

METHOD FOR PRODUCING CASEIN AND USES THEREOF

The invention pertains to the food industry and relates to new methods for producing casein compositions and uses thereof, in particular for producing dairy substitute (notably vegan cheese), while limiting environmental impact.

Background

The use of milk as a protein, sugar and lipid-rich nutrient, has become almost universal in traditional societies. In addition, milk transformation into various derivatives to obtain dairy products, is one of the most ancient examples of human agro-industries. Today, the cheese market represents 20 million tons of product per year, for a value of about \$140bn.

However, dairy products, are associated with several issues or concerns, in terms of health, as well as environmental and ethical considerations. These concerns have highlighted the need for dairy product substitutes alleviating these various issues. Health issues include lactose intolerances, allergies, and others, such as a high content in saturated fatty acids, known to have a potential negative impact on health.

In addition, environmental and ethical concerns related to food products of animal origin have raised over the last decades. The burden of animal breeding for a 7 billion population (11 billion in 2050) has become increasingly high. The environmental consequences are severe. They are today mostly considered in terms of anthropogenic greenhouse gas (GHG) emissions, water consumption, pollution by effluents, and land occupation.

Cattle breeding is today considered as one of the first sources GHG emissions, with an estimated 7.1 Gigatonnes of CO2-equivalent per year, representing 14,5% of all anthropogenic greenhouse gas (GHG) emissions.

A very strong water consumption is also associated with animal farming. In addition, farming effluents also have a strong environmental impact. Although the impact of crop fertilizers on groundwater should not be neglected, the impact of animal farming effluents is often massive and has proven to be disastrous in many parts of the world. Furthermore, livestock takes up to nearly 80% of global agricultural land, yet produces less than 20% of the world's supply of calories, showing the very high level of stress brought by animal farming on land resources.

Finally, animal welfare has become an increasingly important concern. The scale-up of meat and dairy production and processing has turned it into an intensive industrial process, which is increasingly perceived as ethically not acceptable.

Therefore, there is a strong need for dairy products substitutes alleviating the issues listed above.

Plant-based substitutes are a potential alternative to conventional dairy products. However, these products, which are often derived from starch, soy, almond, or coconut milk, can be far from mimicking the taste of dairy products. In addition, they are anyway fundamentally different in terms of composition.

Therefore, there is today a need for dairy substitutes, and in particular, cheese substitutes that are

- (i) free of undesired compounds
- (ii) similar to the original product in aspect, texture and taste
- (iii) equivalent or superior to the original products in terms of nutrition

Cows' milk typical composition is described in Table 1 and more detailed compositions of cow's and other animals milk, including the listing of the different lipids, proteins, salts, vitamins and other nutrients can be found from a large number of sources (https://en.wikipedia.org/wiki/Milk#Cow's_milk; Haug et al (2007) Bovine milk in human nutrition – a review, Lipids Health Dis.; Volume 6, pages 25; Dominguez-Salasa et al (2019) Contributions of Milk Production to Food and Nutrition Security; Encyclopedia of Food Security and Sustainability, Volume 3, pages 278-291).

Type of molecule	mass in 1 liter in grams
Water	902
Lactose	49
Fat	39
Caseins	28
Other proteins	5
Salts and vitamins	9
Total	1032

Table 1: Cow's milk composition

Lipids and carbohydrates (but lactose) can be recovered from plant, calcium from inorganic sources, from animal sources or from plants (such as sea weeds). In the case of proteins, other sources need be considered as animal proteins differ in amino-acid content from plant proteins. Milk's proteins can also be produced by fermentation, another source of ingredients of a non-animal origin.

Furthermore, proteins isolated from milk are also individually used as nutritional supplements and other applications. Therefore, milk proteins made by fermentation, independently of other milk components can also be used for other applications than the making of dairy substitutes.

Production by fermentation is based on the growth of microorganisms (bacteria or fungi) producing a compound of interest in a fermenter, usually followed by the recovery and purification of the compound of interest. Such microorganisms are generally transgenic, *i.e.* transformed with a genetic construct for expression of the desired protein(s) or compound obtained by metabolism of the microorganisms. The making of proteins by fermentation is a process that has widely been used in the food industry. Several studies have described the production of milk's constitutive proteins or homologues by fermentation in various microorganisms (see below) and the assembly of ingredients including fermentation proteins to make dairy substitutes has been described several times (US6270827; US5,942,274; WO2018039632; WO2020223700; WO2020081789, WO2020219596, WO2022098835).

Production by fermentation is usually conducted with a sugar feedstock, to support the growth of the microorganism and the production of the desired compound. Depending on the capacity of the microbe to metabolize the different sugars, one can use glucose or sucrose or other sugars such as lactose.

For example *Escherichia coli* is known to consume glucose very efficiently, but can also consume a series of other sugars, including glucose, maltose, lactose, trehalose, fructose, xylose and arabinose with a hierarchy of preference.

Many *Escherichia coli* strains, including BL21, cannot consume sucrose. However, other strains such as *Escherichia coli* W (ATCC 9637) can efficiently use it as sugar source (Sabri et al. (2013). Appl Environ Microbiol. 79:478-87). Two gene clusters can be responsible of sucrose consumption in *Escherichia coli*. The *scr* regulon codes for a sucrose phosphotransferase system (Schmid et al. (1988). Mol. Microbiol. 2:1-8) whereas the *csc* regulon encodes a sucrose utilization

system that does not depend on phosphotransferase (Jahreis et al. (2002). J. Bacteriol. 184:5307–5316). An *Escherichia coli* strain that does not consume sucrose, such as BL21 for example, can be transformed into a strain consuming sucrose for example by introduction of genes from the csc regulon (Bruschi et al. 2012. Biotechnol Adv. 20 ;30(5):1001-10).

Microorganism growth and metabolism requires not only sugar feedstock, but also nitrogen input. For this, various compounds can be added, including liquid or gaseous ammonia, urea, amino acids, peptones, and yeast extract.

Alternative feedstocks derived from dairy, such as lactoserum (whey) have also been used in fermentation with various microorganisms comprising *Escherichia coli* (González-Siso (1996) The biotechnological utilization of cheese whey: A review. Bioresource technology vol. 57, no 1, p. 1-11; Viitanen et al. (2003), Cheese whey-induced high-cell-density production of recombinant proteins in *Escherichia coli*. Microbial Cell Factories volume 2:2; Guimaraes et al. (2010) Fermentation of lactose to bio-ethanol by yeasts as part of integrated solutions for the valorisation of cheese whey. Biotechnology Advances 28: 375-384; Christensen et al. (2011) Production of bioethanol from organic whey using *Kluyveromyces marxianus*. J Ind Microbiol Biotechnol volume 38, pages 283–289; Pasotti et al. (2017) Cheese whey-induced high-cell-density production of recombinant proteins in *Escherichia coli* BMC Biotechnol. 17:48; Amaro et al. (2019) Prospects for the Use of Whey for Polyhydroxyalkanoate (PHA) Production. Front. Microbiol. Volume 10: 992-; Hausjell et al. (2019) Valorisation of cheese whey as substrate and inducer for recombinant protein production in *E. coli* HMS174(DE3). Bioresource Technology Reports volume 8:100340; Louasté and Elourtassi (2020) Succinic acid production from whey and lactose by *Actinobacillus succinogenes* 130Z in batch fermentation. Biotechnology Reports volume 27:e00481; Carranza-Saavedra et al. (2021), Kinetic analysis and modeling of L-valine production in fermentation batch from *E. coli* using glucose, lactose and whey as carbon sources. Biotechnology Reports volume 31, e00642; Chaparro et al. (2021), Whey as an Alternative Nutrient Medium for Growth of *Sporosarcina pasteurii* and Its Effect on CaCO₃ Polymorphism and Fly Ash Bioconsolidation. Materials volume 14: 2470; Mobayed et al. (2021) Effect of by-products from the dairy industry as alternative inducers of recombinant β -galactosidase expression. Biotechnol. Letter 43(3):589-599; Zou and Chang (2022) Past, Present, and Future Perspectives on Whey as a Promising Feedstock for Bioethanol Production by Yeast. Journal of Fungi, volume 8, pages

395-; de Diviitis et al. (2023) Cheese-whey permeate improves the fitness. *Biotechnology for Biofuels and Bioproducts*, volume 16:30).

Lactosermum is a byproduct or waste of the cheese-making process, but also any other process, such as Greek yoghurt production, wherein some whey is drained out of the product after curdling. Whereas cheese production can result in 10L of lactosermum for 1 kg of cheese (Mollea et al. (2013) Chapter 24: Valorisation of cheese whey, a by-product from the dairy industry. Pages 549–588 in *Food Industry*. I. Mazzalupo, ed. InTech Open, London, UK), production of 1 kg of Greek yoghurt can result in about 2 to 3 kg of acid whey (Erickson, B. E. (2017) *Chem. Eng. News*, 95(6), 26-30).

The valorization of lactosermum, and notably acid lactosermum is an important issue in the dairy industry (Gonzales-Siso (1996) The biotechnological utilization of cheese whey: a review. *Bioresour Technol.* 57:1–11; Marwaha and Kennedy (2007) Whey-pollution problem and potential utilization. *Int J Food Sci Technol.*;23:323–36; Rocha-Mendoza et al (2021) Acid whey trends and health benefits. *J. Dairy Sci.* volume 104, pages 1262–1275). Some companies may be able to valorize lactosermum (in particular soft whey, but also acid whey, sometimes), but often acid lactosermum is discarded as a waste.

Lactosermum contains proteins (the whey proteins, about 20% of total milk's proteins), and has a complex composition including minerals and lactose. Whey is characterized by its green-yellowish color, resulting from the presence of riboflavin (vitamin B12; González-Siso (1996) The biotechnological utilization of cheese whey: A review. *Bioresource technology* vol. 57, no 1, p. 1-11;). In fact, whey (lactosermum) contains a variety of minerals, including calcium, phosphorus, magnesium, sodium, and potassium, and is also rich in water-soluble vitamins such as B1 (thiamine), B2 (riboflavin), B6 (pyridoxine), B12 (cobalamin), and C (ascorbic acid). There are two main types of lactosermum: sweet lactosermum and acid lactosermum.

Sweet lactosermum is a byproduct associated with most cheese production, after enzymatic curdling using natural or synthetic rennet. It has a pH around 6 to 7 (González-Siso (1996) The biotechnological utilization of cheese whey: A review. *Bioresource technology* vol. 57, no 1, p. 1-11), and has a higher protein content and lower salts than acidwhey

Acid lactoserum is associated with acid curdling of milk. Acidic curdling can result from the activity of lactic bacteria, lowering the pH by the production of organic acids, or from the addition of organic (lactic, citric, acetic) or mineral (sulfuric, chlorhydric) acids. Acid whey is a natural by product of cottage cheese and Greek yoghurt two products production has increased significantly. Its valorization is difficult for several inherent reasons (Rocha-Mendoza et al (2021) Acid whey trends and health benefits. J. Dairy Sci. volume 104, pages 1262–1275), and its use in fermentation represents a solution to address this issue.

Lactoserum can vary in composition, depending on the production process. For example, in acid lactoserum, lactose concentration can vary from about 2 to about 5% (weight/volume) (Rocha-Mendoza et al (2021) Acid whey trends and health benefits J. Dairy Sci. volume 104, pages 1262–1275; O'Donoghue and Murphy (2023) Comprehensive Reviews in Food Science and Food Safety, 22:2652-2677).

Various whey derivatives may be obtained, in order to valorize whey proteins and lactose.

Lactoserum permeate is obtained after lactoserum proteins are removed using ultrafiltration (O'Donoghue and Murphy (2023) *op.cit.*). Lactoserum permeate can be used as a source of dairy solids in various food, beverage, or feed applications.

In delactosed lactoserum permeate, a large part of lactose is removed, although lactose can still represent 60% of dry weight, and thus remain a significant part of the remaining composition.

Lactoserum can also be concentrated, in order to facilitate transportation.

We will refer indifferently to such products as lactoserum or whey, whether they are raw (or whole) lactoserum, permeates, or delactosed permeates, as long as they still contain at least about 2% of lactose (w/w, wet weight), preferably at least 3%, preferably at least 4%, preferably at least 5%, preferably at least 8%. In some embodiments, and when concentrated lactoserum is used, it may contain at least 30% lactose (w/w wet weight), or at least 32% lactose or 35% lactose or more.

Using this type of feedstock does not qualify for the making of animal-free products, given the origin of whey. Nevertheless, the use of a waste as feedstock can be very helpful to reach good economics and advantageous life cycle analysis.

During fed-batch fermentation, pH can be adapted to keep within a certain range, and for this addition of acidic or basic compounds can be used, including hydrochloric acid, sulfuric acid, lactic acid, and phosphoric acid (acidic compounds)

or liquid or gaseous ammonia, sodium hydroxyde, or potassium acid (basic compounds).

All these solutions are not exclusive and can be combined.

5 The inventors have proposed to use lactoserum as a source of carbon for the culture of microorganisms expressing caseins, in particular during fed-batch culture. When such product is used, the inventors have shown that the environmental impact is decreased as compared to the impact observed when using glucose, sucrose or sucrose molasses as the feedstock for microorganisms
10 growth (with Isopropyl β -D-1-thiogalactopyranoside (IPTG) used to induce casein production if appropriate). As indicated above, in view of the composition of lactoserum, using such product also provides vitamins and minerals to the culture medium, which is of interest for maintaining long-term cell (notably bacterial) cultures.

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It is reminded that a fed-batch process is a method for the cultivation of microorganisms, such as bacteria, yeast, or cells, in a bioreactor to produce substances of interests. This process offers greater control over the culture environment and can lead to higher yields and product concentrations compared
20 to batch cultures.

The process begins with the inoculation of a small quantity of microorganisms (the inoculum) into the bioreactor containing a growth medium. This medium contains essential nutrients (such as sugars, nitrogen sources, minerals, and vitamins) for the microorganisms to grow and produce the desired product.

25 The bioreactor initially operates in a batch mode, with all the necessary nutrients are added at the beginning of the cultivation. During this phase, the microorganisms grow, consume nutrients, and may also produce the target product. The culture medium doesn't contain an excess of nutrients, to avoid overgrowth, production of deleterious by-products by the microorganisms, and to ensure better control.

30 As the culture progresses, the concentration of nutrients in the bioreactor decreases, upon consumption by the microorganisms. Additional nutrients are gradually introduced into the bioreactor during the fed-batch phase. The rate and amount of nutrient addition as well as various parameters such as temperature, pH, dissolved oxygen, agitation speed, and nutrient feed rates are closely monitored,
35 controlled, and adjusted as needed to optimize cell growth and product formation

based on factors like cell density, nutrient levels, and product formation, to maintain optimal growth conditions and extend the production phase. In continuous fed-batch processes, nutrients are continuously provided. In other fed-batch processes, nutrients are provided from time to time.

- 5 Once the desired product concentration is achieved or after a given period of time, the culture is harvested. Harvesting is performed before decrease of the amount of microorganisms, as death of such may lead to formation of undesired products. The product can be purified and further processed.

10 The advantages of a fed-batch process include: controlled growth of the microorganisms and product formation by adjusting nutrient feed rates, higher yields by providing nutrients in a controlled manner, extended production phase, reduced byproduct formation.

15 In one embodiment, lactoserum is present in the culture medium at the start of the culture (inoculation phase), alone or preferably with another sugar, notably glucose, saccharose, fructose, in particular in the form of molasse.

20 In another embodiment, lactoserum is not present in the culture medium at the start of the culture (inoculation phase). In this embodiment, another sugar is present in the culture medium, notably glucose, saccharose, fructose, in particular in the form of molasse.

 In one embodiment, lactoserum is introduced as the carbon source during at least part of the fed-batch period. In one embodiment, no other sugar is provided with lactoserum. In another embodiment, lactoserum is introduced with another sugar, as indicated above.

- 25 In one embodiment, lactoserum is present in the starter culture medium together with a sugar other than lactose (notably glucose), and the fed-batch process comprises provision of additional nutrients, wherein no sugar is provided with the additional nutrients for a least a period of time (ranging from 15 minutes to 1 hour, preferably from 20 minutes to 45 minutes, preferably around 30 minutes), and a
30 sugar another than lactose (notably glucose) is further provided again with the additional nutrients, after this period of time. In one embodiment, lactoserum is not further provided with the sugar another than lactose (notably glucose). In another embodiment, lactoserum is further provided with the sugar another than lactose (notably glucose).

In one embodiment, lactoserum is not present in the starter culture medium, which contains only a sugar other than lactose (notably glucose). In this embodiment, the fed-batch process comprises provision of additional nutrients, wherein lactoserum is the only sugar is provided with the additional nutrients for a
5 least a period of time (ranging from 15 minutes to 1 hour, preferably from 20 minutes to 45 minutes, preferably around 30 minutes). In this embodiment, a sugar other than lactose (notably glucose) may be further provided again with the additional nutrients, after this period of time. In one embodiment, lactoserum is not further provided with the sugar other than lactose (notably glucose). In
10 another embodiment, lactoserum is further provided with the sugar other than lactose (notably glucose).

One goal is to use the lactose present in the lactoserum as the sole sugar to be used by the microorganisms for some time during cell culture. When expression of the casein is induced by lactose, this also ensures the switch to casein production
15 by the microorganisms. When the production of the caseins has started, it is possible to reintroduce glucose or another sugar (even lactose as a sugar).

