 1, and simultaneously inoculated in to

a milk medium. The amounts of inoculum were 1.0% for *L. acidophilus* and 0.0005% for YIT 0100. After being stirred, the medium was incubated at $37 \pm 2^\circ\text{C}$ for 48 hours, the acidity was then above 2.75 and the numbers of viable cells were 5.0×10^8 /ml for *L. acidophilus* and 3.0×10^8 /ml for YIT -0100. After being cooled to 20°C and maintained at the same temperature for 1 hour the culture was processed as in Example 1 for manufacturing beverages containing viable bacteria. The beverage was subjected for a sensory test and the absence of a diacetyl odor and astringency thereof was noted.

WHAT WE CLAIM IS:—

1. A method for manufacturing a lactic acid beverage which comprises lactic acid fermentation of a medium comprising animal milk using *Lactobacillus helveticus* YIT -0100 (FERM—P 2946) or *L. helveticus* YIT -0019 (FERM —9 No. 3083).

2. A method according to claim 1 wherein the fermentation uses a mixed strain culture of one or both of the said *L. helveticus* strains with a strain of a complementary lactic acid bacillus.

3. A method for manufacturing lactic acid beverages according to claim 2, wherein said complementary lactic acid bacillus is *Lactobacillus casei* (PS — 1) (FERM —P No. 1766).

4. A method for manufacturing lactic acid beverages, the medium being substantially as described in any one of the preceding claims.

5. A lactic acid beverage when manufactured by a method according to any one preceding claim.

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Agents for the Applicants.

Example 2.

A mixed strain culture of PS — 1 and YIT -0019 was prepared as in Example 1. The amounts of inoculum were 0.5% for PS — 1 and 0.005% for YIT -0019. The incubation was continued for 40 hours to give an acidity of 2.75 and viable cell numbers of 3.0×10^8 /ml for PS — 1 and 5.0×10^8 /ml for YIT -0019. The acidity increased to 2.8 during ageing. The culture was homogenized and sugar syrup, flavoring materials and fruit juices were added to prepare a stock liquid for lactic acid beverages containing viable bacteria. The stock liquid was diluted with 1.5 times its volume of sterile water to manufacture a beverage for drinking.

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WHAT WE CLAIM IS:—

1. A method for manufacturing a lactic acid beverage which comprises lactic acid fermentation of a medium comprising animal milk using *Lactobacillus helveticus* YIT -0100 (FERM—P 2946) or *L. helveticus* YIT -0019 (FERM—9 No. 3083).
2. A method according to claim 1 wherein the fermentation uses a mixed strain culture of one or both of the said *L. helveticus* strains with a strain of a complementary lactic acid bacillus.
3. A method for manufacturing lactic acid beverages according to claim 2, wherein said complementary lactic acid bacillus is *Lactobacillus casei* (PS — 1) (FERM — P No. 1766).
4. A method for manufacturing lactic acid beverages, the method being substantially as described in any one of the preceding claims.
5. A lactic acid beverage when manufactured by a method according to any one preceding claim.

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