FORM 2

THE PATENTS ACT, 1970
(39 of 1970)

The Patent Rules, 2006

Complete Specification

(See section 10 and rule 13)

A PROCESS FOR THE PREPARATION OF CURD

COUNCIL OF SCIENTIFIC & INDUSTRIAL RESEARCH
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The following specification particularly describes the nature of this invention and the manner in which it is to be performed

FIELD OF THE INVENTION:

The present invention relates to a novel process for the preparation of a milk product, curd. Particularly, bacteria attached membranes is used for the preparation of curd. These membranes can also be used for addition of different supplements such as Histidine during curd production.

BACKGROUND THE INVENTION:

Milk and other dairy products were considered as important foods as early as 4000 BC. In the whole world it is one of the most important components of the human diet. Milk is considered to be the only foodstuff which contains almost all essential human nutrients. Especially it is an important source of protein, calcium, and the B-group vitamins (thiamin, riboflavin, niacin, vitamin B6, and folate), and provides vitamin A, vitamin C, magnesium, and zinc as well.

The production of acid milk products has long been known in all countries. The most ordinary way to get acid curdled milk is actually spontaneous acidification. This course relies on lactic acid bacteria, naturally present in milk as adventitious contaminants, which grow and produce the lactic acid required to coagulate the milk. Sometimes, the lactic acid bacteria are accompanied by yeasts or moulds, which give special features to the fermented product. By this way, several communities around the world established their own characteristic fermented milks. In Caucasian countries milk is fermented by a co-culture of lactic acid bacteria and yeasts, named the product kefir. In Siberia and south Russia, along with alcohol the fermentation of horse milk by yeasts and lactic acid bacteria results in koumiss. Similarly another alcoholic acid milk in America is mazun. Accordingly the important milk product Egypt and leben is yoghurt, in Italy gioddu and in Western Europe buttermilk are well known. Whereas in Scandina the well known fermented milks are viili and langfil.

Various materials viz. ceramic, polymers, etc. are used for the preparation of membranes. These membranes are common in use due to its simplicity in the preparation and cost effectiveness. There are many kinds of bacteria which have been recovered from different membranes. The inherent characteristic of these bacteria are to attach the membranes even when there are no transmembrane pressure differentials. The attachment between bacteria and

membrane occurs due to the accumulation of trace organic nutrients at the solid-liquid interface which is enough for bacteria to survive. The attachment does not involve any particular stereospecific macromolecular binding sites and usually, it is not substrate specific. Hence, numerous kinds of bacteria get attached to different polymeric membrane surfaces; it does not need any particular interaction. This is the reason bacteria get readily appended to the membrane. Generally, various bacterial and membrane characteristics influence this attachment phenomenon. Bacterial features that are responsible for adhesion to membrane include cell surface charge, cell wall hydrophobicity, cell surface structure, and the type of extracellular polymeric substances (EPS) produced by it. The bacterial adhesion to the membrane not only depends on its nature but also on the membrane. The characteristic property like surface hydrophobicity, surface charge, chemical composition, and roughness also influences the attachment.

Histidine is one of the standard amino acids in proteins, and which plays a critical role in plant growth and development. Human body cannot synthesize histidine. For that reason in human body it is coming as supplement. *Corynebacterium glutamicum* is a Gram-positive, aerobic, rod-shaped, and non-sporulating soil bacterium which is extensively used for industrial L-Histidine production.

Reference may be made to an article entitled "Interaction between dairy yeasts and lactic acid bacteria strains during milk fermentation" by P. Á. Martínet al. published in *Food Control,* 2008, 19, 62-70 reports that lactic acid bacteria species were mostly responsible for milk acidification whereas yeast strains produced most of the volatile maltic compounds. Balancing with these compounds different cheese varieties and other dairy products can be formed.

Reference may be made to an article entitled ''The Use of Lactic Acid Bacteria Starter Culture in the Production of Nunu, a Spontaneously Fermented Milk Product in Ghana'' by F. Akabanda et al. published in *International Journal of Food Science,* 2014, 721067-721078 reported that lactic acid bacteria isolated from Ghanaian traditional fermented milk product have desirable technological properties and successfully used as starter cultures for Nunu fermentation.

Reference may be made to an article entitled ''Lactic acid bacteria and yeasts involved in the fermentation of amabere amaruranu, a Kenyan fermented milk'' by B. Nyambane et al. published in *Food Sci Nutr.*, 2, 692–699 reports a Microorganisms involved inamabere amaruranu fermentation were found to consist of lactic acid bacteria from the three general Lactobacillus, Leuconostoc and Streptococcus.