In view of the concentration of lactose in the lactoserum that is not concentrated, one favorable embodiment is to use a medium containing lactoserum (up to 60%, preferably up to 55%, in particular around 45% to 55%, notably around 50% v/v)
20 as the starter medium, and also containing a sugar other than lactose, that induces CCR (see below), such as glucose. The microorganisms shall then use and consume the other sugar. At a time specified by one of skill in the art (generally when the microorganisms are in the second half of the exponential growth phase), no further sugar other than lactose is provided, so that the microorganisms will
25 consume the lactose present in the medium from the start of the culture (which was not consumed as the other sugar was used by the microorganisms as the source of carbon). Lactose catabolism thus starts and CCR is lifted (thus allowing consumption of the lactose present in the medium and induction of casein production when it is lactose inducible). Lactose is then consumed, and further
30 carbon provision can be obtained by feeding with any sugar (glucose, lactose, fructose, saccharose), without further provision of lactoserum.

In some embodiments, lactoserum is used as the only source of sugar (lactose present in the lactoserum).

In other embodiments, lactoserum is used with other feedstocks, such as glucose (which can be obtained from corn or wheat), saccharose (available from sugar beets or sugar cane). Such other feedstocks may be in the form of pure sugar, molasses or others forms.

5 It is preferred to use lactoserum together with other feed stocks. Indeed, due to the fact that concentration of lactose in lactoserum is generally low, using such product as the sole feedstock would require addition of a large volume of lactoserum to obtain the adequate lactose amount.

10 In addition, since lactoserum contains lactose, it can trigger expression of the transgenes expressing the caseins, when a lactose-inducible promoter is used (Viitanen et al. (2003), Cheese whey-induced high-cell-density production of recombinant proteins in *Escherichia coli*. *Microbial Cell Factories* volume 2:2; Hausjell et al. (2019) Valorisation of cheese whey as substrate and inducer for recombinant protein production in *E. coli* HMS174(DE3). *Bioresource Technology Reports* volume 8:100340; Mobayed et al. (2021) Effect of by-products from the dairy industry as alternative inducers of recombinant β -galactosidase expression. *Biotechnol. Letter* 43(3):589-599; de Diviitis et al. (2023) Cheese-whey permeate improves the fitness. *Biotechnology for Biofuels and Bioproducts*, volume 16:30;

20 In one embodiment, it is proposed to use have both glucose and lactoserum in the culture medium early in the growth of the microorganisms (up to the end of the exponential growth or the beginning of the stationary growth), whereas glucose will be preferably used, due to the carbon catabolic repression (CCR), and to switch to lactose catabolism, once the appropriate growth of the microorganisms have been achieved and when expression of the recombinant casein is desired (Görke and Stülke *Nat Rev Microbiol* 6, 613–624 (2008); Postma et al *Microbiol Rev.* 1993 Sep;57(3):543-94). It may be possible to feed the culture with a low level of glucose, which will maintain appropriate carbon supply to maintain vigor of the microorganism growth without any switch back to only glucose use, and hence

30 continuing protein expression. Although other sugars could be used (such as sucrose or galactose), use of glucose is preferred, at least at the start of the culture (exponential growth), to induce CCR, in particular when lactose inducible promoters are used to trigger expression of the casein(s).

Lactose is a disaccharide composed of glucose and galactose. Its

35 metabolization generally depends on its hydrolysis in glucose and galactose. Some

Escherichia coli strains such as BL231(DE3) cannot consume the galactose moiety, because of a mutation that results in the lack of enzymes involved in galactose metabolization by the Leloir pathway. Consequently, in order to fully use lactose as a provider of carbon for the bacteria, with maximal yield, it is possible and preferred to use strains with a proficient Leloir pathway (Hausjell et al. (2019) Valorisation of cheese whey as substrate and inducer for recombinant protein production in *E. coli* HMS174(DE3). Bioresource Technology Reports volume 8:100340), or to restore this pathway in mutated strains by genetic engineering. Such strains able to metabolize D-galactose and form glucose are generally designed as *gal*⁺ strains.

Furthermore, the inventors used adapted purification procedures, to produce the caseins at a grade compatible with use in the food industry, with a high level of purity, but are also easily scalable at industrial level, and at costs compatible with exploitation, in particular energy costs.

In particular, recombinant caseins can be isolated from the biomass and from the culture broth, by heating the composition, as disclosed in WO2022253816.

Using the methods herein disclosed herein makes it possible to obtain casein compositions with an environmental impact that is reduced when compared to the impact observed when glucose, sucrose or sucrose molasses is used as the carbon source for culturing the microorganisms (i.e. when lactoserum, and especially acid lactoserum or acid lactoserum permeate is not used in the process), in particular as compared to glucose. In addition, it makes it possible to provide to the dairy industry a way to recycle wastes, which currently must be treated and disposed of. When microorganisms are used with lactose-induced expression of caseins, IPTG is used for inducing expression of the proteins, at the time casein expression would be induced by the lactose of the lactoserum.

Environmental impact is multifactorial and must be considered in terms of climate change, pollution of freshwater, marine water, soils and air, impact on ecosystems, use of fossil resources, depletion of material resources, water use and others. Criteria can include

- (i) Climate change, measured in kgCO₂eq (eq for equivalent)
- (ii) Acidification, in mol H⁺-eq

- (iii) Ecotoxicity, in comparative toxic unit for ecosystems (CTUe)
- (iv) Use of non-renewable energy resources (in MJ or KWh)
- (v) Eutrophication of freshwater (in kg P-eq) (kg phosphorus equivalent)
- (vi) Eutrophication of marine water (in kg N-eq) (kg of Nitrogen equivalent)
- 5 (vii) Eutrophication of terrestrial land (accumulated excedence in mol N-eq)
- (viii) Human toxicity, carcinogenic (in comparative toxic unit for human, CTUh)
- (ix) Human toxicity, non-carcinogenic (in comparative toxic unit for human, CTUh)
- (x) Ionizing radiation, impact on human health (human exposure efficiency relative to U_{235} kNq U_{235} -eq)
- 10 (xi) Land use soil quality index (ss)
- (xii) Use of material resources (kg Sb-Eq)
- (xiii) Ozone depletion potential (in kg CFC11-eq)
- (xiv) Particulate matter formation (disease incidence)
- 15 (xv) Photochemical oxidant formation (kg NMVOC-eq)
- (xvi) Water use (m^3 world eq. deprived)

The method is of interest to provide improvements for at least one of these criteria, in particular climate change, acidification, eutrophication (v, vi and/or vii) and/or water use. Notably, improvement of the impact on climate change and at least one other criterion can be achieved.

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The invention thus also relates to a method for reducing environmental impact of a casein composition prepared by fermentation of transgenic microorganisms, comprising

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- a. providing microorganisms that have been transformed with at least one nucleic acid coding for a casein,
- b. culturing said microorganisms, using a medium comprising lactoserum so as to express and produce casein,
- 30 c. thereby providing a microorganism composition wherein the pH of the composition is equal or above 6.5 and preferably below 9
- d. heating the microorganism composition so as to reduce the amount of the other proteins in soluble fraction of the composition, wherein heating is performed at a temperature at a temperature equal or above 75°C,
- 35 e. recovering the soluble fraction from the heated cell composition of iv),

thereby obtaining a casein composition in the soluble fraction, wherein the environmental impact is reduced as compared to the environmental impact of a casein composition obtained by the same steps, except that glucose or sucrose or molasses are used in place of lactoserum in b.

- 5 In particular, the reduction in environmental impact is observed for at least one criteria selected from (i) Climate change, (ii) acidification, (iii) Ecotoxicity, (iv) Use of non-renewable energy resources, (v) Eutrophication of freshwater, (vi) Eutrophication of marine water, (vii) Eutrophication of terrestrial land, (viii) Human toxicity, carcinogenic, (ix) Human toxicity, non-carcinogenic, (x) Ionizing radiation, 10 impact on human health, (xi) Land use, (xii) Use of material resources, (xiii) Ozone depletion potential, (xiv) Particulate matter formation, (xv) Photochemical oxidant formation, and (xvi) Water use, preferably (i) climate change.

- The casein composition can also be processed to obtain dairy substitutes, such 15 as cheese, yoghurt, that result from curdling, but also ice cream, reconstituted milk and the like, the environmental impacts of such dairy substitutes being also reduced as compared to the environmental impacts observed when using recombinant caseins from microorganisms grown in presence of glucose, sucrose or sucrose molasses as the carbon source. It has generally a better environmental 20 impact for essentially all criteria as compared to the same dairy substitutes made using recombinant caseins from microorganisms grown in presence of glucose as sole carbohydrate feedstock. Environmental impact is also improved for many criteria when using recombinant caseins from microorganisms grown in presence of sucrose, and even when using recombinant caseins from microorganisms grown 25 in presence of sucrose molasses.

- In the context of the present invention, a "casein" is any casein protein or mixture of casein protein. Thus, a "casein" is an alpha-S1 casein, an alpha-S2 casein, a beta casein, or a kappa casein. In some extend, it can also designate any mixture 30 of such proteins. One can use the term "casein" or "caseins" to discuss casein proteins in general.

In the context of the invention, the term "between" includes the limits.

In the context of the present invention, the term "dairy substitute" means a food product having the essential features of a dairy product, obtained using cow milk,

(such a cheese, yogurt, ice cream...), in terms of nutritional value, aspect, texture and taste.

In the context of the present invention, the term "cheese substitute" means a food product having the essential features of cheese in terms of nutritional value, aspect, texture and taste.

The term "fresh cheese" designates a cheese with a moisture higher than 80% on a fat-free basis (ratio of water vs. total mass of product without fat), and a protein content comprised between 2% and 15% of total weight (ratio of protein mass vs. total product mass).

The term "soft cheese" or "semi-soft cheese" designates a cheese with a moisture comprised between 62% and 80% on a fat-free basis (ratio of water vs. total mass of product without fat), and a protein content comprised between 15% and 30% of total weight (ratio of protein mass vs. total product mass). A soft cheese has a moisture comprised between 67% and 80%, on a fat-free basis, and a semi-soft cheese has a moisture comprised between 62% and 67%, on a fat-free basis.

In the context of the present invention, the term "liquid pre-curd composition" or "LpCC" designates the composition containing at least a casein and at least one other ingredient comprising at least a component among water, calcium, lipids, an carbohydrate, before the addition of rennet and ferments and curdling (in particular coagulation of casein). The casein concentration in the LpCC is higher than that in milk. The concentration of the other ingredient in the LpCC is also higher than that in milk. This makes it possible to save some water when using a LpCC with recombinant caseins as disclosed in the present application, as the LpCC doesn't represent an artificial (cow's) milk. The respective concentrations of proteins, lipids, calcium salts and/or carbohydrates are adjusted to fit the final desired compositions of cheese substitutes

In the context of the present invention, the term "curdling agent" designates a chemical or biochemical composition capable of triggering curdling. Curdling can be achieved by the addition of acid solutions, by the addition of starter cultures (ferments which growth in the presence of carbohydrates triggers a decrease of pH), by heat treatment, by addition of calcium chelating agent, by the addition of natural or recombinant rennet, by the addition of rennet substitutes, such as animal proteases, or vegetal curdling enzymes), or by a combination of these processes. A curdling agent may be any additive, compound, composition, or treatment which addition results in curdling, alone or in combination with another or others additives,

compounds, compositions, or treatments. When preparing animal-free edible compositions, it is preferred to use an acidifying agent (such as an acid or starter cultures), in order to avoid using rennet, so that the curdling agent does not comprise any element of animal origin. It is advantageous when the acidifying agent comprises a lactic bacterium or lactic bacteria, but acidic chemicals can also be used.

In the context of the present invention, the term "curd" means a composition wherein casein coagulates or precipitate, under the action of the curdling agent, and which can be separated from a liquid phase, if any, by draining, for example on a cheese cloth. The curd also comprises other ingredients, including water and lipids (when present),

In the context of the present invention, the term "ferment" designates a composition containing at least one microbial strain, added during the process of production of the cheese substitute. Lactic ferments are responsible for lactic fermentation, resulting notably in an acidification of the medium. As a consequence, they can be used as a curdling agent.

Other ferments are used in cheese production for the processing of the curd, resulting in modifications of texture, taste, smell, and chemical composition (with notably the cleavage of proteins into smaller peptides). These ferments can be called "ferments for maturation", "maturation ferments" or "ripening ferments" and are generally not used for production of fresh cheeses. Such ferments for maturation can be added together with the curdling agent.

In the context of the present invention, the term "of non-animal origin" means a compound or composition which has not been directly derived from an animal, produced from animal cells in culture, or isolated from animal products such as milk. Compounds or compositions produced by fermentation of microbes are thus "of non-animal origin" even though some products of animal origin, bacto peptone for example, can be involved during fermentation. Therefore, in the context of the invention, a protein that is naturally produced in animals will be called of non-animal origin when it is produced in microbial (such as bacterial or yeast) cells or in plant cells, even though its sequence or structure may be identical to the sequence or structure of the protein that would be isolated from animal.

In the context of the present invention, the term "animal-free" means a compound or composition which has not been derived from an animal, from animal

cells in culture, or from animal products such as milk, and whose production process does not involve any feedstock or additive of animal origin.

In the context of the invention, the term "texturing agent" means any gelling agent, including emulsifier such as lecithin, and hydrocolloids such as cassia gum, sesbania gum, tamarind gum, guar gum, fenugreek gum, Arabic gum, agar agar (or agar-agar), carrageenans, tragacanth gum, xanthan gum, carob (locust bean) gum, cellulose gum.

In a first aspect, is herein disclosed a method for obtaining a casein composition, comprising

- i. providing microorganisms that have been transformed with at least one nucleic acid coding for a casein,
- ii. culturing said microorganisms, using a medium comprising lactoserum so as to express and produce casein,
- 15 iii. thereby providing a microorganism composition wherein the pH of the composition is equal or above 6.5 and preferably below 9
- iv. heating the microorganism composition so as to reduce the amount of the other proteins in soluble fraction of the composition, wherein heating is performed at a temperature at a temperature equal or above
- 20 75°C,
- v. recovering the soluble fraction from the heated cell composition of iv), thereby obtaining a casein composition in the soluble fraction.

During step (ii), lactoserum can be used in combination with other sugars, such as glucose, saccharose, or even purified lactose.

Said method makes it possible to obtain a reduced environmental impact for at least one of one of the criteria mentioned above (in particular the Green House Gas (GHG) emissions, in mass of CO₂eq), as compared to performing the process using glucose, or using sucrose or using sucrose molasses in place of lactoserum in (ii). The impact can be particularly reduced when acid lactoserum (or derivatives, such as permeate) is used as the lactoserum in step (ii).

In particular, when compared to the use of glucose, it is possible to obtain a reduction of at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, or potentially at least 30%, depending on the criteria that is looked at.

Reduced environmental impacts can also be observed for dairy substitutes made with caseins obtained by the methods herein disclosed.

5 It is reminded that microorganisms, in particular bacterial cells present an exponential (or log) phase, in which cells are doubling after each generation time. Metabolic activity is high in this phase, and energy (nutrients) in the culture medium is directed to such doubling activity. After the exponential time, the population growth experienced in the log phase begins to decline as the cell growth reaches
10 a plateau, or stationary phase, where the number of dividing cells equal the number of dying cells.

In the context of the process herein disclosed, it is preferred to have lactoserum, as a feedstock to be used by the microorganisms, preferably in the middle or late exponential phase or at the beginning of the stationary phase (this is when feed
15 supply of a sugar other than lactose is avoided, and feed supply is made with lactoserum or lactoserum is present within the medium from the inoculum phase), and sometimes to maintain this provision during the production of the caseins. However, early induction streps can also be envisioned. One of skill in the art can determine the end of the exponential phase or the start of the stationary phase, for
20 a culture of microorganism, which depends on the doubling time of the microorganisms, the concentration of the starter culture, and the duration of growth. Measuring the Optical Density (OD) of the culture may also provide information as to the growth phase of the microorganism. When *Escherichia coli* is used, as a microorganism, one can envisage a duration of exponential phase of between 16-
25 20 hours, followed by a stationary phase during which expression of the protein is performed of about 7-10 hours.

The microorganism composition is a liquid solution that contains casein and other proteins. It also presents a soluble fraction and a non-soluble fraction. This
30 composition also contains transgenic microorganisms such as bacteria that produce caseins as a result of the presence, in their genome of transgene(s) coding for casein. Such bacteria (whether lyzed or not) are present in the non-soluble fraction.

The composition containing the transgenic microorganism is obtained by
35 fermentation, using various sugars and nitrogen sources as feedstocks. In one

embodiment, fermentation is conducted in a medium containing glucose, sucrose or lactose in addition to lactoserum. In a preferred embodiment, glucose, sucrose or lactose are used as a carbon source. In a some embodiments, fermentation is conducted in a medium using molasses or vinasses as a carbon source. In one
5 embodiment, fermentation is conducted in a medium containing molasses as a carbon source. In another embodiment, liquid or gaseous ammonia, urea, amino acids, peptones, or yeast extract are present or added in the medium as a nitrogen source. In one embodiment, fermentation is conducted in a medium containing ammonia, urea, amino acids, peptones, or yeast extract as a nitrogen source. In
10 one embodiment, fermentation is conducted on a medium containing lactoserum as the main feedstock (main source of carbon). In one embodiment, fermentation is conducted in a medium containing acid lactoserum or acid lactoserum permeate. In one embodiment, fermentation is conducted in a medium containing lactoserum and another sugar as additional sugar source. In a preferred embodiment,
15 fermentation is conducted on a medium containing acid lactoserum (or acid lactoserum permeate) and glucose, sucrose or lactose as additional sugar source. Glucose can be used as the other carbon source.

