

Frozen starter of yogurt

The present invention relates to a process for the preparation of a frozen starter for the direct inoculation of milk, and the frozen starter obtained by this process.

It is known that a culture of lactic bacteria can be preserved by freezing with a view to using it for making yogurt. Such a culture, also termed starter, can either be reactivated by means of a preculture before being used for inoculating milk, or be used directly, in the frozen form, for inoculating milk to make it into yogurt.

Thus, US 3975545 describes the preparation of a frozen starter of yogurt which is reactivated before using it for inoculating milk. In this process, lactic bacteria are grown in a synthetic medium, concentrated, a cryoprotectant, namely glycerophosphoric acid, is added, and the bacteria are then frozen at -20°C . This starter is subsequently reactivated by preculturing it in a synthetic medium or in milk together with the cryoprotectant, and it is then used for inoculating milk to make it into yogurt.

Equally, EP 0378478 describes the preparation of a frozen starter for the direct inoculation of milk. In this process, lactic bacteria are grown in a synthetic nutrient medium which comprises, in particular, polyphosphate salts for solubilizing the casein, and the culture is concentrated and then frozen. Such a starter, once frozen, comprises approximately 10^9 to 10^{10} colonies of live cells per gram, or cfu/g (cfu being the abbreviation of colony-forming unit), and is typically used for direct inoculation at a concentration of 0.01-0.1%.

However, the lactic bacteria in a frozen starter used for direct inoculation have been subjected to stress factors which affect the capability of fermenting milk in a normal way.

These stress factors can be due, for example, to culturing bacteria in a synthetic nutrient medium



whose composition differs more or less widely from the composition of milk. Thus, the bacteria develops a metabolism which is poorly adapted to fermenting milk. Equally, the bacteria are concentrated, for example by centrifugation, and are therefore also disturbed. Finally, a cryoprotectant is traditionally added to the culture before freezing so as to protect the cells from freezing. However, these cryoprotectants can also have an inhibitory effect on bacterial growth.

10 Once the live bacteria of such a starter have thus been disturbed, only a fraction of them is really capable of fermenting milk and thus of producing lactic acid. It was thus found that less than 10% of these inoculated bacteria efficiently ferment milk. It would
15 therefore be highly interesting to increase the level of fermenting activity of a frozen starter.

 It is an aim of the present invention to overcome the disadvantages of the prior art and thus to propose a frozen starter for the direct inoculation of
20 milk having a level of fermentation activity of over 10%, preferably over 95%.

 To this end, in the process for preparing a frozen starter for the direct inoculation of milk with a view to making it into yogurt, lactic bacteria are
25 grown in milk at a temperature of 40-45°C until a pH of 4.3-5.5 is reached, and the culture is then frozen directly.

 The invention furthermore relates to the frozen starter and to its use for making yogurt.

30 Thus, the process for the preparation of a frozen starter according to the present invention has the advantage of dispensing with a synthetic culture medium and with a device for concentration. This process is therefore particularly uncomplicated and
35 simple to carry out.

 Moreover, it has surprisingly been found that the frozen starter according to the present invention has a level of fermentation activity of over 95%, which means that amongst the live bacteria which have

survived freezing, more than 95% are capable of rapidly fermenting milk, which leads to the formation of lactic acid.

Thus, the starter which is provided by the present invention is capable of fermenting milk until it has reached a pH which is traditional for yogurt, that is to say in the order of 4.4 to 4.8, as rapidly as can be done with a traditional frozen starter for direct inoculation, while the latter contains at least 10 times more live cells.

Finally, this process can be carried out without using a cryoprotectant, in particular in the case of preparing a frozen starter of streptococci.

In the text which follows, the term "streptococci" comprises the lactococci and the streptococci according to the novel nomenclature (Bergey's Manual).

To carry out the present process, at least one strain of streptococci and/or at least one strain of lactobacilli, for example, can thus be grown in milk. Amongst the streptococci, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactic* biovar *diacetylactis*, and *Streptococcus thermophilus* are thus preferably used. Equally, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus helveticus*, *Lactobacillus casei* subsp. *casei*, and *Lactobacillus delbrueckii* subsp. *lactis*, for example, (Bergey's Manual of Systematic Bacteriology, vol 2, 1986) can be used from amongst the lactobacilli.