Reference may be made to an article entitled "Enhanced bacterial affinity of PVDF membrane: its application as improved sea water sampling tool for environmental monitoring" by S. B. Kumar et al. published in *Environmental Science and Pollution Research*, 2017, 24, 5831–5840 reports for the preparation of PVDF membrane and check their check their affinity for the attachment of selected gram-positive (Bacillus subtilis) and gram-negative (Escherichia coli) bacteria.

Reference may be made to an article entitled "Biofouling potentials of microporous polysulfone membranes containing a sulfonated polyether-ethersulfone/polyethersulfone block copolymer: correlation of membrane surface properties with bacterial attachment" by T. Knoellet al. published in *Journal of Membrane Science*, 1999, 157, 117–138 reports that enhancement of Mycobacterium attachment to the modified polysulfone membranes compared to Flavobacterium attachment which is related to the surface hydrophobicity of the modified polysulfone membranes.

Reference may be made to an article entitled "The role of cell-surface interactions in bacterial initial adhesion and consequent biofilm formation on nanofiltration/reverse osmosis membranes" by O. Habimanaet al. published in *Journal of Membrane Science,* 2014, 454, 82-96 reports that the interactions between bacterial cells and NF/RO membranes are important for biofouling.

Reference may be made to US 20070184532, which reports a method for producing L-histidine using bacterium of Enterobacteriaceae family.

Reference may be made to US3849250, which reports a process for producing L-arginine by fermentation.

OBJECTIVE OF THE INVENTION:

- The main objective of the present invention is to provide a novel process for the preparation of curd.
- Another objective of the present invention is to prepare *Corynebacterium glutamicum* curd strips.
- Another object of the present invention is to provide bacteria attached PVDF membrane as a strip for the preparation of curd.
- Yet another objective of the present invention is to provide a process for the addition of different important supplements released by the bacteria during curd formation.
- Yet another objective of the present invention is to prepare curd like milk product containing histidine with the help of *Corynebacterium glutamicum* curd strips.

BRIEF DESCRIPTION OF THE DRAWINGS:

Fig. 1: Curd formation by the curd strip

Fig. 2: Bacterial attached membrane (Curd strip)

DETAILED DESCRIPTION OF THE INVENTION:

The present invention relates to a process for the preparation of curd. The present invention also relates to prepare *Corynebacterium glutamicum* curd strips made of PVDF membrane for the preparation of sweet, fermented milk product containing histidine. The design of the present invention retains the curd forming bacteria on the membrane. The membrane can then be directly dipped into the processed milk for the preparation of curd. The simple method of using strips for the curd preparation makes it advantageous as they are ready-to-use anytime.

In another embodiment, the bacteria attached to the membrane can be of varied type depending on the type of curd or any milk product.

In another embodiment, the strip prepared can be used for the production of any fermented milk product like yogurt and kefir etc.

In yet another embodiment, the thickness of the membrane is between 130-135 μm (30-35 μm membrane thickness and 100 μm support thickness).

In yet another embodiment, the membrane selected for the bacterial detection is polyvinylidene fluoride (PVDF) (20 % w/w) (573 KDa, Solef, Solvay, France).

In yet another embodiment, *Corynebacterium glutamicum* curd strip was prepared to add extra nutrient into the curd i.e. histidine.

In yet another embodiment, the invention provides a process for the preparation of curd comprising the following steps:

a. providing cooled boiled milk;
b. providing *corynebacterium glutamicum*;
c. growing *corynebacterium glutamicum* in nutrient broth media for 20-30h at temperature 25-40°C followed by centrifugation with 3000-5000 rpm;
d. washing the cells obtained in step (c) by centrifugation with 3000-5000 rpm;
e. resuspending the cells obtained in step (d) in 10% skimmed milk;
f. dipping PVDF membranes into bacterial suspension obtained in step (e) for attaching the bacteria onto the membrane;
g. lyophilizing the bacterial attached membranes obtained in step (f) for 6-12 h to get *corynebacterium* curd strips.
h. dipping the strip obtained in step (g) into the milk obtained in step (a);

i. incubating the liquid obtained in step (h) for 10-20 hours at a temperature ranging between 20-40° C to obtain curd.

In yet another embodiment, the invention provides a membrane which is made up of 20% w/w PVDF polymer concentration prepared in DMF as a solvent and taking water as a non-solvent medium.

In yet another embodiment, the invention provides a PVDF polymer whose concentration is in the range of 6-24% w/w.

In yet another embodiment, the invention provides a membrane wherein, the thickness of membrane is in the range of 30 to 50 µm.