Before heating, or during growth of the microorganisms, pH can be adjusted by
20 the addition of as liquid or gaseous ammonia, sodium hydroxide, potassium hydroxide or calcium hydroxide or other basic compounds. pH is superior to 6,5, and preferably inferior to 9.

The microorganisms are transgenic and contain at least one transgene (a
25 nucleic acid) coding for a casein that is introduced present in the microorganisms. This transgene can be present within the microorganisms' genome or be present outside of the genome (on a plasmid, a cosmid, an artificial chromosome such as a bacterial artificial chromosome or a yeast artificial chromosome).

In a specific embodiment, the microorganisms are bacterial cells, in particular
30 *Escherichia coli*. In another embodiment, the microorganisms are fungi cells (including yeast cells). In another embodiment, the microorganisms are eukaryotic cells, in particular plant cells. In particular, prokaryotic hosts suitable for expressing caseins include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*, *Lactococcus lactis* and various species within the genera *Lactococcus*, *Pseudomonas*,
35 *Streptomyces*, and *Staphylococcus*. Eukaryotic host suitable for expressing

caseins include fungi, such as *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Pichia pastoris*, or *Trichoderma reesei*). Plant cells may also be used for producing protein.

It is preferred that the casein is produced in non-animal cells, preferably in
5 bacterial cells.

The microorganisms are transformed with at least one nucleic acid for expression of a casein. Such nucleic acid contains the elements necessary for the transcription and translation of the transgene coding for casein. In particular, the n
10 nucleic acid shall contain a promoter sequence, a sequence coding for casein and a terminator sequence.

In some embodiments, the microorganisms are transformed with one nucleic acid coding for one casein (and produce such casein). It is preferred when expression of the nucleic acid (production of the protein) is induced by lactose, i.e.
15 the nucleic acid coding for the casein is expressed in the presence of lactose metabolized by the microorganism (lactose-induced expression).

In other embodiments, the microorganisms express two caseins. In such embodiments, the microorganisms may be transformed with two transgenes, each for the expression of a given casein. It is preferred when expression of at least one
20 transgene is a lactose-induced expression. Preferably expression of the two transgenes are lactose-induced expression (using identical or different systems). In another embodiments, the two transgenes are linked together in an operon (the caseins are thus simultaneously produced). It is preferred when expression of the operon is a lactose-induced expression.

One also envisages embodiments, where the microorganisms express more than two caseins. In such embodiments, each transgene coding for a given casein may be under the control of its own promoter (preferably for a lactose-induced expression), or part or all of the transgenes may be co-expressed in an operon (preferably for a lactose-induced expression).
25

In some embodiments, the microorganism culture contains a mixture of some microorganisms transformed with one nucleic acid coding for one casein and of other microorganisms transformed with one nucleic acid coding for another casein.
30

Lactose induced expression of a gene is known in the art. It is often based on
35 the lactose operon (*lac* operon), which contains an operator region (*lac o*), to which

binds the lac repressor, *lacI*, in the absence of lactose, thereby preventing transcription of the genes downstream the *lac o* region. In the presence of lactose (allolactose, isomer of lactose), the conformation of the *lacI* protein changes and it doesn't bind to *lac o* anymore, thereby allowing transcription of the sequences downstream the operator. The sequence of the lac operator TTGTGAGCGGATAACAA (SEQ ID NO: 5). The system (that comprises the *lac o* operator and the *lacI* repressor) can be used for expression of genes downstream the *lac o* operator, with a promoter upstream the *lac o* operator being either the native promoter of the lac operon, or another promoter (such as the T7 promoter).

10 The lac operon comprises a regulatory region, including a promoter (P), where the RNA polymerase binds and initiates transcription, an operator (O) to which binds the lac repressor, which prevents transcription of the structural genes in the absence of lactose, and the *lacI* gene, encoding the lac repressor, which binds to the operator and blocks transcription in the absence of lactose.

15 One can note that lactose inducible promoters and systems have been described in the art. One can cite the lac promoter that regulate expression of the lactose operon (lac operon), an operon required for the transport and metabolism of lactose, in *E. coli* and many other enteric bacteria. One can also cite the P(*bgaL*) promoter (Hartman et al, Appl Environ Microbiol. 2011 Jan;77(2):471-8), the P_{lacA} and P_{lacLM} promoters disclosed in Zhang et al (Biochemical Engineering Journal Volume 151, 15 November 2019, 107316), or the P_{lacA} promoter disclosed in Heiis et al (Microb Cell Fact 15, 50 (2016)).

20

In some embodiments, the microorganisms are able to use galactose as a carbon source. In particular, it is preferred when the microorganisms express the galETKM operon from *E. coli* K12 strain, necessary for galactose consumption. It is reminded that the galETKM operon contains four genes; *galE* encodes an epimerase, *galT* encodes a uridylyltransferase; *galK*, encodes a galactokinase; *galM* facilitates the interconversion between β -galactose and α -galactose.

25

Thus, in some embodiments, the culture is performed in bacteria, with the caseins under the control of a lactose-inducible promoter (for instance the promoter fused to the *lac o* region and the bacteria expression the *lacI* repressor), wherein the bacteria also possess the genetic machinery needed for galactose consumption. In this embodiment, the bacteria are thus able to efficiently consume glucose, lactose (after the CCR has been ended) and galactose generated from lactose consumption.

30

35

In some embodiments, one can cite a method for culturing microorganisms, comprising:

5 providing microorganisms that have been transformed with at least one nucleic acid coding for a casein, wherein the nucleic acid is under the control of a lactose-inducible promoter

culturing the microorganisms in the presence of glucose, and optionally of lactoserum, preferably up to the exponential growth phase

10 reducing the amount of glucose, in particular when the microorganisms are in the exponential growth phase

culturing the microorganisms, using a medium comprising lactoserum so as to induce expression of the gene coding for the casein and production of casein, (depletion of glucose would abolish catabolic repression of the lactose-inducible promoter)

15 optionally further culturing the microorganisms in the presence of glucose after induction of the expression and production of casein

optionally recovering the composition of cultured microorganisms having expressed and produced casein. This method comprises the various steps described elsewhere herein. It makes it possible to induce the expression of the casein when the cells are fit for such expression (essentially during the exponential growth phase, around the middle of this phase), and to provide the best sources of carbon for the cells, while reducing the costs and environmental impact. One would thus obtain a culture of microorganisms (preferably bacteria) expressing casein.

25 In particular, one can perform a method for culturing microorganisms, comprising:

i. providing microorganisms that have been transformed with at least one nucleic acid coding for a casein, wherein expression of said nucleic acid is inducible by lactose,

30 ii. culturing said microorganisms, in a culture medium comprising glucose as a source of carbon, wherein expression of the nucleic acid is not induced

iii. depleting the culture medium of glucose when the microorganisms are in the exponential phase growth, wherein lactoserum is present in the culture medium at the time of said depletion, thereby providing the lactose present in the lactoserum as a source of carbon, and wherein expression of the nucleic acid is induced by
35 the lactose present in the lactoserum

iv. further culturing the microorganisms in the presence of a source of carbon.

In one embodiment, glucose is not present as a source of carbon in iv.

In one embodiment, lactoserum is not present as a source of carbon in iv.

In one embodiment, sources of carbon in iv comprise both glucose and
5 lactoserum.

In one embodiment, the microorganisms also metabolize galactose as a source of carbon, as disclosed elsewhere.

In one embodiment, the nucleic acid coding for a casein is under the control of a promoter fused with the *laco* operator region of the lactose operon, and wherein
10 the microorganisms express the *lacI* repressor of said operon. This ensures catabolic repression of the expression in ii. Thus, in particular in this embodiment, it is possible for glucose and lactoserum to be present in the culture medium of ii.

In one embodiment, lactoserum is not present in the culture medium of ii and is added in iii.

15 In one embodiment, depletion of glucose in iii is performed by consumption of the glucose by the microorganisms, in the absence of refeed of glucose (it is indeed generally necessary to continuously provide glucose or another source of carbon during culture; stopping this refeed would lead to total consumption of the glucose previously provided, and the microorganisms would thus start using lactoserum as
20 the source of carbon).

In one embodiment, the microorganisms are bacteria, notably *E. coli* bacteria.

This method, when comprising the steps below, makes it possible to obtain a casein composition:

thereby providing the microorganism composition as obtained, wherein the pH
25 of the composition is equal or above 6.5 and preferably below 9

heating the microorganism composition so as to reduce the amount of the other proteins in soluble fraction of the composition, wherein heating is performed at a temperature at a temperature equal or above 75°C,

recovering the soluble fraction from the heated cell composition,
30 thereby obtaining a casein composition in the soluble fraction.

As indicated above, a specific embodiment encompasses the situation when the starting composition (composition containing the casein and other proteins) has been obtained from a bacterial culture. In such embodiment, and as indicated

above, the bacteria have been transformed with one or more nucleic acid coding for one or more casein.

In particular, in one embodiment, the bacteria have been transformed with one or more nucleic acid coding for a beta casein.

5 In particular, in one embodiment, the bacteria have been transformed with one or more nucleic acid coding for an alpha-S1 casein.

In particular, in one embodiment, the bacteria have been transformed with one or more nucleic acid coding for an alpha-S2 casein.

10 In particular, in one embodiment, the bacteria have been transformed with one or more nucleic acid coding for a beta casein and an alpha-S1 casein.

In particular, in one embodiment, the bacteria have been transformed with one or more nucleic acid coding for a beta casein, an alpha-S1 casein and an alpha-S2 casein, or a combination of two out of them.

15 It is to be noted that, when multiple proteins are produced, the bacteria can be transformed with different nucleic acids (each one coding for a different protein) or with a unique nucleic acid (which contains the elements allowing production of the various proteins, such as operon). Such methods of transforming microorganisms and bacteria in particular, for production of one or of multiple proteins are known in the art.

20 Consequently, the recovered casein composition contains casein, preferably selected from beta casein, alpha-S1 casein, alpha-S2 casein and mixture of these caseins, notably selected from beta casein, alpha-S1 casein and mixture of these caseins. As indicated, the microorganisms are transformed with a nucleic acid coding for alpha-casein and another nucleic acid coding for beta casein, wherein
25 the two genes are under the control of a lactose inducible promoter.

It is preferred when the microorganisms are not transformed with a nucleic acid coding for kappa-casein. Consequently, it is preferred when no kappa casein is produced by the microorganisms, and when no kappa casein is present in the various compositions and solutions.

30

In the method herein disclosed, the lactoserum serves as an important source of carbon needed for the growth of the microorganisms,

In one embodiment, culture of the microorganisms is initiated in presence of a sugar other than lactose, as described above, in particular in the presence of
35 glucose. When bacteria, in particular *E. coli* bacteria are used, as the

microorganisms, glucose is present in an amount sufficient to induce CCR (carbon catabolic repression). Indeed, the presence of both glucose and lactose results in a regulatory scenario where the lac operon is repressed. The high priority given to glucose utilization through catabolite repression keeps cAMP levels low, essentially
5 reducing or abolishing the transcriptional activity of the lac operon.

In such occurrence, bacteria are using glucose as the source of carbon and lactose metabolism is repressed. It is reminded that lactose metabolism involves the cleavage of one molecule of lactose to one molecule of glucose and one molecule of galactose. Such metabolism is repressed in the presence of glucose,
10 as the carbon source in the culture medium, as the catabolites produced by the breakdown of glucose prevent the activation of the production of enzymes needed for lactose metabolism. However, once these enzymes are activated, repression no longer occurs (Görke and Stülke Nat Rev Microbiol 6, 613–624 (2008); Postma et al. Microbiol Rev. 1993 Sep;57(3):543-94).

15 In particular, it is preferred when glucose (or a sugar other than lactose) is used at the start of the culture, and during the early exponential phase of growth as it will ensure proper exponential growth of the bacteria.

At a later point of the exponential growth, or at the beginning of the stationary growth, lactoserum shall be used as a source of lactose for inducing expression of
20 the caseins (if a lactose inducible system is used), or as a feedstock for stationary growth of the proteins.

In order to trigger the switch to the lactose metabolism and lactose consumption (stop CCR), the amount of glucose is limited (glucose could even be depleted from the culture medium). Once this switch has occurred, it is possible to
25 add glucose again in the culture medium or another sugar such as saccharose, fructose, galactose, notable in the form of molasse in the culture medium to complete the sugar supply.

In one embodiment, lactoserum is absent from the culture medium at the beginning of cultivation and is added, when the amount of the glucose is limited
30 and when the switch to lactose consumption (and induction of protein expression) is desired (during exponential phase, or at start of stationary phase).

In another embodiment, lactoserum is added, with glucose, at the beginning of cultivation, and during the beginning of the exponential phase but it is not consumed as the concentration of glucose is maintained at a level high enough to
35 induce CCR. Consequently, the switch to lactose consumption is achieved by

reducing the glucose feed, for a given period of time (30 minutes may be enough), bacteria then using the lactose present in the lactoserum. Glucose may then be fed again, a low amount, so that there is no accumulation of glucose in the medium (this can be verified by sampling the medium and measuring glucose concentration). Generally, stopping glucose feed to suppress CCR and later feeding again with glucose would not lead to a reappearance of CCR.

Thus, lactoserum is present in the culture medium when the microorganisms are in the late exponential phase and/or stationary phase. In one embodiment, lactoserum is present in the culture medium when the microorganisms are in the exponential phase. In another embodiment, lactoserum is absent of the culture medium when the microorganisms are in the exponential phase.

it is also possible to use other sugars than glucose for starting the culture such as glycerol, as described by others (Viitanen et al. 2003, Cheese whey-induced high-cell-density production of recombinant proteins in *Escherichia coli*, Microbial Cell Factories 2:2; de Diviitis et al. (2023) Cheese-whey permeate improves the fitness. Biotechnology for Biofuels and Bioproducts, volume 16:30) to add lactoserum later (notably to induce expression of caseins) at an appropriate point of culture. Choice of the sugars and specific operating procedures to add lactoserum can easily be designed by one of skill in the art.

In one embodiment, the culture of the microorganisms is performed in a fed-batch reactor, by providing feed at periodic intervals. In another embodiment, the culture is performed in a continuous mode with continuous feed and withdrawal of product.

In another embodiment, the culture is performed in batch mode.

It is reminded that, during fed-batch fermentation, there is controlled addition of substrate and supplements into the fermentation medium, in contrast with batch mode, where all substrates are present in the bioreactor at the beginning of the process.

The "feed flow" describes the amount of culture medium, preferably comprising the microbial or bacterial host cell, that is transferred to the production reactor in a distinct period of time and may be expressed as volume per time, e.g. liters per minute. The feed flow may be regulated e.g. by an adjustable or controllable pump.

It is preferred when the concentration of the lactose in the culture medium is higher than 1%, and preferably between 1% and 5% (w/v, for 1g/100mL to 5g/100mL), when the switch to lactose consumption occurs.

5 In one embodiment, the lactoserum is raw or whole lactoserum, i.e. the liquid obtained from curdling or straining of milk. It contains some whey proteins.

In another embodiment, the lactoserum is permeate lactoserum, obtained by ultrafiltration of whole lactoserum, removing the lactoserum's serum proteins.

10 In one embodiment, one uses acid lactoserum (or derived permeate), obtained by coagulating milk through acidification with lactic acid bacteria. In this embodiment, a base is added to the culture medium to maintain the pH of the solution containing the microorganisms around pH 7.

In another embodiment one uses sweet lactoserum.

15 In one embodiment, the composition containing casein and other proteins (as well as potentially microorganisms, obtained from the culture of the microorganisms) is thus flown from the first container via a device containing a pipe or a tube, such tubular exchanger having the functions of both displacing the composition and heating it. There is thus a heat transfer (or heat exchange) during
20 the passing of the composition within the pipe (corresponding to the heating step iv), so as to heat the composition, such transfer being performed on at least a fraction of the pipe. Such heating precipitates other proteins present in solution, while the casein remains in the soluble fraction.

25 The soluble fraction may be recovered by performing a centrifugation of the liquid composition flowing from the pipe.

The concentration of casein, and/or the ration of casein versus other proteins is higher in the recovered soluble fraction than in the starting composition, the casein thereby being enriched in this soluble recovered fraction.

30 As indicated, the composition of i) contains a soluble fraction and an insoluble fraction. The soluble fraction refers to a fraction that is not pelleted upon centrifugation. The insoluble fraction is the pellet obtained after centrifugation. Centrifugation may be performed at about 3000 g for 20-30 minutes. At industrial scale, continuous flow centrifugation, or another method such as filtration may be used to recover the soluble fraction. In the case of filtration, membranes of different
35 types can be used.

The desired technical effect is obtained by heating during the passing in the tube, and preferably in the absence of organic solvents, or of addition thereof.

The device may contain, before the charcoal zone, a zone (such as a chamber or a length of a pipe) for pre-heating the composition before flowing within the pipe at the desired temperature. For instance, when the composition is at room temperature, the pre-heating may raise the composition temperature at about 80°C. The device may also present, after the heating pipe zone, a zone for cooling the composition, at temperature below 100°C (preferably about 60-80°C at most) for being able to recover the soluble fraction.

10

The ratio of casein is increased in the soluble fraction, when the relative amount of casein increases as compared to the total amount of proteins.

The other proteins are non-casein proteins. heating thus makes it possible to obtain a composition enriched in casein. Enrichment of casein in the composition refers to an increase of casein in the soluble fraction as compared to the amount present in the soluble fraction of the starting composition before flow through the pipe.

15

In one embodiment, the microorganism composition is washed to remove the culture medium before heating in iv).