To prepare the starter of the present invention, it is possible, in particular, to inoculate the milk with a fresh preculture of each bacterial genus or species used and/or with a fresh preculture comprising a mixture of the bacterial genera and species used. Moreover, the preculture medium can be a synthetic nutrient medium conventionally used for the laboratory culture of lactic bacteria, for example the MRS medium (De Man et al, 1960) or a milk-based medium, for example the MSK medium, which comprises 10% of

reconstituted powdered skimmed milk and 1/10,000 parts of a commercial yeast extract. One contemplates therefore all possibilities of inoculation with one or more fresh precultures, each of which comprises one or more strains of lactic bacteria which can be from a different genus or species. The inoculum of each fresh preculture is thus added in an amount which depends on the fermentation time required for achieving the final pH, and on the strain, or strains, of lactic bacteria used. Finally, it is preferred to prepare the fresh preculture by making several successive precultures of strains which have initially been frozen or lyophilized, and the milk is inoculated with this last preculture, in general the third, taken at the stage of coagulation of the preculture medium.

Other forms of direct inoculation, for example with lyophilized cultures, can also be used.

Preferably, 3-10% of glycerol are added, prior to freezing, to a culture which comprises, in particular, lactobacilli. Such a culture can thus comprise, in addition to the lactobacilli, another species or genus of bacteria, for example one or more strains of streptococci. The addition of glycerol makes it possible to increase the survival of these lactobacilli when freezing and thus to obtain a starter of the present invention having a level of fermentation activity of above 95%. However, it must be noted that such an addition is not a necessary condition for obtaining a starter according to the present invention which has a level of fermentation activity of over 10%.

Equally, lactic bacteria can be frozen in a traditional manner, for example between -20 and -90°C.

Finally, the milk may be sterile skimmed milk or sterile reconstituted skimmed milk, that is to say reconstituted from milk powder and sterilized at 110-120°C for 20-40 minutes, for example. This milk can furthermore comprise 1% of yeast extract so as to improve growth of the bacteria when preparing the starter.

In a first preferred embodiment of the process according to the present invention to obtain a starter of streptococci having a maximum level of fermentation activity, streptococci are grown at a temperature of 41-44°C until the pH has reached 4.9 to 5.2 so as to obtain a cell density in the order of $2 \cdot 10^8$ to $5 \cdot 10^8$ cfu/g, and the culture is then frozen directly.

It is thus possible to grow a plurality of species or strains of streptococci in the milk. The inoculum of streptococci can be either a fresh preculture comprising the species or strains of streptococci used in the form of a mixture, and/or a plurality of fresh precultures of each species or strain of streptococcus used. In particular, at least one strain of *Streptococcus thermophilus* is grown in this manner.

In a second preferred embodiment of the process according to the present invention to obtain a starter of lactobacilli having a maximum level of activity, the lactobacilli are grown at a temperature of 41-44°C until a pH of 4.6 to 5 is reached so as to obtain a cell density in the order of 10^8 to $5 \cdot 10^8$ cfu/g, and the culture is then frozen directly.

It is thus possible to grow a plurality of species or strains of lactobacilli in the milk. The inoculum of lactobacilli can be either a fresh preculture comprising the species or strains of lactobacilli used in the form of a mixture, and/or a plurality of fresh precultures of each species or strain of lactobacillus used. In particular, at least one strain of *Lactobacillus delbruckii subsp. bulgaricus* is grown in this manner.

In a third preferred embodiment of the process according to the present invention for obtaining a mixed starter comprising a mixture of lactobacilli and streptococci having a maximum level of fermentation activity, said bacteria are grown at a temperature of 41-44°C until a pH of 4.5 to 4.9 has been reached so as to obtain a cell density in the order of $3 \cdot 10^8$ to $5 \cdot 10^8$

cfu/g, and the culture is then frozen directly.

The inoculum can also be a fresh preculture comprising the genera, species or strains of bacteria used in the form of a mixture and/or a plurality of
5 fresh precultures of each genus, species or strain of bacterium used. In particular, this cultivated mixture of bacteria can comprise 50-90% of streptococci relative to the lactobacilli, for example.

The frozen starter is also an object of the
10 present invention. In fact, it will be found, surprisingly, that more than 95% of the cells which have survived freezing are capable of producing lactic acid.

The use of the frozen starter for making yogurt is also an object of the present invention. In fact,
15 such a starter ferments milk as rapidly as a traditional starter can do, while the latter contains at least 10 times more live cells. Thus, it is preferred to use the starter, which forms an object of the present invention, at a concentration of 0.01 to
20 0.1% in milk.

The process and the frozen starter according to the present invention are illustrated in greater detail in the examples which are given hereinbelow by way of illustration. The percentages are given by weight.
25 These examples are preceded by a brief description of the drawings.

Drawings

Figure 1: Preparation of a set yogurt. The pH
30 of two milks which are fermented with cocktails of different frozen starters is shown as a function of the fermentation time.