In yet another embodiment, the invention provides a strip containing lyophilised form of *corynebacterium* prepared by the method steps 1 a-g.

In yet another embodiment, the invention provides a kit for curd preparation consisting of a strip.

In yet another embodiment, the invention provides a probiotic curd containing *corynebacterium* sp. prepared by using the method.

EXAMPLES

The following examples are given by way of illustration therefore should not be construed to limit the scope of the Invention.

Example 1

Membrane preparation

Membranes were prepared using 20% w/w PVDF as a base membrane. All the membranes were prepared by phase inversion technique. Membrane preparation involves two phases: (a) Preparation of solution and (b) Membrane Casting. Both test as well as control membrane

polymers were dissolved in solvent N, N- dimethyl formamide (DMF), keeping temperature of about 50-60℃ with constant stirring for overnight at around 100-120 rpm. The solution was kept overnight for the removal of air bubbles.

The polymer solution was then poured onto the polyester fabric which was attached to the glass plate. Then the solution was uniformly spread over the fabric using a casting blade. After spreading of the polymer solution the whole system was immediately transfer to the non-solvent bath containing reverse osmosis (RO) water. The membrane prepared was then removed from the water and allowed to air dry. Subsequently, the dried membrane was cut into 2 × 2 cm^2 pieces and autoclaved at 121° C for 15 min.

Example 2

Preparation ôf curd strips

Curd was collected from the local market for the experiment. The curd was then centrifuged at 1000 rpm. 1mL of its supernatant was inoculated into Nutrient broth and kept it (on shaking) for overnight for the growth of bacteria present in the curd. The curd bacteria grown in the curd was washed with PBS (2-3 times) to remove all the traces of growth media. The washed bacteria were resuspended in 10% skimmed milk (cryoprotectant) and then the sterile 20% PVDF membrane was added to the prepared bacterial suspension. The bacteria were allowed to attach to the membrane for 24 h on shaking (100 rpm) at 37°C. The bacterial attached membrane was finally lyophilized to make the curd strips.

For *Corynebacterium*, the bacterium (ATCC 21607) was grown in same nutrient medium for 28 h. The washed cells were again resuspended in 10% skimmed milk. The cut and autoclaved membranes were then kept into the prepared bacterial suspension for attachment

as mentioned above. The membranes with attached bacteria were then lyophilized for the preparation of *Corynebacterium* curd strips.

Example 3

Curd preparation with the help of prepared curd strips

For curd the preparation, processed milk (Amul gold) was purchased from the local market. The milk was then boiled and allowed to cool.

Three strips of curd bacteria and *Corynebacterium* were added into 30 mL of milk separately and incubated at room temperature. Curd formation was observed after 16 h for curd bacteria strip and 20 h for *Corynebacterium* strip.

Advantages of the invention:

1. A simple technique for the preparation of curd.
2. Various types of bacteria (depending on the requirement) can be attached on any polymeric membrane which will also release required supplement during curd formation.
3. A simple technique to prepare curd in consistent manner every time
4. A cost effective curd strip which is simple to prepare and use.
5. No requirement of keeping/storing the curd inocula every time.
6. Spillage proof, portable curd strips.
7. Ready-to-use.

We Claim:

1. A process for the preparation of curd comprising the following steps:
    a. providing cooled boiled milk;
    b. providing *corynebacterium glutamicum*;
    c. growing *corynebacterium glutamicum* in nutrient broth media for 20-30h at temperature 25-40°C followed by centrifugation with 3000-5000 rpm;
    d. washing the cells obtained in step (c) by centrifugation with 3000-5000 rpm;
    e. resuspending the cells obtained in step (d) in 10% skimmed milk;
    f. dipping PVDF membranes into bacterial suspension obtained in step (e) for attaching the bacteria onto the membrane;
    g. lyophilizing the bacterial attached membranes obtained in step (f) for 6-12 h to get *corynebacterium* curd strips.
    h. dipping the strip obtained in step (g) into the milk obtained in step (a);
    i. incubating the liquid obtained in step (h) for 10-20 hours at a temperature ranging between 20-40° C to obtain curd.
2. The process as claimed in claim 1 (f) wherein the membrane is made up of 20% w/w PVDF polymer concentration prepared in DMF as a solvent and taking water as a non-solvent medium.
3. The process as claimed in claim 1 (f) wherein the PVDF polymer concentration is in the range of 6-24% w/w.
4. The process as claimed in claim 1 (f) wherein, the thickness of membrane is in the range of 30 to 50 μm.