20

As an illustration, it is possible to perform centrifugation of the microorganism cells (preferably bacteria) after culture, washing of the pellet, and resuspension in an appropriate liquid or fluid (appropriate buffer and more preferably in water, more preferably in the absence of organic solvents). The buffer is at a pH that is not acidic (equal or above 6.5), preferably close to neutral (between 6.5 and 7.5), or basic (preferably below 9). Indeed, caseins can precipitate at acidic pH. It is also preferred when the buffer is not ionic, or with a low ionic strength.

25

The resuspended composition is thus submitted to the heating step of iv).

In one embodiment, the microorganisms of the microorganism composition have been lysed to obtain a liquid composition before iv), wherein the liquid composition is in a first container. In another embodiment, the microorganisms in the microorganism composition are not lysed and heating in iv) lyses the microorganisms.

30

Lysis of the microorganisms may be performed, using any method known in the art, preferably using a chemical (such as detergent) or enzymatic lysis using for instance lysozyme or proteinase K, or mechanically, using a French press.

5 It is possible to optionally perform one further step chosen among addition of activated charcoal, chemical resins, membrane filtration (ultrafiltration, nanofiltration, or reverse osmosis), centrifugation, chromatography or precipitation of casein, to the soluble fraction recovered in v). The soluble fraction may notably be recovered by performing a centrifugation of the liquid composition flowing from
10 the pipe, using regular or disc stack centrifuges Alternatively, it can be obtained by microfiltration or diafiltration.

Activated charcoal can be added to the soluble fraction and stirring is performed. Activated charcoal can be used to adsorb impurities (in particular organic impurities or chlorine) present in the soluble fraction, and that were not
15 removed when recovering the soluble fraction in iii). Stirring with activated charcoal is preferably done, and the activated charcoal is removed before another method (such as the ones disclosed below) is performed on the soluble fraction.

Chromatography methods are widely used for the purification methods, and include notably affinity chromatography, ion exchange chromatography,
20 hydrophobic interaction chromatography and others.

Membrane filtration, including notably ultrafiltration and nanofiltration is commonly used in protein purification as well (Saxena et al. (2009) Membrane-based techniques for the separation and purification of proteins: An overview. Advances in colloids and Interface Science Volume 145, pages 1-22), and
25 membrane filtration techniques are widely used in the dairy industry. Interestingly, they can be used to separate the different caseins from each other (see above).

Finally, the specific properties of caseins (Post et al. (2012) Effect of temperature and pH on the solubility of caseins: Environmental influences on the dissociation of caseins. J. Dairy Sci. Volume 95 : pages 1603–1616), and notably,
30 their propensity to precipitate in acidic conditions can be used for further purification.

In a preferred embodiment, further processing said soluble fraction is done by precipitation of caseins in acidic conditions. After precipitation, caseins can be resuspended, and using an appropriate basic buffer, they can also be resolubilized
35 (Post et al. (2012) Effect of temperature and pH on the solubility of caseins:

Environmental influences on the dissociation of caseins. J. Dairy Sci. Volume 95 : pages 1603–1616). In a preferred embodiment, further processing of said soluble fraction is done by precipitation of caseins at pH ranging from pH=4 to pH=5. In a more preferred embodiment, precipitation of caseins is conducted at pH of about 4.6. Various acids can be used including lactic acid, hydrochloric acid, and sulfuric acid.

Casein can then be recovered by methods such as centrifugation, or tangential filtration. A washing step can be added, with water adjusted at the pH used for precipitation. Separation of the washed casein can be made using the method as cited above.

Precipitation in acidic conditions is particularly interesting when the casein is isolated from a culture of microorganisms, as it makes it possible to remove or degrade any nucleic acid that may be present in the casein composition. Presence of DNA or RNA of the microorganisms may prove to be detrimental (at least from a regulatory point of view) when the casein is to be used for obtaining edible compositions intended to be ingested by human beings (such as cheese substitutes). pH is in the range of, and at about 4.6, and temperature ranging from 80°C to 140°C and times may vary between a few minutes and 2 hours.

Alternatively, recombinant DNA can be eliminated using nucleases such as DNAses that can be eliminated by heating.

When using microorganisms such as Gram- bacteria, one also could add other steps for elimination of lipopolysaccharides (LPS), and treatment at high temperature in acidic or basic conditions, the use of activated charcoal at high temperature, or filtration methods (10-20kDa separation, or various extraction methods could be used).

pH can then be neutralized to obtain caseinates. For this, various basic compounds can be added such as liquid or gaseous ammonia, sodium hydroxide, potassium hydroxide or calcium hydroxide. Targeted pH can range between 6.5 and 9/. The kinetic of pH adjustment can be continuous, or following a series of plateaus, in order to achieve a stable target pH. before drying, caseins can be concentrated by filtration or evaporation. Caseins can then be desiccated by spray drying, flash drying, or other methods known in the field.

In a preferred embodiment, pH is adjusted using sodium hydroxide. In another embodiment, pH is adjusted using potassium hydroxide. In another embodiment, pH is adjusted using calcium hydroxide.

5 In a specific embodiment, the casein composition is desiccated. When obtained from a bacterial culture, such composition shall contain about 15-30% casein (w/w) and carbohydrates in the desiccated composition.

It is preferred when a little water is left, to favor future rehydration. Consequently, desiccation is to be understood as reducing the amount of water. In
10 some embodiments, the amount of water is about 50% or less (w/w).

The insoluble fraction, containing most of the biomass produced during fermentation, can be separately treated and valorized. Such biomass can be used in feed or food applications, after using the same treatment as for caseins to
15 remove recombinant DNA: pH is in the range of 4.6, with temperature ranging from 80°C to 140°C, and time may vary between a few minutes and 2 hours; alternatively, recombinant DNA can be eliminated using nucleases such as DNAses that can be eliminated by heating.

20 The composition of i) in the methods disclosed above is thus heated when flowing through the pipe, at a temperature (the heating temperature) comprised higher than 75°C. More preferably, the temperature is higher than about 80°C, more preferably higher than about 85°C, more preferably higher than about 90°C, more preferably higher than about 95°C, most preferably higher than about 100°C,
25 or higher than 100°C. It may be advisable to use temperatures equal or higher than 105°C, or equal or higher than 110°C. It is possible to use temperatures as high as 140°C or even 150°C. It is preferred to heat the composition for 3 minutes, at most or even 1 minute at most. In this case, an adequate temperature is preferably between 95°C and 115°C, preferably between 100°C and 115°C (limits included).
30 A temperature of about 110°C would be appropriate. Such temperatures make it possible to obtain the technical and functional effects of removal of the other proteins, therefore reducing their amount and/or moving the casein from the insoluble to the soluble fraction, and while heating for a short duration (at most five minutes, preferably at most two minutes, but rather 90 seconds or less, or 75
35 seconds or less, more preferably 60 seconds or less, or even 45 seconds or less).

The examples show that durations of 30 seconds or even 2 seconds can be applied. It is however preferred to heat the composition flowing within the pipe for at least 5 seconds or at least 10 seconds.

Pressure within the pipe is generally higher than 3 bars, more preferably 4 bars or more, more preferably 4.5 bars or more. It is generally 7 bars or less, preferably 6.5 bars or less, preferably 6 bars or less. A pressure between 4.5 and 5.5 bars (limits included) is well adapted.

The term "about", when referring to a measurable value, is meant to encompass variations of $\pm 3\%$ from the specified value, as such variations are appropriate to perform the disclosed methods.

The examples show various conditions that make it possible to obtain purification of caseins, from recombinant *E. coli*. Although these examples also mention the specific conditions, one can note specific couples temperature/holding time-length at that temperature (110°C/75 sec; 110°C/30 sec; 120°C/30 sec; 140°C/30 sec; 140°C/2 sec) that are disclosed to respond to the technical problem of purifying caseins. It may be advisable not to heat the composition too much and to use longer times than very short times. Various conditions (using heating temperatures from 95°C-140°C and holding times within the heated chamber from 2-120 seconds) all made it possible to obtain reasonably concentrated caseins in the soluble fraction post heating treatment, with good purity.

It is advisable to pre-heat the composition arising from the first container before performing the heating step within the pipe. This is performed in a chamber out of the first container or in a pipe that conducts the composition from the first container to the pipe where it is heated at the desired temperature for the desired amount of time. The pre-heat temperature may be 20°C or 30°C less than the targeted heating temperature, or between about 20°C et about 60°C less than the heating temperature. Pre-heating between 75°C and 90°C (such as at 80°C) is well adapted. The total duration of treatment (Pre-heating + heating + chambering (maintaining at the heating temperature) + pre-cooling + cooling) was about 150-350 seconds, preferably 150-320 seconds, or 200-320 seconds.

In order to obtain the desired temperature within the pipe, and especially when heating by heat-exchange is performed, it is heated at a temperature higher than such desired temperature (generally about 5°C higher) as this takes into account the temperature gradient from the outside of the pipe to the center of the pipe. Heating can be performed by any of the following methods:

Direct steam injection: injecting steam directly into the pipes to heat them. The steam is generated in a boiler and directed into the pipes, where it transfers its heat to the pipe walls, heating the product flowing through.

5 Indirect heating by heat exchange: double-walled pipes are used. The composition flows through the inner tube, while steam circulates in the space between the two walls. The heat from the steam transfers through the pipe wall, heating the product.

Electric heating: heating elements are built into the pipes to heat them, electricity being used to generate heat directly in the pipe walls.

10 Induction beam heating: This method uses electromagnetic induction to heat the pipes, by Joule effect, by use of an alternating electric current induced in the pipe walls with the aid of a magnetic field.

15 In some embodiments, the composition is circulated within the pipe with a flow rate comprised between 15 and 30 L/h.

The composition is generally cooled and delivered to a second container after flowing. In some embodiments, the soluble fraction, that has been recovered by centrifugation and/or filtration after flowing and heating is delivered to the second
20 container. Further purification or concentration of casein (for instance by ways of evaporation) and/or of quantification of the casein in the soluble fraction can then be performed.

Such method makes it possible to obtain a casein composition where the caseins represent at least 60 %, preferably at least 62 %, preferably at least 65 %, preferably at least 67 %, preferably at least 70 % of the total proteins of the soluble
25 fraction. In particular, the ratio of casein as compared to the total proteins is increased in the soluble fraction as compared to the ratio in the microorganism composition.

30 In summary, the methods herein disclosed thus make it possible to obtain a casein composition with a low environmental impact. Such a casein composition susceptible to be obtained (or obtained) by a method herein disclosed is a further subject of the invention. Such composition can be characterized as defined above.

35 In milk, the casein is in the micellar form found in milk, wherein micelles include alpha-S1, alpha-S2, beta and kappa caseins assembled in the same particules. In

the context of the present invention, casein may not be assembled in the micellar form. However, this doesn't prevent ability to obtain a curd from the casein composition, by addition of an appropriate curdling agent.

Indeed, as developed in the examples, such casein composition can be used
5 for producing cheese substitutes, and in particular animal-free cheese substitutes, *i.e.* cheese substitutes that don't contain any products of animal origin (in particular when the casein has been isolated from bacterial or yeast culture).

In summary, simple and short (less than 5 minutes, or preferably less than 2 minutes) heating of a composition containing casein and other proteins through a
10 pipe makes it possible to enrich the composition in casein by reducing the amount of most of the other proteins in the soluble fraction. Heating also makes it possible to solubilize casein found in the insoluble fraction of bacterial cultures. The obtained casein, although not in the optimal condition for forming micelles, can appropriately curd upon the action of a curdling agent. Such findings were unexpected.

15 In particular, as shown in WO2022253816, curdling can be achieved with the recombinant caseins made in bacteria, and in the absence of kappa caseins, even though they don't possess post-translation modifications (such as phosphorylations) and the fact that kappa caseins are known to play an important role in the formation of dairy casein micelles.

20

In one embodiment is disclosed a method for obtaining a dairy substitute, comprising performing the method herein disclosed, allowing to obtain a casein composition, mixing the casein composition with at least one other ingredient comprising at least one component selected from the group consisting of proteins,
25 water, calcium, lipids, and carbohydrate to obtain a liquid pre-curd composition (LpCC), and further processing the liquid pre-curd composition (LpCC) to obtain a dairy substitute. Further processing the LpCC notably includes providing a curdling agent to the LpCC to obtain a curd. Such curd can then be drained (and the obtained lactoserum may be used again in methods herein disclosed, therefore providing circular use), molded and aged to obtain a cheese substitute. The curd
30 can be obtained and processed with lactic bacteria (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) to obtain yogurt substitutes.

Such embodiment is disclosed notably in WO2022058573 and
35 WO2022253816.

As indicated, said curdling agent is preferably not rennet, but is an acidifying agent, notably a lactic bacteria, or lactic, citric or acetic acid. The casein composition may contain only alpha-S1 casein (as the casein protein), or only alpha-S2 casein (as the casein protein), or only beta casein (as the casein protein),
5 or only alpha-S1 and beta casein (as the casein proteins), or only alpha-S2 and beta casein (as the casein proteins), or only alpha-S1 and alpha-S2 casein (as the casein proteins), or alpha-S1, alpha-S2 and beta casein.

The curd may be processed to obtain an edible composition, notably a cheese substitute, as disclosed in WO2022058573 and WO2022253816. These two
10 documents are herein incorporated by reference, in particular the parts describing the obtention of a LpCC or of an edible composition, starting from a composition containing caseins, notably produced in bacteria and/or without kappa casein, in particular parts pertaining to obtaining a cheese substitute having the essential features of fresh cheese and a cheese substitute having the essential features of
15 soft cheese or semi-soft cheese. The parts of these documents and in particular of WO2022253816, pertaining to calcium and other salts, lipids, emulsifying and gelling agents, carbohydrates, vitamins, curdling agents, ferments, moisture, and processing of the curd are also incorporated by reference.

The teachings pertaining to the composition of the LpCC, depending on the
20 type of cheese substitute that is desired, are also incorporated by reference, as well as the amount of gelling agent to add (notably pages 24 to 28 of WO2022253816).

It is reminded that a gelling agent (in particular agar-agar) can be added in with the other ingredients before curdling or after curdling.

25 It is preferred when the other ingredients added in b) are all of non-animal origin.

The ingredients added in ii) comprise

- i. Optionally proteins other than caseins
- ii. Lipids
- 30 iii. Water, and
- iv. Carbohydrates; in preferred embodiments, the carbohydrates do not comprise lactose, in order for the edible composition to be more acceptable to lactose-intolerant customers.

The choice and amount of the ingredients is adjusted so that the composition of the LpCC is adapted to the eventual composition desired for the edible composition, taking into account the loss of water due to curdling and aging (which can be modulated by one of skill in the art by modifying the duration and conditions of maturation).

In particular, processing of the LpCC comprises

- vi. adding at least one curdling agent to the liquid composition so as to obtain a curd, and
- vii. further processing said curd in order to obtain a dairy substitute, wherein the dairy substitute is a cheese substitute or a yoghurt substitute.

Such further step vi) may comprise acidic precipitation of casein. In particular, acidic precipitation is performed at a pH between 4 and 5 (such as pH=4.6), and preferably at room temperature (about 20°C), although it could also be conducted at about 90°C. Multiple other steps of purification can be performed.

Further processing of the curd may include the steps of

- a) mixing a structuring agent with the curd, wherein the structuring agent is selected in the group consisting of gelling agents, texturing agents, emulsifying agents and mixtures thereof, to obtain a supplemented curd, and
- b) molding and draining the supplemented curd.

One can also cite a method comprising

- a) Providing a casein composition, comprising a recombinant casein as herein disclosed
 - b) Mixing said casein composition with at least one other ingredient comprising at least one component selected from the group consisting of water, calcium, lipids, and carbohydrate, so as to obtain a liquid pre-curd composition (LpCC),
 - c) Adding at least one curdling agent to the liquid pre-curd composition so as to obtain a curd
 - d) Further processing the curd (in particular by draining) to obtain an edible composition, wherein a gelling agent (notably agar-agar) is added in b) and/or in d).
- In one embodiment, the gelling agent is added in b).
- In another embodiment, the gelling agent is added in d).

In another embodiment, the gelling agent is added in b) and in d).

In this embodiment, it is preferred when the edible composition

- i. has a protein content comprised between 2 and 15% (weight content)
- ii. has a moisture content superior to 80%, on a fat-free basis.

- 5 This embodiment is thus well adapted for production of a fresh cheese substitute. Further processing the curd may further include molding the drained curd, and/or aging the dried curd.

- 10 The environmental impact of dairy substitutes, or any other dairy substitutes made with caseins obtained by the methods herein described, can be significantly reduced for at least one of the criteria listed above, as compared with a dairy substitute obtained by the same method, but wherein lactoserum is not added to produce casein by fermentation, and wherein the lactose from lactoserum is replaced with an equivalent mass of glucose, or an equivalent mass of sucrose, provided as such or provided in sucrose molasses. In particular, the environmental impact is reduced of at least 5%, or at least 10%, or at least 20 %, or at least 25 %, or at least 30 % for at least one of the factors as described above (in particular climate change).

20 DESCRIPTION OF THE FIGURES

Figure 1: Structure of plasmid expressing beta and alpha-S1 caseins.