Figure 2: Preparation of a stirred yogurt. The pH of two milks which are fermented with cocktails of
35 different frozen starters is shown as a function of the fermentation time.

Example 1

A frozen starter of a commercial yogurt strain

of *Streptococcus thermophilus* is prepared as follows.

A skimmed milk is reconstituted using 10% of skimmed milk powder, 1% of yeast extract is added, and the mixture is sterilized to 115°C for 30 minutes and then cooled to a temperature of approximately 42°C.

A frozen traditional strain of *S. thermophilus* is reactivated by several successive precultures in MSK-medium which has been sterilized at 155°C for 30 minutes. The sterile reconstituted milk is subsequently inoculated at a ratio of 1% with the third preculture taken at the stage where the medium coagulates, and the mixture is incubated at a temperature of approximately 42°C until a pH of 5.1 is reached and then cooled to a temperature of 4°C. Finally, the batch is frozen at -75°C.

Example 2

A frozen starter of a commercial ropy yogurt strain of *Lactobacillus delbruckii subsp. bulgaricus* is prepared as follows.

The same skimmed and sterilized milk as described in Example 1, which comprises 1% of yeast extract, is used. A commercial strain of frozen *L. delbruckii subsp. bulgaricus* is reactivated by several successive precultures in sterile MSK medium. The sterile reconstituted milk is subsequently inoculated at a ratio of 5% with the third preculture taken at the stage where the medium coagulates and the mixture is incubated at a temperature of approximately 42°C until a pH of 4.8 is reached and subsequently cooled to a temperature of 4°C. Then, 5% of sterile glycerol are added, and the mixture is frozen at -75°C.

Example 3

A frozen mixed starter comprising a mixture of two commercial strains of *Streptococcus thermophilus* and a traditional ropy strain of *Lactobacillus delbruckii subsp. bulgaricus* is prepared as follows.

The same skimmed and sterilized milk as

described in Example 1, which also comprises 1% of yeast extract, is first prepared.

To this milk there is added 1% of a fresh preculture of each strain of *S. thermophilus* prepared
5 as described in Example 1 and 2% of a fresh preculture of the strain *L. delbruckii subsp. bulgaricus* as prepared as described in Example 2. The milk is subsequently incubated at a temperature of approximately 42°C until a pH of 4.7 is reached. The milk is
10 cooled to a temperature of 4°C. Then, 5% of sterile glycerol is added and the mixture is frozen at -75°C.

Example 4

Set-style yogurts are prepared by direct inoculation
15 with frozen starters as described in Examples 1 and 2.

A milk is prepared starting from full-cream milk with 3.7% of fat and 2.5% of skimmed milk powder. 40 l of this milk are sterilized at 92°C for 6 minutes, subsequently homogenized at 75°C and 150 bar (two
20 stages) and finally cooled to a temperature of approximately 42°C.

This milk is now fermented with a cocktail of three frozen starters comprising, respectively, a strain *S. thermophilus* CNCM I-1422, a strain
25 *S. thermophilus* CNCM I-1424 and a strain *L. delbruckii subsp. bulgaricus* CNCM I-1420. In each case, these strains are used conventionally in the industrial production of yogurt and have been deposited at the Collection Nationale de Cultures de Microorganismes
30 (CNCM), Institut Pasteur, 28 rue du Dr Roux, 75724 Paris Cedex 15, France on May 18th, 1994, in accordance with the Budapest Treaty. The starters comprising strains CNCM I-1422 and CNCM I-1424 were prepared as described in Example 1, and the starter comprising
35 strain CNCM I-1420 was prepared as described in Example 2.

To the milk there are now added 7 ml of the frozen starters comprising strains CNCM I-1422 and CNCM I-1424 and 6 ml of frozen starter comprising strain

CNCM I-1420, the milk is incubated at 42°C until a pH of approximately 4.65 is reached and subsequently cooled to 4°C.

Equally, pasteurized full-cream milk is fermented with another cocktail of three frozen starters, each comprising a strain *S. thermophilus* CNCM I-1421, a strain *S. thermophilus* CNCM I-1423 and a strain *L. delbruckii* subsp. *bulgaricus* CNCM I-800 respectively. These strains have in each case been conventionally used in the industrial production of yogurt and were deposited at the Collection Nationale de Cultures de Microorganismes (CNCM), Institut Pasteur, 28 rue du Dr Roux, 75724 Paris Cedex 15, France, on 18.05.94 in the case of strains CNCM I-1421 and I-1423 and on 4.10.88 in the case of strain CNCM I-800, in accordance with the Budapest Treaty. The starters comprising strains CNCM I-1421 and CNCM I-1423 were prepared as described in Example 1 and the starter comprising strain CNCM I-800 were prepared as described in Example 2.