5. A strip containing lyophilised form of *corynebacterium* prepared by the method steps 1 a-g as claimed in claim 1.
6. A kit for curd preparation consisting of a strip as claimed in claim 5.
7. A probiotic curd containing *corynebacterium* sp. prepared by using the method as claimed in claim 1.
8. A probiotic curd as claimed in claim 7 useful in Histidine deficiency.

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ABSTRACT

A PROCESS FOR THE PREPARATION OF CURD

The present invention relates to a novel process for the preparation of milk product curd using *corynebacterium glutamicum* and method of preparation of bacterial strip containing *corynebacterium glutamicum*. Bacteria attached membranes is used for the preparation of curd. Bacterial strip containing *corynebacterium glutamicum* can also be used for addition of Histidine supplement during curd production.

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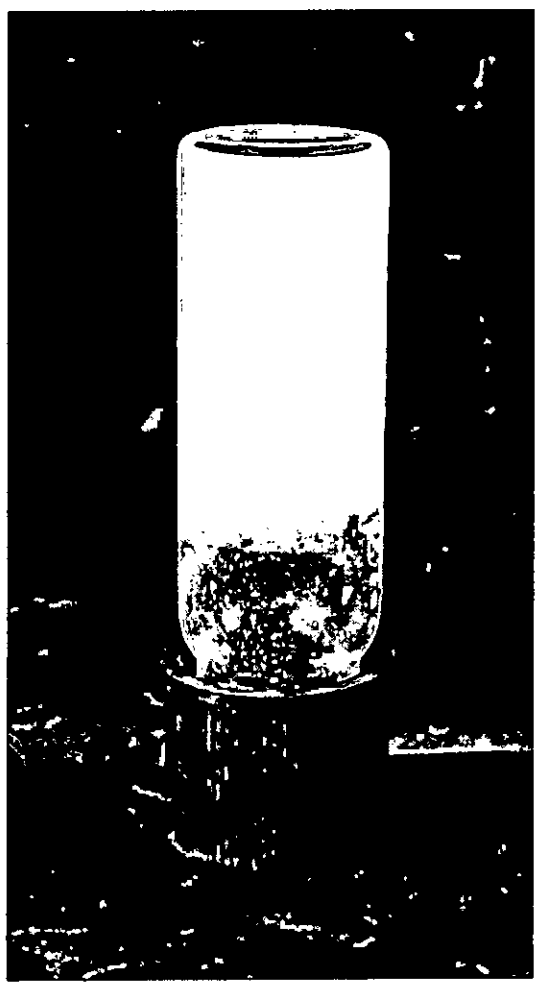

Fig. 1: Curd formation by the curd strip

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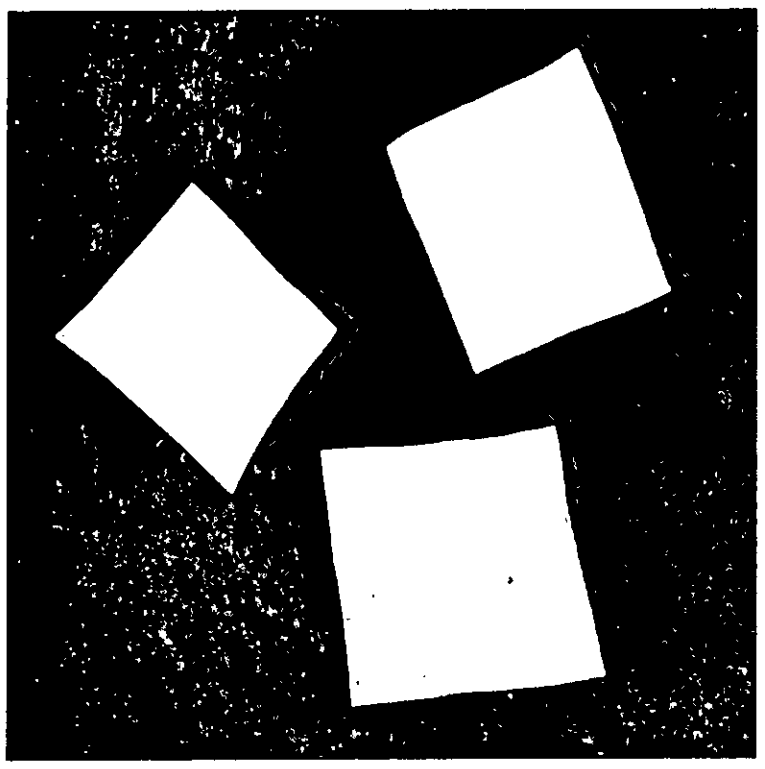

Fig. 2: Bacterial attached membrane (Curd strip)

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