- Figure 2:** SDS-PAGE analysis of the α_{s1} and β caseins in the lysate, before and after thermal treatment, in soluble and insoluble fractions. **Lane 1:** Size marker, Precision Plus Protein™ Unstained Standards (Bio-Rad, 1610363EDU) - **Lane 2:** Cell suspension before thermal treatment, soluble fraction) - **Lane 3:** Lysate after thermal treatment (Trial 1), soluble fraction - **Lane 4:** Lysate after thermal treatment (Trial 2), soluble fraction - **Lane 5:** Lysate after thermal treatment (Trial 3), soluble fraction - **Lane 6:** Lysate after thermal treatment (Trial 4), soluble fraction - **Lane 7:** Lysate after thermal treatment (Trial 5), soluble fraction - **Lane 8:** Cell suspension before thermal treatment, insoluble fraction - **Lane 9:** Lysate after thermal treatment (Trial 1), insoluble fraction - **Lane 10:** Lysate after thermal treatment (Trial 2), insoluble fraction - **Lane 11:** Lysate after thermal treatment (Trial 3), insoluble fraction - **Lane 12:** Lysate after thermal treatment (Trial 4), insoluble fraction - **Lane 13:** Lysate after thermal treatment (Trial 5), insoluble fraction.

Figure 3: SDS-PAGE analysis of the α_{s1} and β caseins in the lysate, before and after thermal treatment, in soluble and insoluble fractions. Analysis of 3-fold diluted samples. For each panel, **Lane 1:** Size marker, Precision Plus Protein™ Unstained Standards (Bio-Rad, 1610363EDU) - **Lane 2:** Cell suspension before thermal treatment, soluble fraction - **Lane 3:** Cell suspension before thermal treatment, insoluble fraction - **Lane 4:** Lysate after thermal treatment, soluble fraction - **Lane 5:** Lysate after thermal treatment, insoluble fraction. Panel A: 110°C, 75 seconds; B: 110°C, 30 seconds; C: 120°C, 30 seconds; D: 140°C, 30 seconds; E: 140°C, 2 seconds.

Figure 4: Monitoring of growth and glucose concentration in the culture medium in various culture conditions. Growth was monitored by optical density (OD) at 600nm (A). Uninduced culture in glucose is represented by dotted lines, culture with glucose induced with IPTG by dashed lines, and culture with glucose and lactoserum by solid lines.

Figure 5: Analysis of casein production by SDS-PAGE in various induction conditions. Samples were prepared as described in Example 4. **T1, T2, T3:** total lysates from uninduced culture in glucose (**T1**), culture with glucose induced with IPTG (**T2**) culture with glucose and lactoserum (**T3**, as described in example 4). **P1, P2, P3:** insoluble fraction (pellet) of lysates from uninduced culture in glucose (**P1**), culture with glucose induced with IPTG (**P2**) culture with glucose and lactoserum (**P3**). **S1, S2, S3:** soluble fractions (supernatant) of lysates from uninduced culture in glucose (**S1**), culture with glucose induced with IPTG (**S2**) culture with glucose and lactoserum (**S3**). **A:** purified Alpha S1 and Alpha S21 caseins from Sigma 1g/L. **A:** purified beta casein from Sigma 1g/L. **M:** molecular weight marker. Band sizes were indicated left of the gel.

Figure 6: Comparison of the environmental impact of casein production using different feedstocks. Use of Glucose is presented as the reference basis (100%).

Figure 7: SDS page of the clarified cell lysate from samples; (1)BR1 – SO_gal0, (2) BR2 – SO_gal1, (3) BR3 – SO_gal2, (4) BR4 – SO_gal3 without lactoserum and (5) BR5 – SO_gal3 with lactoserum. As control, solutions of commercial alpha casein (A) and beta casein (B).

Figure 8: SDS page of the insoluble fraction of the lysate from samples; (1)BR1 – SO_gal0, (2) BR2 – SO_gal1, (3) BR3 – SO_gal2, (4) BR4 – SO_gal3

without lactoserum and (5) BR5 – SO_gal3 with lactoserum. As control, solutions of commercial alpha casein (A) and beta casein (B).

Figure 9: Lactose (A), Glucose (B) and Galactose (C) concentrations in the culture growth.

5

EXAMPLES

Example 1: Production of alpha-S and beta caseins

Synthetic genes coding for alpha-S1 and beta casein (related to natural genes P02662, and P02666, respectively), were modified, in order to remove the signal peptide, and to optimize codon usage for expression in Escherichia coli, and the sequence of the proteins encoded by the new synthetic and the sequence of the new synthetic open reading frames (SEQ ID NO: 2 and SEQ ID NO: 4) are shown in Table 2, last column. The two synthetic open reading frames were cloned together into pET25b+, in replacement of the NdeI-HindIII small fragment of this vector, in a same operon under the control of the T7 promoter of pET25b+, with (i) both synthetic open reading frames under the control of a T7 ribosome binding site and terminated by two TAA stop codons and (ii) the beta casein open reading frame being proximal to the promoter and the alpha-S1 open reading frame distal to the promoter (Figure 1). The resulting plasmid was transformed into BL21(DE3) strains (Novagene). Individual transformed clones were isolated, and checked for each synthetic gene. One clone was used to inoculate LB medium.

Name and Uniprot reference of protein precursor	sequence of casein precursor	sequence of recombinant caseins in examples
Alpha-S1 casein P02662	MKLLILTCLVAVALARPKHPIK HQGLPQEVLNENLLRFFVAPFP EVFGKEKVNELSKDIGSESTED QAMEDIKQMEAESISSSEEIVP NSVEQKHIQKEDVPSERYLGYL EQLLRLKKYKVPQLEIVPNSAE ERLHSMKEGIHAQQKEPMIGV	MRPKHPIKHQGLPQEVLNENL LRFFVAPFPPEVFGKEKVNELSK DIGSESTEDQAMEDIKQMEAE SISSEEIVPNSVEQKHIQKED VPSERYLGYLEQLRLKKYKVP QLEIVPNSAEERLHSMKEGIHA QQKEPMIGVNQELAYFYPELF

	NQELAYFYPELFRQFYQLDAY PSGAWYYVPLGTQYTDAPSFS DIPNPIGSENSEKTTMPLW (SEQ ID NO: 1)	RQFYQLDAYPSGAWYYVPLG TQYTDAPSFS DIPNPIGSENSE KTTMPLW (SEQ ID NO: 2)
Beta casein P02666	MKVLILACLVALALARELEELN VPGEIVESLSSESSEITRINKKIE KFQSEEQQTEDELQDKIHFPF AQTQSLVYPFPGPIPNSLPQNI PPLTQTPVVVPFLQPEVMGV SKVKEAMAPKHKEMPFPKYPV EPFTESQSLTLTDVENLHLPLP LLQSWMHQPHQPLPPTVMFPP QSVLSLSQSKVLPVPQKAVPY PQRDMPIQAFLLYQEPVLGPV RGPFPPIV (SEQ ID NO: 3)	MRELEELNVPGEIVESLSSEE SITRINKKIEKFQSEEQQQTED ELQDKIHPPFAQTQSLVYPFPGP IPNSLPQNIPLTQTPVVVPFL QPEVMGVSKVKEAMAPKHKE MPFPKYPVEPFTESQSLTLTD VENLHLPLPLLQSWMHQPHQP LPPTVMFPPQSVLSLSQSKVL PVPQKAVPYPQRDMPIQAFLL YQEPVLGPVRGPFPIIV (SEQ ID NO: 4)

Table 2: Sequences of natural caseins (precursors) and of the related recombinant proteins. In natural caseins, signal peptides are indicated in bold. In recombinant caseins, the methionine resulting from cloning into the pET expression system is indicated in bold.

5

In order to produce a batch of alpha-S1 and beta caseins, the resulting strain was cultivated as follow:

Five concentrated stocks of the strain of each 1 mL, stored in 10 % DMSO at -80°C, were thawed and used to inoculate five precultures of a volume of 1 L each, respectively (0.1 % v/v inoculation rate), in a revivification medium Y15 composed of yeast extract (15 g/L) and NaCl (5 g/L), and supplemented with 100 µg/mL of ampicillin. The five precultures were grown in 4 L or 5 L erlenmeyers at 30°C, for 9 hours, at 170 rpm (orbital diameter: 25 mm). After 9 hours, optical densities (60nm) in the range of 3 were achieved.

10

2.4 L of the first preculture were used to inoculate a volume of 120 L (2% v/v inoculation rate), in an optimized culture media supplemented with 100 µg/mL of ampicillin and with 15 g/L of glucose. Growth parameters (oxygen pressure, stirring,

15

pressure, aeration rate and pH regulation) were optimized to achieve an optical density (600nm) in the range of 15.

90 L of this culture were used to inoculate a fermentor with an initial volume of 1500 L (6 % v/v inoculation rate), in an optimized culture media, supplemented with 100 µg/mL of ampicillin. Growth parameters (glucose feed, oxygen pressure, stirring, pressure, aeration rate and pH regulation) were optimized to achieve an optical density (600nm) in the range of 90. IPTG was added at a final concentration of 0,2mM when optical density reached 20. This resulted in around 2 tons of culture broth with 3,4% of dry biomass.

10

Example 2: Thermal bacterial lysis by ultra-high temperature processing (UHT)

A protocol was set for extraction and solubilization of α_{s1} and β caseins from cells at ultra-high temperature.

Biomass concentration and washing

The culture medium obtained as described in Example 1 was concentrated using a self-cleaning disk-stack centrifuge (centrifugal force : 20000 G, temperature < 20 °C). 298 kgs of concentrated cells were collected.

The concentrated biomass was then washed three times with osmosis water, using the same centrifuge. The concentrated biomass was mixed with water, until obtaining a homogeneous suspension. The suspension was then separated by centrifugation, and the first fraction of washing water was eliminated. This washing procedure was repeated twice; to finally obtain a pellet of concentrated cells of 239.1kg.

25

Thermal bacterial lysis

Washed concentrated biomass was then resuspended in 4,2 volumes of osmosis water, until having a homogeneous suspension. 60 L of this resulting suspension were stored at +4°C. The suspension was then divided in four fractions of 10 kgs, and one fraction of 8.15 kgs. The pH of each suspension was adjusted at 8.5, using a 2 M solution of sodium hydroxide (NaOH).

The five fractions were then heated by monitoring a holding time at a given temperature in a pasteurization chamber), using a UHT Pasteurizer (Pasteurizer, OMVE, HTST/UHT System, HT220-DSI, configuration : tubular heat exchanger with a flow rate through the chamber of 16.66-27.2 L/h and a holding time (time

35

within the chamber) of 2-75 seconds. The temperature in the pasteurization chamber was 110-140°C.

Five different conditions were tested on the five bacterial suspensions, in order to determine an optimal and fast thermal treatment, allowing efficient bacterial lysis, *E.coli* proteins precipitation and caseins solubilization. The different set heating temperatures were slightly higher than the temperatures applied in the pasteurization chamber, to ensure that those last temperatures would be reached.

- Trial 1** : temperature = 110 °C, holding time = 75 seconds
- Trial 2** : temperature = 110°C, holding time = 30 seconds
- Trial 3** : temperature = 120 °C, holding time = 30 seconds
- Trial 4** : temperature = 140 °C, holding time = 30 seconds
- Trial 5** : temperature = 140 °C, holding time = 2 seconds, at micro-pilot scale

In each case, the temperature of the product was controlled during the heating step and at the exit of the pasteurization chamber, while the supply pressure was measured during all the process.

In a view to investigate the efficiency of these five different heat treatments, samples were collected before and after each trial and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a polyacrylamide gel (4-20% Criterion™ TGX Stain-Free Protein Gel, Bio-Rad, 5678093) with Precision Plus Protein™ Unstained Standards (Bio-Rad, 1610363EDU) as ladder.

For each trial, 1 mL of bacterial suspension was taken before and after the applied heat treatment, and centrifuged (13000 rpm, 5 minutes, RT), to separate the soluble and insoluble fractions. The supernatant was separated from the pellet, and mixed in a 1:1 ratio with the 2X sample buffer, composed of 0,1 M Tris-HCl, 4% v:v SDS, 0,2% w:v bromophenol blue and 20 % v:v glycerol. The cell pellet was mixed with 1 mL of a 2% v:v SDS solution until obtaining a homogeneous suspension, and mixed in the same ratio with the 2X sample buffer previously described. Each resulting solution was heated at 95°C for 5 minutes and loaded in the wells of a polyacrylamide gel, using a running buffer composed of Tris-HCl (25 mM), glycine (250 mM) and SDS (0,1% v:v). The gel was then visualized under UV

light, in a Gel-Doc™ EZ Imager (Bio-Rad). Diluted samples (3-fold) were also used for better quantification. The ratio of casein content vs total protein content was determined *via* the analysis of each SDS PAGE gels using the software Imagelab, by comparing bands' intensity of caseins and bands' intensity of other proteins from

5 *E.coli*.

Results are summarized in Figures 2 and 3, with undiluted (Figure 2) and diluted (Figure 3) samples. In all tested conditions for pasteurization, *E.coli* cells were efficiently lysed and a large majority of α_{s1} and β caseins were solubilized:

10 total amounts of caseins amounts were very similar before and after pasteurization, but these caseins were essentially in the insoluble fraction before pasteurization (Figure 3, lane 8) and in the soluble fraction after pasteurization (Figure 3, lanes 3-7), indicating the process yield was very high (at least >80%).

15 The proportion of casein vs. total protein in the soluble fraction of the lysate (Figure 3, lanes 4 in each panel) was significantly the same in the five trials, ranging from ranging from 75 % to 77 %. This ratio appeared to be significantly higher than in the insoluble faction of cells before lysis (Figure 3, lanes 3 in each panel), which displayed many additional proteins bands, as can be seen on Figure 3 (lanes 3 in

20 each panel). This ratio was a fortiori higher than in the cell composition (insoluble + soluble) before lysis, showing that the lysate soluble fraction was enriched in caseins as compared with the initial composition.

In addition, the average α_{s1} casein/ β casein ratio was also very similar between

25 the five trials, ranging from 47% to 53%, similar to the initial composition in the cell before treatment, showing that in the condition we used, there was no bias regarding the purification of α_{s1} casein vs. β casein.

Example 3: Thermal lysis of bacteria

30 As described in WO2022253816, synthetic genes coding for alpha-S1, alpha-S2 and beta casein (related to natural genes P02662, P02663 and P02666, respectively), were modified, in order to remove the signal peptide. They were cloned into plasmids and the resulting plasmids were transformed into BL21(DE3) strains. Individual transformed clones were isolated, and for each synthetic gene,

35 one clone was used to inoculate LB medium.

Cells were lysed using two different protocols, and total extracts as well as samples from soluble and insoluble fractions were analyzed by SDS page. Caseins were monitored in the soluble and insoluble fractions of cell extracts using two different protocols. Cells transformed with empty vector were used as control.

5 Protocol 1:

Cell pellets obtained from a 100 mL culture were lysed by resuspension in lysis buffer (50 mM Tris HCl pH 7.5, 1 mg/mL lysozyme, 0,03 mg/mL Dnase). The suspension was incubated on ice for 30 min and then sonicated for 10 seconds (10% amplitude, Q Sonica XL-2000). 10 µL of the total fraction, were taken and
10 kept for SDS-PAGE analysis. The soluble and insoluble fraction were separated by centrifugation at 3220 g and 4°C for 20 min. The supernatant was recovered and supplemented with 10% glycerol for preservation. The pellet was further resuspended in Tris 50 mM with SDS 2% and supplemented with 10% glycerol for preservation. 10 µL of each fraction were taken for SDS-PAGE.

15 Protocol 2:

Cell pellets obtained from a 100 mL culture were lysed by resuspension in Bugbuster (Millipore) with 0,4% Lysonase (Millipore). The mix was incubated at room temperature for 5 min and then centrifuged at 3220 g and 4°C for 20 min. The supernatant was recovered and 10 µL of the supernatant were taken and reserved
20 for SDS-PAGE analysis. The pellet was further resuspended in SDS 2% and 10 µL of the suspension, representing the insoluble fraction, were then taken and preserved for SDS-PAGE analysis.

Alpha-S1 casein was found in both the soluble (S) and insoluble (P) fractions, while alpha-S2 was found essentially in the insoluble fraction, and beta caseins
25 largely (protocol 1) or totally (protocol 2) in the insoluble fraction, as often observed with overexpression of recombinant proteins in E. coli. This suggests that in E. coli, caseins may be present in inclusion bodies. The different quantitative outcomes can be due to different solubilities of beta casein or different stabilities of inclusion bodies depending on the protocol. Nevertheless, recovery of caseins from the
30 soluble fraction of the extracts would result in loss of a large part or of all of the recombinant proteins using these protocols.

The impact of lysis by heating on caseins was tested with the recombinant strains described above. A clone transformed with empty vector was used as control.

Cultures were centrifuged and cell pellets were resuspended in 1 volume of sterile water, centrifuged and washed with another volume of water, and resuspended in 1 volume of water. 1 mL samples of this cell suspension were treated by heating at 95°C for 0 (non-heated), 10, 20, 30, 60, 90 and 120 minutes, resulting in cell lysis, and the soluble and insoluble fractions were separated by centrifugation. The insoluble fraction was resuspended in 1 mL of buffer (50 mM Tris HCl pH 7.5, 300 mM NaCl, 10 mM MgCl₂, 2 mM DTT, 0.5% Triton and Sigmafast Protease Inhibitor (Sigma)) and 10 µL aliquots of both soluble and insoluble fractions were analyzed by SDS-PAGE. In such conditions a 10 µL sample of soluble fraction and a 10 µL sample of insoluble fraction represent about the same amount of total cell extract. With no heating, no cell lysis, or only residual cell lysis in water occurred. The pellet contains basically the entire cells, including the caseins, and this sample actually represents total cell extracts.

It was confirmed that lysis by temperature impacted the distribution of caseins in the soluble and insoluble fractions.

By (or before) 10 minutes of heating at 95°C, alpha-S1 casein was almost entirely found in the soluble fraction, whereas it was found in both the soluble and insoluble fraction in similar amounts without heating.