To the milk there are now added 7 ml of the frozen starters comprising strains CNCM I-1421 and CNCM I-1423 and 6 ml of frozen starter comprising strain CNCM I-800, the milk is incubated at 42°C until a pH of approximately 4.65 is reached and subsequently cooled to 4°C.

The acidification curves are shown in Figure 1, and the table which follows illustrates the characteristics of the products obtained.

Starter cocktail (CNCM)	Acidification time to pH 4.65	pH of the product after 1 day (at 4°C)	pH of the product after 24 days (at 4°C)	Tasting on day 24
I-1420 I-1422 I-1424	6 h	4.34	4.1	Good taste A little acidic Short texture
I-800 I-1421 I-1423	5 h 40 min	4.35	4.2	Good taste Smooth texture

Example 5

Stirred yogurts are prepared by direct inoculation with frozen starters as described in Examples 1 and 2.

5 The milk is prepared from full-cream milk comprising 3.7% of fat and 2.5% of skimmed milk powder. 40 l of this milk are sterilized at 105°C for 2 minutes, subsequently homogenized at 75°C and 300 bar (first stage) and finally cooled to a temperature of approxi-
10 mately 43°C.

 This milk is then fermented with a cocktail of three frozen starters comprising strain CNCM I-1422, strain CNCM I-1424 and strain CNCM I-800 respectively. To this end, 7 ml of the frozen starters described in
15 Example 4 comprising strains CNCM I-1422 and CNCM I-1424 and 6 ml of frozen starter described in Example 4 comprising strain CNCM I-800 are added to the milk. This milk is subsequently incubated at 43°C until a pH of approximately 4.65 is reached and subsequently
20 cooled to 4°C while stirring.

 Equally, full-cream milk is fermented with another cocktail of three frozen starters comprising strain CNCM I-1421, strain CNCM I-1423 and strain CNCM I-1420 respectively. To this end, again, 7 ml of the
25 frozen starters described in Example 4 comprising strains CNCM I-1421 and CNCM I-1423 and 6 ml of the frozen starter described in Example 4 comprising strain CNCM I-1420 are added to the milk. This milk is subsequently incubated at 43°C until a pH of
30 approximately 4.65 is reached and then cooled to 4°C.

 The acidification curves are shown in Figure 2, and the table which follows illustrates the characteristics of the products obtained.

Starter cocktail (CNCM)	Acidification time to pH 4.65	pH of the product after 1 day (at 4°C)	pH of the product after 24 days (at 4°C)	Tasting on day 24
I-1422 I-1424 I-800	7 h 15 min	4.49	4.3	Very good taste Aromatic Smooth and creamy texture
I-1420 I-1421 I-1423	6 h 45 min	4.55	4.32	Good taste Aromatic Creamy texture

Example 6

5 The pasteurized milk of Example 4 is inoculated with 0.05% of the cocktail of frozen starters of Example 5 which comprises strains CNCM I-1422, I-1424 and I-800 in equal proportions, and this milk is then incubated at 42°C until the pH has reached 4.65.

10 For comparison purposes, the same milk is inoculated with 3% of a traditional fresh culture comprising the same strains of *S. thermophilus* and *L. delbrückii* subsp. *bulgaricus* in equal proportions, and the milk is then incubated at 42°C until a pH of 4.65 is reached.

15 For comparison purposes, the same milk is inoculated with 0.02% of a traditional concentrated frozen starter comprising the same strains *S. thermophilus* and *L. delbrückii* subsp. *bulgaricus* in equal proportions, and the milk is then incubated at 20 42°C until a pH of 4.65 is reached.

The table hereinbelow shows the results obtained for the three types of fermentation.

Culture characteristics	Fresh culture	Cocktail of frozen starters	Traditional cocktail of concentrated and frozen starters
Number of live cells in the starters (CFU/g)	$3 \cdot 10^8$	$2.5 \cdot 10^8$	10^{10}
Direct inoculation (%)	3	0.05	0.02
Number of live cells in the milk after inoculation (CFU/g)	$9 \cdot 10^8$	$1.3 \cdot 10^9$	$2 \cdot 10^9$
Latent period observed	10 min	1h	1h
Anticipated time for reaching pH 4.65 (approximately $5.3 \cdot 10^8$ CFU/g) for 100% cell activity	3h 7min	7h	5h
Real time to reach pH 4.65	3h 10 min	7h 5min	6h 55min
Level of cell activity (L)	(100%)	>95%	<10%

Assuming that the mean generation time of the cells is 30 minutes and that the level of activity of cells from fresh cultures is referred to as 100%, it is possible approximately to determine the relative level of fermentation activity of cells from frozen starters using the equation which follows.

$$L = 109 \times (5.310^8 / (1.02338)^{(\text{real time} - \text{latent period, in minutes})}) / (\text{CFU/g in the inoculation milk})$$

It is thus found that the two types of cocktail of frozen starters have an identical latent period, certainly due to adaptation of the cells to their new environment.