Casein beta was progressively shifted to the soluble fraction, which contained large amount of this recombinant protein by (or before) 10 minutes of heating, and the major part by 30 minutes. Without heating, beta casein was found mostly or entirely in the insoluble fraction.

The distribution of casein alpha-S2 (entirely in the insoluble fraction without heating) appeared to be less heat-sensitive, and it was still found in great part in the insoluble fraction by 120 minutes of heating. Nevertheless, it appeared in the soluble fraction by 20 minutes of heating at 95°C, and was slightly decreasing in the insoluble fraction over time.

Many other protein bands from the insoluble fraction were diminishing as well over time, but without being shifted into the soluble fraction. In the soluble fraction of the heated samples, only a few faint protein bands could be seen in addition to the casein bands.

These results indicate that lysis by heating is a good way to perform fast purification of caseins, and notably of alpha-S1 and beta casein.

Example 4 : Production of recombinant casein in a 1L fermenter

Acid lactoserum was used as a feedstock, in conjunction with glucose. In order to generate acid lactoserum, semi-skimmed pasteurized milk was incubated with 0,1% of lactic bacteria (Choozit MBT LYO 20 DCU) at 34°C, for about 8 hours, until pH reached a value of about 4,6, to induce curdling. The curd was drained for 48 hours, to recover the lactoserum, and 0,77 kg of lactoserum was recovered for each liter of milk, with a pH of 4,6 and a lactose concentration of 51,3 g/L. This lactoserum was then filtered with a 0.22 µm filter and autoclaved. SDS-PAGE analysis indicted that no residual casein could be observed in this lactoserum.

A culture of recombinant strain described above was used to inoculate a 1L fermenter (Multifors 2 Microbilia 1,4L TV, Infors), in a medium lactoserum and glucose, as carbohydrate sources.

A culture medium supplemented with 50% of lactoserum was used. This medium was also supplemented with 100 µg/ml ampicilline and 2 g/L glucose. In this medium, lactose concentration was about 25,7g/L.

0.5L of medium was inoculated, with a preculture of the recombinant clone, to reach an initial OD₆₀₀ of 0.25 . The fermentation was conducted in a fed-batch mode, at pH=7.2; T=37°C; pO₂=15%, with the culture being fed with a solution containing 600 g/L of D-Glucose. Ampicillin was added after 14 hours (100 µg/ml of culture).

Lactose is capable of inducing the expression of caseins from the pET vector in BL21. However, at the beginning of the run, glucose was capable of repressing lactose uptake, by carbon catabolic repression (Görke, B., Stülke, J. Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nat Rev Microbiol* 6, 613–624 (2008); Postma PW, Lengeler JW, Jacobson GR. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. *Microbiol Rev.* 1993 Sep;57(3):543-94). Thus, at the beginning of the run, only glucose is consumed.

After about 14 hours, with OD₆₀₀=40 (Figure 4), the glucose feed was interrupted for 30 min, resulting in a strong decrease in glucose concentration in the culture medium thereby enabling the metabolic switch enabling lactose consumption and casein expression could be induced. After the 30 min, the glucose feed was resumed, but also reduced to enable culture growth without preventing lactose consumption. In this regard, the induction process was different from the one used by Viitanen et al. (Viitanen et al. 2003, Cheese whey-induced high-cell-density production of recombinant proteins in *Escherichia coli*, *Microbial Cell*

Factories 2:2); in this study, induction was achieved by addition of lactoserum in a medium containing no glucose but supplemented in glycerol. The culture was stopped at T=21 h, with OD₆₀₀=100.7. Lactose could not be detected in the culture medium by this time. Cells were harvested by centrifugation, at room temperature.

- 5 As a negative control, the same medium was used, without lactoserum (replaced with water) , but with the same final concentrations for other components. Thus, glucose is the only carbohydrate source, and no casein expression is expected. The culture was stopped at T=21 h, with OD₆₀₀=94.6. Cells were harvested by centrifugation, at room temperature. As a positive control for casein
- 10 expression, we used the same medium without lactoserum (replaced with water), and induction was achieved by the addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG), a lactose analogue, which inducing effect is not inhibited by glucose, at OD=40. The culture was stopped at T=21 h, with OD₆₀₀=80.0. Cells were harvested by centrifugation, at room temperature.
- 15 Conditions are summarized in Table 3.

	Glucose, non induced	Glucose, induced	Lactoserum and glucose, induced
lactoserum	-	-	+
Lactose	-	-	+ (from lactoserum)
Glucose	2g/L in the initial medium, and glucose feed	2g/L in the initial medium, and glucose feed	2g/L in the initial medium, and reduced glucose feed
Induction step	Casein expression remains repressed by glucose	IPTG addition	Induction by lactose, upon glucose depletion when the glucose feed is interrupted

Table 3. Summary of culture conditions, and induction mechanisms in the different conditions

- 20 For analysis, an aliquot of cells was centrifuged and resuspended in 1/2 volume of sterile water. 1 mL samples of this cell suspension were treated by heating at 95°C for 60 minutes, resulting as described previously

(PCT/EP2022/064728) in cell lysis, casein solubilization, and degradation/and or precipitation of other proteins in the soluble factions and the soluble and insoluble fractions were separated by centrifugation. The insoluble fraction was resuspended in 1 mL of a 2% SDS solution and 10 µL aliquots of both soluble and insoluble

5 fractions were analyzed by SDS-PAGE, together with total lysate..

Purified beta casein from milk (Sigma) was used as control, but as observed previously by others displayed a higher apparent molecular weight (Simons et al. Overproduction of bovine beta-casein in *Escherichia coli* and engineering of its main chymosin cleavage site (1993) Protein Engineering 7:763-770).

10 As shown on Figure 5, induction casein expression was efficiently induced both by IPTG in the presence of glucose and in the medium containing lactoserum. Casein production was very efficient with lactoserum. Recombinant casein production was estimated to be in the range of 2.5 g/L I glucose, and 5 g/L with lactoserum, twice more, and 1, times higher, when normalized to cell density (see

15 OD₆₀₀ above).

The amount of consumed sugars was estimated in all samples. In the conditions used, E. coli BL21 is capable of glucose uptake and metabolization. At low glucose concentrations, it is also capable of lactose uptake, and hydrolysis into glucose and galactose; whereas galactose is very poorly consumed, the glucose

20 resulting from lactose is very efficiently metabolized. Therefore, glucose consumption was estimated, making the difference between glucose added in the medium, and glucose resulting from lactose hydrolysis. Estimation was based on the added sugar mass, given that at the end of the experiments, glucose concentration in the medium was negligible.

25 Results are shown in Table 4. As shown in this table, both added glucose and glucose resulting from lactose hydrolysis were efficiently consumed during the fermentation runs, although the consumption of glucose from lactoserum, (from lactose) was very low until glucose concentration dropped. In the conditions we used, glucose resulting from lactose hydrolysis represented 12% of total glucose

30 consumption, showing that lactose had contributed to the carbohydrate feedstock. Galactose resulting from lactose hydrolysis was not consumed.

	Standard conditions	No induction	Lactoserum
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Total added glucose (g)	48.6	48.5	48.5
Total added lactose (g)	-	-	12.3
Mass of glucose that can be derived by hydrolysis of added lactose (g)	-	-	6.5
Glucose in medium at end of experiment (g)	n.d	1	non detected
Galactose in medium at end of experiment (g)	-	-	5.5
Lactose in medium at end of experiment (g)	-	-	Non detected
A: Consumed from added glucose (g) (% of total consumed)	48,6 (100%)	47,5 (98%)	48.5 (88%)
B: Consumed from hydrolysed lactose (% of total consumed)	-	-	6.5 (12%)
Total glucose consumed (g) (% of total consumed)	48.6	47.5	55.0 (100%)

Table 4: sugar consumption

Example 5: Environmental impact

Environmental impact of caseins obtained by bacterial fermentation with various sugars as feedstock can be quantified using a multi-criteria life cycle assessment (LCA) approach. The study was conducted according to European EF 3.1 method (<https://eplca.jrc.ec.europa.eu/LCDN/developerEF.xhtml>).

The life Cycle Inventory (LCI) is based on average primary data from experimental results at laboratory and pilot scale. Data not collected directly from experimental results were based on industrial projections from the production process, notably on utilities and processes, or from its partners.

The impact of various sugar feedstocks was tested.

Glucose was considered as obtained from corn, origin: Europe

Sucrose was considered as obtained from sugar beets, origin: rest of world
(whole world, except Switzerland)

5 Molasses was considered as obtained from sugar beets, origin: rest of world,
and considered to contain 50% sugar by weight in sugar

Whey was considered as obtained from cheese manufacturing process, with a
global (whole world) origin.

10 It was assumed that differences would only consist in the environmental impact
of the feedstock, and that feedstock change would not impact other parameters.

The LCI for each sugar feedstock was calculated using the Ecoinvent 3.9
database according to its origin.

As compared with example 4, differences are:

- 15
- The strain is considered to be gal+, i.e., having a proficient Leloir pathway,
and able to consume the galactose moiety of lactose,
 - When sucrose or molasses are used, the strain is capable of consuming
sucrose, as described above in text,
 - Sugars fed to the strain comprise 100% of glucose or 68,6% of glucose and
20 31,4% ,of another sugar, which can be sucrose, sucrose from molasses or
lactose from acid whey,
 - Fermenter is 350L,
 - A part of the water is recycled in the process.

The purification process comprised the steps of

- 25
- Recovering the biomass and washing it three times as described in
Example 2
 - Heating the washed biomass an hour at 95°C
 - Recovering the supernatant
 - Precipitating the caseins at pH4.6 using HCl and washing the precipitate

30

 - Resuspending the pellet of acid casein, and neutralizing (pH=7) it with
sodium hydroxyde
 - Drying the sodium caseinate by spray drying, to bring moisture down to 5%.

Life Cycle Impact assessment (LCIA), was performed using datasets from the
35 Ecoinvent 3.9 database (Zurich, Switzerland) and Agrybalise (Ademe, France).

The impacts were entirely allocated to the casein composition, and not to the byproducts of the process such as biomass. The acid whey used as carbon feedstock can be considered as a waste or as a valorized by-product from soft cheese making. Therefore, in the first case there is no environmental impact linked to whey use. In the second case, it contributes to the final's product emissions according to the dataset EF 3.1 from ecoinvent 3.9.

Results for the 16 criteria (see above in text for unit definition), expressed in relative value as compared with glucose as sole feedstock, are shown in Figure 6.

10

For acid whey as waste, environmental impacts are significantly inferior to the impact of glucose for all 16 criteria, and inferior or equal to the impact of other sugars, for all criteria but for criteria

- number ix (human toxicity: non carcinogenic) as compared with molasses because of the property of sugar beets derivatives to fix heavy metals during the sucrose production process.
- number v, ix, and to a lesser extent, number iii, as compared with sucrose.

15

For valorized acid whey with an impact, environmental impacts are superior to other sugars for the criteria land use, because of the animal contribution, that is equivalent to large land use, and impacted in this case to the feedstock. However, it is:

20

- lower than glucose for all the other criteria
- lower than sucrose for 11 criteria out of 16 (i, ii, iv, vi, vii, x, xii, xiii, xiv, xv, xvi),
- lower than molasses for 8 criteria out of 16 (ii, v, vi, vii, x, xiv, xv, xvi)

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In summary, using whey as the carbon source in the process for obtaining recombinant casein from microorganism sources has globally a better environmental impact as the one measured when using other carbon sources, for 50% or more of the criteria.

30

Example 6: Strains construction for lactose and galactose consumption

E. coli BL21(DE3) strains is able to consume glucose and partially lactose (galactose generated from lactose is not consumed). New strains E. coli BL21(DE3) were generated in order to express galETKM operon from E.coli K12 strain, necessary for galactose consumption. The galETKM operon contains four genes; *galE* encodes an epimerase, *galT* encodes a uridylyltransferase; *galK*, encodes a galactokinase; *galM* facilitates the interconversion between β-galactose and α-galactose.

The BL21(DE3) strains constructed harbor the gal operon under different promoters on either a low copy plasmid (pSO148 and pSO149) or into the chromosome (see sequences, table 5).

Genetic constructions	Sequences
pSO148 (SEQ ID NO: 6)	TAGTAAAGCCCTCGCTAGATTTTAATGCGGATGTTGCGATTACTTCGCCAA CTATTGCGATAACAAGAAAAAGCCAGCCTTTCATGATATATCTCCCAATTT GTGTAGGGCTTATTATGCACGCTTAAAAATAATAAAAGCAGACTTGACCTG ATAGTTTGGCTGTGAGCAATTATGTGCTTAGTGCATCTAACGCTTGAGTTA AGCCGCGCCGCGAAGCGGCGTCGGCTTGAACGAATTGTTAGACATTATTTG CCGACTACCTTGGTGATCTCGCCTTTCACGTAGTGGACAAATTCTTCCAAC TGATCTGCGCGCGAGGCCAAGCGATCTTCTTCTTGTCCTCAAGATAAGCCTGT CTAGCTTCAAGTATGACGGGCTGATACTGGGCCGGCAGGCGCTCCATTGCC CAGTCGGCAGCGACATCCTTCGGCGCGATTTTGCCGGTTACTGCGCTGTAC CAAATGCGGGACAACGTAAGCACTACATTTGCTCATCGCCAGCCCAGTCG GGCGCGAGTTCCATAGCGTTAAGGTTTCATTTAGCGCCTCAAATAGATCC TGTTCAAGGAACCGGATCAAAGAGTTCTCCGCCGCTGGACCTACCAAGGCA ACGCTATGTTCTCTTGCTTTTGTGTCAGCAAGATAGCCAGATCAATGTCGATC GTGGCTGGCTCGAAGATACCTGCAAGAATGTCATTGCGCTGCCATTCTCCA AATTGCAGTTCGCGCTTAGCTGGATAACGCCACGGAATGATGTCGTCGTGC ACAACAATGGTGACTTCTACAGCGCGGAGAATCTCGCTCTCTCCAGGGGAA GCCGAAGTTTCCAAAAGGTCGTTGATCAAAGCTCGCCGCGTTGTTTCATCA AGCCTTACGGTCACCGTAACCAGCAAATCAATATCACTGTGTGGCTTCAGG CCGCCATCCACTGCGGAGCCGTACAAATGTACGGCCAGCAACGTGCGTTTCG AGATGGCGCTCGATGACGCCAACTACCTCTGATAGTTGAGTCGATACTTCG GCGATCACCGCTTCCCTCATGATGTTTAACTTTGTTTATAGGGCGACTGCCC TGCTGCGTAACATCGTTGCTGCTCCATAACATCAAACATCGACCCACGGCG TAACGCGCTTGCTGCTTGATGCCCCAGGCATAGACTGTACCCCAAAAAA CAGTCATAACAAGCCATGAAAACCGCCACTGCGCCGTTACCACCGCTGCGT TCGGTCAAGGTTCTGGACCAGTTGCGTGAGCGCATACGTAAGTGCATTAC

	AGCTTACGAACCGAACAGGCTTATGTCCACTGGGTTTCGTGCCTTCATCCGT TTCCACGGTGTGCGTCACCCGGCAACCTTGGGCAGCAGCGAAGTCGAGGCA TTTCTGTCTGGCTGGCGAACGAGCGCAAGGTTTCGGTCTCCACGCATCGT CAGGCATTGGCGGCCTTGCTGTTCTTCTACGGCAAGGTGCTGTGCACGGAT CTGCCCTGGCTTCAGGAGATCGGAAGACCTCGGCCGTCGCGGCGCTTGCCG GTGGTGCTGACCCCGGATGAAGTGGTTCGCATCCTCGGTTTTCTGGAAGGC GAGCATCGTTTGTTGCGCCAGCTTCTGTATGGAACGGGCATGCGGATCAGT GAGGGTTTGCAACTGCGGGTCAAGGATCTGGATTTTCGATCACGGCACGATC ATCGTGCGGGAGGGCAAGGGCTCCAAGGATCGGGCCTTGATGTTACCCGAG AGCTTGGCACCCAGCCTGCGCGAGCAGGGGAATTAATCCACGGGTTTTG CTGCCCGCAAACGGGCTGTTCTGGTGTTGCTAGTTTGTTATCAGAATCGCA GATCCGGCTTCAGCCGGTTTTGCCGGCTGAAAGCGCTATTTCTTCCAGAATT GCCATGATTTTTTCCCCACGGGAGGCGTCACTGGCTCCCGTGTTGTCGGCA GCTTTGATTCGATAAGCAGCATCGCCTGTTTCAGGCTGTCTATGTGTGACT GTTGAGCTGTAACAAGTTGTCTCAGGTGTTCAATTTTCATGTTCTAGTTGCT TTGTTTTACTGGTTTCACCTGTTCTATTAGGTGTTACATGCTGTTTCATCTG TTACATTGTCGATCTGTTTCATGGTGAACAGCTTTGAATGCACCAAAAACCTC GTAAAAGCTCTGATGTATCTATCTTTTTTACACCGTTTTTCATCTGTGCATA TGGACAGTTTTCCCTTTGATATGTAACGGTGAACAGTTGTTCTACTTTTGT TTGTTAGTCTTGATGCTTCACTGATAGATACAAGAGCCATAAGAACCTCAG ATCCTTCCGTATTTAGCCAGTATGTTCTCTAGTGTGGTTCGTTGTTTTTGC GTGAGCCATGAGAACGAACCATTGAGATCATACTTACTTTGCATGTCACTC AAAAATTTTGCCTCAAAACTGGTGAGCTGAATTTTTTGCAGTTAAAGCATCG TGTAAGTGTTTTTCTTAGTCCGTTATGTAGGTAGGAATCTGATGTAATGGTT GTTGGTATTTTGTCAACCATTCATTTTTATCTGGTTGTTCTCAAGTTCGGTT ACGAGATCCATTTGTCTATCTAGTTCAACTTGAAAATCAACGTATCAGTC GGGCGGCCTCGCTTATCAACCACCAATTTTCATATTGCTGTAAGTGTTTAAA TCTTTACTTATTGGTTTCAAACCCATTGGTTAAGCCTTTTAAACTCATGG TAGTTATTTTCAAGCATTAACATGAACTTAAATTCATCAAGGCTAATCTCT ATATTTGCCTTGTGAGTTTTCTTTGTGTTAGTTCTTTTAATAACCACTCA TAAATCCTCATAGAGTATTTGTTTTCAAAGACTTAACATGTTCCAGATTA TATTTTATGAATTTTTTAACTGGAAAAGATAAGGCAATATCTCTTCACTA AAAATAATTCTAATTTTTTCGCTTGAGAACTTGGCATAGTTTGTCCACTGG AAAATCTCAAAGCCTTTAACCAGGATTCCTGATTTCCACAGTTCCTCGTC ATCAGCTCTCTGGTTGCTTTAGCTAATACACCATAAGCATTTTCCCTACTG ATGTTTCATCATCTGAGCGTATTGGTTATAAGTGAACGATACCGTCCGTTCT TTCCTTGTAGGGTTTTCAATCGTGGGGTTGAGTAGTGCCACACAGCATAAA ATTAGCTTGGTTTCATGCTCCGTTAAGTCATAGCGACTAATCGCTAGTTCA
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	TAGTCAAAAGCCTCCGACCGGAGGCTTTTGACTGTCCCTTTGATATTGCAT CCCGCGTATATAATATGTCTAATAGAAATAATTTTGTTTAACTTTATACGT AGGGTGTACAGGGGCGATCGCGGGGTCGACGGCTGTTTTGGCGGATGAGAG AAGATTTTCAGCCTGATACAGATTAAATCAGAACGCAGAAGCGGTCTGATA AAACAGAATTTGCCTGGCGGCAGTAGCGCGGTGGTCCCACCTGACCCCATG CCGAACTCAGAAGTGAAACGCCGTAGCGCCGATGGTAGTGTGGGGTCTCCC CATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACGAAAGGCTCAGTC GAAAGACTGGGCCTTTCGTTTTATCTGTTGTTGTGCGGTGAACGCTCTCCT GAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGCGAAGCAACGGCC CGGAGGGTGGCGGGCAGGACGCCGCCATAAACTGCCAGGCATCAAATTAA GCAGAAGGCCATCCTGACGGATGGCCTTTTTGCGTTTCTACAACTCCAGC TGGATCGGGCGCTAGAGTATACATTTAAATGGTACCCTCTAGTCAAGGCCCT TAAGTGAGTCGTATTACGGACTGGCCGTCGTTTTACAACGTCGTGACTGGG AAAACCCTGGCGTTACCCAACCTAATCGCCTTGACGACATCCCCCTTTCG CCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGT TGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGC ATCTGTGCGGTATTTACACCGCATATGGTGCCTCTCAGTACAATCTGCT CTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACCCGCTGACG AGCT
pSO149 (SEQ ID NO: 7)	TAGTAAAGCCCTCGCTAGATTTTAATGCGGATGTTGCGATTACTTCGCCAA CTATTGCGATAACAAGAAAAAGCCAGCCTTTCATGATATATCTCCCAATTT GTGTAGGGCTTATTATGCACGCTTAAAAATAATAAAAGCAGACTTGACCTG ATAGTTTGGCTGTGAGCAATTATGTGCTTAGTGCATCTAACGCTTGAGTTA AGCCGCGCCGCGAAGCGGCGTCGGCTTGAACGAATTGTTAGACATTATTTG CCGACTACCTTGGTGATCTCGCCTTTCACGTAGTGGACAAATTCTTCCAAC TGATCTGCGCGCGAGGCCAAGCGATCTTCTTCTTGTCGAAGATAAGCCTGT CTAGCTTCAAGTATGACGGGCTGATACTGGGCCGGCAGGCGCTCCATTGCC CAGTCGGCAGCGACATCCTTCGGCGCGATTTTGCCGTTACTGCGCTGTAC CAAATGCGGGACAACGTAAGCACTACATTTGCTCATCGCCAGCCAGTCG GGCGGCGAGTTCCATAGCGTTAAGGTTTCATTTAGCGCCTCAAATAGATCC TGTTCAGGAACCGGATCAAAGAGTTCCTCCGCGCTGGACCTACCAAGGCA ACGCTATGTTCTCTTGCTTTTGTGAGCAAGATAGCCAGATCAATGTCGATC GTGGCTGGCTCGAAGATACCTGCAAGAATGTCATTGCGCTGCCATTCTCCA AATTGCAGTTCGCGCTTAGCTGGATAACGCCACGGAATGATGTCGTCGTGC ACAACAATGGTGACTTCTACAGCGCGGAGAATCTCGCTCTCTCCAGGGGAA GCCGAAGTTTCCAAAAGGTCGTTGATCAAAGCTCGCCGCGTTGTTTCATCA AGCCTTACGGTCACCGTAACCAGCAAATCAATATCACTGTGTGGCTTCAGG

	CCGCCATCCACTGCGGAGCCGTACAAATGTACGGCCAGCAACGTCCGGTTCG AGATGGCGCTCGATGACGCCAACTACCTCTGATAGTTGAGTCGATACTTCG GCGATCACCGCTTCCCTCATGATGTTTAACTTTGTTTTAGGGCGACTGCCC TGCTGCGTAACATCGTTGCTGCTCCATAACATCAAACATCGACCCACGGCG TAACGCGCTTGCTGCTTGATGCCCCGAGGCATAGACTGTACCCCAAAAAA CAGTCATAACAAGCCATGAAAACCGCCACTGCGCCGTTACCACCGCTGCGT TCGGTCAAGGTTCTGGACCAGTTGCGTGAGCGCATACGCTACTTGCATTAC AGCTTACGAACCGAACAGGCTTATGTCCACTGGGTTTCGTGCCTTCATCCGT TTCCACGGTGTGCGTCACCCGGCAACCTTGGGCAGCAGCGAAGTCGAGGCA TTTCTGTCTGGCTGGCGAACGAGCGCAAGGTTTCGGTCTCCACGCATCGT CAGGCATTGGCGGCCTTGCTGTTCTTCTACGGCAAGTGCTGTGCACGGAT CTGCCCTGGCTTCAGGAGATCGGAAGACCTCGGCCGTCGCGGCGCTTGCCG GTGGTGCTGACCCCGGATGAAGTGGTTCGCATCCTCGGTTTTCTGGAAGGC GAGCATCGTTTTGTTGCGCCAGCTTCTGTATGGAACGGGCATGCGGATCAGT GAGGGTTTGCAACTGCGGGTCAAGGATCTGGATTTGATCACGGCACGATC ATCGTGCGGGAGGGCAAGGGCTCCAAGGATCGGGCCTTGATGTTACCCGAG AGCTTGGCACCCAGCTGCGCGAGCAGGGGAATTAATCCCACGGGTTTTG CTGCCCGCAAACGGGCTGTTCTGGTGTTGCTAGTTTGTTATCAGAATCGCA GATCCGGCTTCAGCCGGTTTGCCGGCTGAAAGCGCTATTTCTTCCAGAATT GCCATGATTTTTTCCCCACGGGAGGCGTCACTGGCTCCCGTGTTGTGCGCA GCTTTGATTCGATAAGCAGCATCGCCTGTTTCAGGCTGTCTATGTGTGACT GTTGAGCTGTAACAAGTTGTCTCAGGTGTTCAATTTTCATGTTCTAGTTGCT TTGTTTTACTGGTTTCACCTGTTCTATTAGGTGTTACATGCTGTTTCATCTG TTACATTGTCGATCTGTTTCATGGTGAACAGCTTTGAATGCACCAAAAACTC GTAAAGCTCTGATGTATCTATCTTTTTTACACCGTTTTTCATCTGTGCATA TGGACAGTTTTTCCCTTTGATATGTAACGGTGAACAGTTGTTCTACTTTTGT TTGTTAGTCTTGATGCTTCACTGATAGATACAAGAGCCATAAGAACCTCAG ATCCTTCCGTATTTAGCCAGTATGTTCTCTAGTGTGGTTCGTTGTTTTTGC GTGAGCCATGAGAACGAACCATTGAGATCATACTTACTTTGCATGTCACTC AAAAATTTTGCCTCAAAACTGGTGAGCTGAATTTTGCAGTTAAAGCATCG TGTAAGTTTTTCTTAGTCCGTTATGTAGGTAGGAATCTGATGTAATGGTT GTTGGTATTTTGTCAACCATTCATTTTTATCTGGTTGTTCTCAAGTTCGGTT ACGAGATCCATTTGTCTATCTAGTTCAACTTGAAAATCAACGTATCAGTC GGGCGGCCTCGCTTATCAACCACCAATTTTCATATTGCTGTAAGTGTTTAAA TCTTTACTTATTTGGTTTCAAACCCATTGGTTAAGCCTTTTAAACTCATGG TAGTTATTTTCAAGCATTAACATGAACTTAAATTCATCAAGGCTAATCTCT ATATTTGCCTTGTGAGTTTTCTTTGTGTTAGTTCTTTTAATAACCACTCA TAAATCCTCATAGAGTATTTGTTTTCAAAGACTTAACATGTTCCAGATTA
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	TATTTTATGAATTTTTTTAACTGGAAAAGATAAGGCAATATCTCTTCACTA AAAAC TAATTCTAATTTTTTCGCTTGAGAACTTGGCATAGTTTGTCCACTGG AAAATCTCAAAGCCTTTAACC AAAGGATTCCTGATTTCCACAGTTCTCGTC ATCAGCTCTCTGGTTGCTTTAGCTAATACACCATAAGCATTTTCCCTACTG ATGTT CATCATCTGAGCGTATTGGTTATAAGTGAACGATAACCGTCCGTCT TTCCTTG TAGGGTTTTCAATCGTGGGGTTGAGTAGTCCACACAGCATAAA ATTAGCTTGGTTTCATGCTCCGTTAAGTCATAGCGACTAATCGCTAGTTCA TTTGCTTTGAAAACAAC TAATTCAGACATACATCTCAATTGGTCTAGGTGA TTTTAATCACTATACCAATTGAGATGGGCTAGTCAATGATAATTACTAGTC CTTTTCCTTTGAGTTGTGGGTATCTGTAAATTCGTCTAGACCTTTGTGGA AAACTTGTAATTCGTCTAGACCCTCTGTAAATTCGCTAGACCTTTGTGT GTTTTTTTTGTTTATATTCAAGTGGTTATAATTTATAGAATAAAGAAAGAA TAAAAAAGATAAAAAGAATAGATCCCAGCCCTGTGTATAACTCACTACTT TAGTCAGTTCCGCAGTATTACAAAAGGATGTCGCAAACGCTGTTTGCTCCT CTACAAAACAGACCTTAAACCCCTAAAGGCTTAAGTAGCACCCCTCGCAAGC TCGGGCAAATCGCTGAATATTCCTTTTGTCTCCGACCATCAGGCACCTGAG TCGCTGTCTTTTTCGTGACATTCAGTTCGCTGCGCTCACGGCTCTGGCAGT GAATGGGGGTAAATGGCACTACAGGCGCCTTTTATGGATTCATGCAAGGAA ACTACCCATAATACAAGAAAAGCCCGTCACGGGCTTCTCAGGGCGTTTTAT GGCGGGTCTGCTATGTGGTGCTATCTGACTTTTTGCTGTTTCAGCAGTTCCCT GCCCTCTGATTTTCCAGTCTGACCACTTCGGATTATCCCGTGACAGGT CAT TCAGACTGGCTAATGCACCCAGTAAGGCAGCGGTATCATCAACAGGCTTAC CCGTCTTACTGTCGGAATTC AAATCATAAAAAATTTATTTGCTTTCAGGA AAATTTTTCTGTATAATAGATTCATAAATTTGAGAGAGGAGTTTTCACACA GGAGGTTAATTAAGGGGCGGCCGCCCGGCGCGCCGCCATCGATGCGCCCG GGGCGCCTGCAGGGGGGACTCCTGTTGATAGATCCAGTAATGACCTCAGAA CTCCATCTGGATTTGTT CAGAACGCTCGGTTGCCCGGGCGTTTTTTATT GGTGAGAATAGTACTATTTAAATAGTCAAAGCCTCCGACCGGAGGCTTTT GACTGACCTATTGACAATTAAAGGCTAAAATGCTATAATTCCACTAATAGA AATAATTTTGTTTAACTTTACCATGGGGGGCTAGCGGGAAGCTTGGGCTCG AGATGAGAGTTCTGGTTACCGGTGGTAGCGGTTACATTGGAAGTCATACCT GTGTGCAATTACTGCAAACGGTCATGATGTCATCATTTCTTGATAACCTCT GTAACAGTAAGCGCAGCGTACTGCCTGTTATCGAGCGTTTAGGCGGCAAAC ATCCAACGTTTGTTGAAGGCGATATTCGTAACGAAGCGTTGATGACCGAGA TCCTGCACGATCACGCTATCGACACCGTGATCCACTTCGCCGGGCTGAAAG CCGTGGGCGAATCGGTACAAAACCGCTGGAATATTACGACAACAATGTCA ACGGCACTCTGCGCCTGATTAGCGCCATGCGCGCCGCTAACGTCAAAAAC TTATTTT TAGCTCCTCCGCCACCGTTTATGGCGATCAGCCCAAATTC CAT
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	ACGTTGAAAGCTTCCCGACCGGCACACCGCAAAGCCCTTACGGCAAAGCA AGCTGATGGTGGAACAGATCCTCACCGATCTGCAAAAAGCCCAGCCGGACT GGAGCATTGCCCTGCTGCGCTACTTCAACCCGGTTGGCGCGCATCCGTCCG GCGATATGGGCGAAGATCCGCAAGGCATTCCGAATAACCTGATGCCATACA TCGCCCAGGTTGCTGTAGGCCGTCGCGACTCGCTGGCGATTTTTGGTAACG ATTATCCGACCGAAGATGGTACTGGCGTACGCGATTACATCCACGTAATGG ATCTGGCGGACGGTCACGTCGTGGCGATGGAAAACTGGCGAACAAGCCAG GCGTACACATCTACAACCTCGGCGCTGGCGTAGGCAACAGCGTGCTGGACG TGGTTAATGCCTTCAGCAAAGCCTGCGGCAAACCGGTTAATTATCATTTTG CACCGCGTCGCGAGGGCGACCTTCCGGCCTACTGGGCGGACGCCAGCAAAG CCGACCGTGAAGTGAAGTGGCGCGTAACGCGCACACTCGATGAAATGGCGC AGGACACCTGGCACTGGCAGTCACGCCATCCACAGGGATATCCCGATTAAAG GAACGACCATGACGCAATTTAATCCCGTTGATCATCCACATCGCCGCTACA ACCCGCTCACCGGGCAATGGATTCTGGTTTCACCGCACCGCGCTAAGCGCC CCTGGCAGGGGGCGCAGGAAACGCCAGCCAAACAGGTGTTACCTGCGCACG ATCCAGATTGCTTCCTCTGCGCAGGTAATGTGCGGGTGACAGGCGATAAAA ACCCCGATTACACCGGGACTTACGTTTTCACTAATGACTTTGCGGCTTTGA TGTCTGACACGCCAGATGCGCCAGAAAGTCACGATCCGCTGATGCGTTGCC AGAGCGCGCGCGGCACCAGCCGGTGATCTGCTTTTCACCGGATCACAGTA AAACGCTGCCAGAGCTCAGCGTTGCAGCATTGACGGAAATCGTCAAAACCT GGCAGGAGCAAACCGCAGAACTGGGGAAAACGTACCCATGGGTGCAGGTTT TTGAAAACAAAGGCGCGCGATGGGCTGCTCTAACCCGCATCCGCACGGTC AGATTTGGGCAAATAGCTTCCTGCCTAACGAAGCTGAGCGGAAGACCGCC TGCAAAAAGAATATTTTGCCGAACAGAAATCACCAATGCTGGTGGATTATG TTCAGCGCGAGCTGGCAGACGGTAGCCGTACCGTTGTCGAAACCGAACACT GGTTAGCCGTCGTGCCTTACTGGGCTGCCTGGCCGTTGAAACGCTACTGC TGCCCAAAGCCACGTTTTACGGATCACCGATTTGACCGACGCCAGCGCA GCGATCTGGCGCTGGCGTTGAAAAAGCTGACCAGTCGTTATGACAACCTCT TCCAGTGCTCCTTCCCCTACTCTATGGGCTGGCACGGCGCGCCATTTAATG GCGAAGAGAATCAACACTGGCAGCTGCACGCGCACTTTTATCCGCCTCTGC TGCGCTCCGCCACCGTACGTAAATTTATGGTTGGTTATGAAATGCTGGCAG AGACCCAGCGAGACCTGACCGCAGAACAGGCAGCAGAGCGTTTGCGCGCAG TCAGCGATATCCATTTTCGCGAATCCGAGTGTAAGAAATGAGTCTGAAAG AAAAACACAATCTCTGTTTGCCAACGCATTTGGCTACCCTGCCACTCACA CCATTCAGGCGCCTGGCCGCGTGAATTTGATTGGTGAACACACCGACTACA ACGACGGTTTCGTTCTGCCCTGCGCGATTGATTATCAAACCGTGATCAGTT GTGCACCACGCGATGACCGTAAAGTTCGCGTGATGGCAGCCGATTATGAAA ATCAGCTCGACGAGTTTTCCCTCGATGCGCCCATTGTGCGACATGAAACT
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	ATCAATGGGCTAACTACGTTTCGTGGCGTGGTGAAACATCTGCAACTGCGTA ACAACAGCTTCGGCGGCGTGGACATGGTGATCAGCGGCAATGTGCCGCAGG GTGCCGGGTAAAGTTCTTCCGCTTCACTGGAAGTCGCGGTGCGAACCGTAT TGCAGCAGCTTTATCATCTGCCGCTGGACGGCGCACAAATCGCGCTTAACG GTCAGGAAGCAGAAAACAGTTTGTAGGCTGTAAGTGCAGGATCATGGATC AGCTAATTTCCGCGCTCGGCAAGAAAGATCATGCCTTGCTGATCGATTGCC GCTCACTGGGGACCAAAGCAGTTTCCATGCCCCAAAGGTGTGGCTGTCTGCA TCATCAACAGTAACTTCAAACGTACCCTGGTTGGCAGCGAATACAACCCC GTCGTGAACAGTGCGAAACCGGTGCGCGTTTCTTCCAGCAGCCAGCCCTGC GTGATGTCACCATTGAAGAGTTCAACGCTGTTGCGCATGAACTGGACCCGA TCGTGGCAAACGCGTGCGTCATATACTGACTGAAAACGCCCGCACCGTTG AAGCTGCCAGCGCGCTGGAGCAAGGCGACCTGAAACGTATGGGCGAGTTGA TGGCGGAGTCTCATGCCTCTATGCGCGATGATTTCGAAATCACCGTGCCGC AAATTGACACTCTGGTAGAAATCGTCAAAGCTGTGATTGGCGACAAAGGTG GCGTACGCATGACCGGCGGCGGATTTGGCGGCTGTATCGTCGCGCTGATCC CGGAAGAGCTGGTGCTGCCGTACAGCAAGCTGTGCTGAAACAATATGAAG CAAAAACAGGTATTAAGAGACTTTTTACGTTTGTAACCATCACAAAGGAG CAGGACAGTGCTGAACGAAACTCCCGCACTGGCACCCGATGGTCAGCCGTA CCGACTGTTAACCTTTCGTAACAACGCAGGGATGGTAGTCACGCTGATGGA CTGGGGTGCGACTTTACTTTCCGCCCCGATTTCCGCTTTCCGATGGCAGCGT CCGCGAGGCGCTGCTCGGCTGTGCCAGCCCGGAATGCTATCAGGATCAGGC CGCGTTTCTGGGGGCTCTATTGGTCGTTATGCCAACCGTATCGCCAATAG CCGTATACCTTTGACGGTGAAACCGTGACGCTTTCCGCAAGTCAGGGCGT TAACCAGCTGCACGGCGGGCCGGAAGGGTTCGACAAACGTCGCTGGCAGAT TGTGAACCAGAACGATCGTCAGGTGCTGTTTGCCCTGAGTTCAGATGATGG TGATCAGGGCTTCCCGGTAATCTCGGCGCGACGGTGCAATATCGTCTGAC CGACGATAACCGTATCTCCATTACTTATCGCGCCACAGTTGATAAACCTTG CCCGGTGAATATGACTAATCACGTCTATTTCAATCTTGACGGCGAGCAGTC TGACGTGCGCAATCACAGTTGCAGATTCTGGCGGACGAATATCTGCCGGT TGATGAAGGCGGCATTCCGCACGACGGCCTGAAATCTGTGCGCCGAACGTC TTTTGATTTCCGCAGCGCCAAAATCATCGCCAGTGAGTTTCTTGCCGACGA CGATCAGCGCAAAGTGAAAGGTTACGATCACGCATTCTTGTTACAGGCCAA AGGCGATGGCAAGAAAGTGGCGGCGCATGTCTGGTCAGCAGATGAAAAATT GCAGCTGAAGGTCTACACCACCGCTCCGGCTCTGCAATTCTACTCCGGCAA CTTCCTCGGCGGCACACCGTCGCGGGGAACCGAACCTTACGCCGACTGGCA AGGGCTGGCTCTGGAAAGCGAGTTTCTACCGGACAGCCCGAACCCCTGA ATGGCCGCAACCGGACTGCTTCTGCGTCCTGGCGAAGAGTATTCCAGCCT GACGGAATATCAGTTTATTGCTGAGTAATGTTACGCCCTCGTCGGATCCGG
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	GTCTAGAGGGGTTTAAACGGGACGCGTACTGGCCTAGCAAACACAGAAAAA AGCCCGCACCTGACAGTGCGGGCTTTTTTTTCCCGCGGATTTAAATAGTC AAAAGCCTCCGACCGGAGGCTTTTGACTGTCCCTTTGATATTGCATCCCGC GTATATAATATGTCTAATAGAAATAATTTTGTTTAACTTTATACGTAGGGT GTACAGGGGCGATCGCGGGTTCGACGGCTGTTTTGGCGGATGAGAGAAGAT TTTCAGCCTGATACAGATTAAATCAGAACGCAGAAGCGGTCTGATAAAACA GAATTTGCCTGGCGGCAGTAGCGCGGTGGTCCCACCTGACCCCATGCCGAA CTCAGAAGTGAAACGCCGTAGCGCCGATGGTAGTGTGGGGTCTCCCCATGC GAGAGTAGGGAAGTCCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAG ACTGGGCCTTTCGTTTTATCTGTTGTTTGTGCGGTGAACGCTCTCCTGAGTA GGACAAATCCGCCGGGAGCGGATTTGAACGTTGCGAAGCAACGGCCCGGAG GGTGGCGGGCAGGACGCCCGCCATAAACTGCCAGGCATCAAATTAAGCAGA AGGCCATCCTGACGGATGGCCTTTTTGCGTTTCTACAACTCCAGCTGGAT CGGGCGCTAGAGTATACATTTAAATGGTACCCTCTAGTCAAGGCCTTAAGT GAGTCGTATTACGGACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAAC CCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGC TGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGC AGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTG TGCGGTATTTACACCGCATATGGTGCACCTCTCAGTACAATCTGCTCTGAT GCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGAGCT
galETKM construction in ss9 locus (SEQ ID NO: 8)	GCAGAGTGGTTAACCTTGCAGACCGCCCGGTTGCCCGTACATTGGCGGCA TCAGAATGCCGTGGTGAGTATTGTCCGGTAAGAATCGTGGCGCGGG TTGGGGAAGAACTCGGTTGAGAATACGCCGAAGTTAAAATCAGCCCCCTGGC TGGCAACGGCGTCGATATCTGGTGTAGGGTTACCCACCGCCACGCCCGCGC CGTTAAAGCCAACATCCATCCAGCCCACATCATCCAGCAAGAAAACAACCA CGTTCGGTTTCTTACCGTTTTTTTTCTCAAGTTCTGCCAGCTTCTGCTGGG TTTCTTTGTCTTGTGCAGGATGCTGCATCACCGGCATCATGTTGTGCGCAA TAGTGGTCGCCGGTTTAACCGATACTGGTTTGGGTGATCGTATCCGGCAA AGCCTTTGCGTGCGGTGGCGGTTGACGGGGTATCTGCTGCGTTGGCCATGA GTGGAAGAGCGCGGCGACAGCTACAACAAGAAGTTTGGGTGAAAACGAAA ATTCCATGCAAAATGCTCCGGTTTCATGTCGTCAAAATGTTGACGTAATTA AGCATTGATAATTGATAATTGAGATCCCTCTCCCTGACAGGATGATTGCAT AAATAATAGTGATGAAAATAAATTATTTATTTATCCAGAAAATGAATTGGA AAATCAGGAGAGCGTTTTCAATCCTACCTCTGGCGCAGGTGATATGTAAGG CGGTGATGTTATATCGCGTTGATTATTGATGCTGTTTTAGTTTTAACGGC AATTAATATATATGTTATTAATTGAATGAATTTTATTATTATTATATATA TGTGTAGAATCGTCGCAGGAGAAATAAAGCGCAATCATTCATAAACCCCTC TGTTTTATAATCACTTAATCGCGCATAAAAAACGGCTAAATTCTTGTGTAA