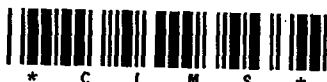
However, these two types of starter cocktail do not ferment the milk in the same manner. In fact, the cocktail of traditional frozen starters ferments the milk in 6 hours 55 minutes, while it should ferment it in 5 hours if a mean generation time of 30 minutes is used as reference. In contrast, the cocktail of starters according to the present invention ferments the milk virtually in the anticipated time.

It is thus found that over 95% of the lactic bacteria from the starter according to the present invention ferment the milk, while less than 10% of lactic bacteria from the traditional frozen starter ferment this milk.

This is why, as a function of the initial

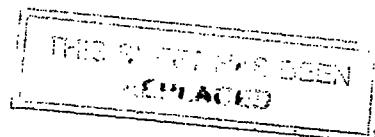
inoculation percentage, the starter which is an object of the present invention is capable of fermenting a milk until it has reached a pH which is traditional for yogurt, that is to say to pH 4.4 to 4.8, as rapidly as
5 this can be done by a traditional frozen starter (by direct inoculation). To this end, a milk is inoculated preferably with 2.5 times more starter according to the present invention compared with a traditional frozen starter.

10 Finally, for an inoculation percentage which equals that of the traditional frozen starter, the starter according to the present invention ferments the milk a little less rapidly than this can be done by a traditional frozen starter, as can be seen from the
15 table hereinabove. However, the loss in time is minimal, in the order of 20 minutes, and is largely compensated for by the gain in cost price of the starter of the present invention and the ease with which it can be produced.



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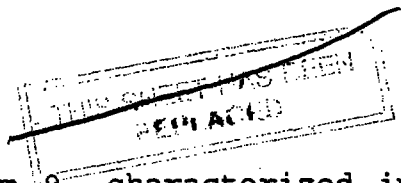


Patent claims

1. Process for the preparation of a frozen starter for the direct inoculation of milk in order to make it into yogurt, characterized in that lactic bacteria are grown in milk at a temperature of 40-45°C until a pH of 4.3-5.5 is reached and the culture is subsequently frozen directly.
2. Process according to Claim 1, characterized in that at least one strain of streptococci and/or at least one strain of lactobacilli is/are grown.
3. Process according to Claim 2, characterized in that the milk is inoculated with one or more fresh precultures comprising in each case one or more strains of lactic bacteria which can belong to a different genus or species.
4. Process according to Claim 2, characterized in that 3-10% of glycerol are added to a culture of lactobacilli before freezing it.
5. Process according to Claim 1, characterized in that the milk additionally comprises 1% of yeast extract.
6. Process according to Claim 1, characterized in that the culture is frozen at between -20 and -90°C.
7. Process according to one of Claims 1 to 6, characterized in that streptococci are grown at a temperature of 41-44°C until a pH of 4.9-5.2 is reached so that a cell density in the order of $2 \cdot 10^8$ to $5 \cdot 10^8$ CFU/g is reached and the culture is then frozen directly.
8. Process according to one of Claims 1 to 6, characterized in that lactobacilli are grown at a temperature of 41-44°C until a pH of 4.6-5 is reached so that a cell density in the order of 10^8 to $5 \cdot 10^8$ CFU/g is reached and the culture is then frozen directly.
9. Process according to one of Claims 1 to 6, characterized in that lactobacilli and streptococci are grown at a temperature of 41-44°C until a pH of 4.5-4.9 is reached so that a cell density in the order of $3 \cdot 10^8$ to $5 \cdot 10^8$ CFU/g is reached and the culture is then frozen directly.

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10. Process according to Claim 9, characterized in that the mixture of bacteria comprises 50-90% of streptococci relative to lactobacilli.
11. Frozen starter obtained by the process
5 according to one of Claims 1 to 10.
12. Use of the frozen starter according to Claim 11 for producing yogurt.
13. Use according to Claim 12 at the concentration of 0.01 to 0.1% in milk.