	ACGATTCCACTAATTTATTCCATGTCACACTTTTCGCATCTTTGTTATGCT ATGGTTATTTTCATACCATAAGCCTAATGGAGCGAATTATGAGAGTTCTGGT TACCGGTGGTAGCGGTTACATTGGAAGTCATACCTGTGTGCAATTACTGCA AAACGGTCATGATGTCATCATTTCTTGATAACCTCTGTAACAGTAAGCGCAG CGTACTGCCTGTTATCGAGCGTTTAGGCGGCAAACATCCAACGTTTGTGTA AGGCATATTTCGTAACGAAGCGTTGATGACCGAGATCCTGCACGATCACGC TATCGACACCGTGATCCACTTCGCCGGGCTGAAAGCCGTGGGCGAATCGGT ACAAAACCGCTGGAATATTACGACAACAATGTCAACGGCACTCTGCGCCT GATTAGCGCCATGCGCGCCGCTAACGTCAAAAACCTTTATTTTGTAGCTCCTC CGCCACCGTTTATGGCGATCAGCCCAAAATTCATACGTTGAAAGCTTCCC GACCGGCACACCGCAAAGCCCTTACGGCAAAAGCAAGCTGATGGTGGAAAC GATCCTCACCGATCTGCAAAAAGCCAGCCGGACTGGAGCATTGCCCTGCT GCGCTACTTCAACCCGGTTGGCGCGCATCCGTCGGGCGATATGGGCGAAGA TCCGCAAGGCATTCCGAATAACCTGATGCCATACATCGCCAGGTTGCTGT AGGCCGTCGCGACTCGCTGGCGATTTTGGTAACGATTATCCGACCGAAGA TGGTACTGGCGTACGCGATTACATCCACGTAATGGATCTGGCGGACGGTCA CGTCGTGGCGATGGAAAACTGGCGAACAAGCCAGGCGTACACATCTACAA CCTCGGCGCTGGCGTAGGCAACAGCGTGCTGGACGTGGTTAATGCCTTCAG CAAAGCCTGCGGCAAACCGGTTAATTATCATTTTGCACCGCGTCGCGAGGG CGACCTTCCGGCCTACTGGGCGGACGCCAGCAAAGCCGACCGTGAAC TGAA CTGGCGCGTAACGCGCACACTCGATGAAATGGCGCAGGACACCTGGCACTG GCAGTCACGCCATCCACAGGGATATCCCGATTAAGGAACGACCATGACGCA ATTTAATCCCGTTGATCATCCACATCGCCGCTACAACCCGCTCACCGGGCA ATGGATTCTGGTTTCACCGCACCGCGCTAAGCGCCCCTGGCAGGGGGCGCA GGAAACGCCAGCCAAACAGGTGTTACCTGCGCACGATCCAGATTGCTTCCT CTGCGCAGGTAATGTGCGGGTGACAGGCGATAAAAACCCCGATTACACCGG GACTTACGTTTTCACTAATGACTTTGCGGCTTTGATGTCTGACACGCCAGA TGCGCCAGAAAGTCACGATCCGCTGATGCGTTGCCAGAGCGCGCGCGCAC CAGCCGGGTGATCTGCTTTTCACCGGATCACAGTAAACGCTGCCAGAGCT CAGCGTTGCAGCATTGACGGAAATCGTCAAAACCTGGCAGGAGCAAACCGC AGAACTGGGGAAAACGTACCCATGGGTGCAGGTTTTTGAAAACAAAGGCGC GGCGATGGGCTGCTCTAACCCGCATCCGCACGGTCAGATTTGGGCAAATAG CTTCCTGCCTAACGAAGCTGAGCGCGAAGACCGCCTGCAAAAAGAATATTT TGCCGAACAGAAATCACCAATGCTGGTGGATTATGTTACGCGCGAGCTGGC AGACGGTAGCCGTACCGTTGTCGAAACCGAACACTGGTTAGCCGTCGTGCC TTACTGGGCTGCCTGGCCGTTTCGAAACGCTACTGCTGCCCAAAGCCCACGT TTTACGGATCACCGATTTGACCGACGCCAGCGCAGCGATCTGGCGCTGGC GTTGAAAAGCTGACCAGTCGTTATGACAACCTCTTCCAGTGCTCCTTCCC
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	CTACTCTATGGGCTGGCACGGCGCGCCATTTAATGGCGAAGAGAATCAACA CTGGCAGCTGCACGCGCACTTTTATCCGCCTCTGCTGCGCTCCGCCACCGT ACGTAAATTTATGGTTGGTTATGAAATGCTGGCAGAGACCCAGCGAGACCT GACCGCAGAACAGGCAGCAGAGCGTTTGCGCGCAGTCAGCGATATCCATTT TCGCGAATCCGGAGTGTAAGAAATGAGTCTGAAAGAAAAACACAATCTCT GTTTGCCAACGCATTTGGCTACCCTGCCACTCACACCATTTCAGGCGCCTGG CCGCGTGAATTTGATTGGTGAACACACCGACTACAACGACGGTTTCGTTCT GCCCTGCGCGATTGATTATCAAACCGTGATCAGTTGTGCACCACGCGATGA CCGTAAAGTTTCGCGTGATGGCAGCCGATTATGAAATCAGCTCGACGAGTT TTCCCTCGATGCGCCCATTGTGCGACATGAAAATATCAATGGGCTAACTA CGTTCGTGGCGTGGTGAACATCTGCAACTGCGTAACAACAGCTTCGGCGG CGTGGACATGGTGATCAGCGGCAATGTGCCGAGGGTGCCGGGTAAAGTTC TTCCGCTTCACTGGAAGTCGCGGTGGAACCGTATTGCAGCAGCTTTATCA TCTGCCGCTGGACGGCGCACAAATCGCGCTTAACGGTCAGGAAGCAGAAAA CCAGTTTGTAGGCTGTAACGCGGGATCATGGATCAGCTAATTTCCGCGCT CGGCAAGAAAGATCATGCCTTGCTGATCGATTGCCGCTCACTGGGGACCAA AGCAGTTTCCATGCCCAAAGGTGTGGCTGTGTCATCATCAACAGTAACCTT CAAACGTACCCTGGTTGGCAGCGAATAACAACACCCGTCGTGAACAGTGCGA AACCGGTGCGCGTTTCTTCCAGCAGCCAGCCCTGCGTGATGTCACCATTGA AGAGTTCAACGCTGTTGCGCATGAACTGGACCCGATCGTGGCAAAACGCGT GCGTCATATACTGACTGAAAACGCCCGCACCGTTGAAGCTGCCAGCGCGCT GGAGCAAGGCGACCTGAAACGTATGGGCGAGTTGATGGCGGAGTCTCATGC CTCTATGCGCGATGATTTCGAAATCACCGTGCCGCAAATTGACACTCTGGT AGAAATCGTCAAAGCTGTGATTGGCGACAAAGGTGGCGTACGCATGACCGG CGGCGGATTTGGCGGCTGTATCGTCGCGTGATCCCGGAAGAGCTGGTGCC TGCCGTACAGCAAGCTGTGCTGAACAATATGAAGCAAAAAACAGGTATTAA AGAGACTTTTTACGTTTGTAACCATCACAAAGGAGCAGGACAGTGCTGAAC GAAACTCCCGCACTGGCACCCGATGGTCAGCCGTACCGACTGTTAACTTTG CGTAACAACGCAGGGATGGTAGTCACGCTGATGGACTGGGGTGCGACTTTA CTTTCCGCCCGTATTCCGCTTTCCGATGGCAGCGTCCGCGAGGCGCTGCTC GGCTGTGCCAGCCCGGAATGCTATCAGGATCAGGCCGCGTTTCTGGGGGCC TCTATTGGTCGTTATGCCAACCGTATCGCCAATAGCCGTTATACCTTTGAC GGTGAAACCGTGACGCTTTTCGCCAAGTCAGGGCGTTAACAGCTGCACGGC GGGCCGGAAGGGTTCGACAAACGTGCTGGCAGATTGTGAACCAGAACGAT CGTCAGGTGCTGTTGCCCTGAGTTCAGATGATGGTGATCAGGGCTTCCCG GGTAATCTCGGCGGACGGTGCAATATCGTCTGACCGACGATAACCGTATC TCCATTACTTATCGCGCCACAGTTGATAAACCTTGCCCGGTGAATATGACT AATCACGTCTATTTCAATCTTGACGGCGAGCAGTCTGACGTGCGCAATCAC
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	AAGTTGCAGATTCTGGCGGACGAATATCTGCCGGTTGATGAAGGCGGCATT CCGCACGACGGCCTGAAATCTGTCGCCGGAACGTCTTTTGATTTCCGCAGC GCCAAAATCATCGCCAGTGAGTTTCTTGCCGACGACGATCAGCGCAAAGTG AAAGGTTACGATCACGCATTCTTGTTACAGGCCAAAGGCGATGGCAAGAAA GTGGCGGGCGCATGTCTGGTCAGCAGATGAAAAATTGCAGCTGAAGGTCTAC ACCACCGCTCCGGCTCTGCAATTCTACTCCGGCAACTTCCTCGGCGGCACA CCGTCGCGGGGAACCGAACCTTACGCCGACTGGCAAGGGCTGGCTCTGGAA AGCGAGTTTCTACCGGACAGCCCGAACCACCTGAATGGCCGCAACCGGAC TGCTTCTGCGTCTTGGCGAAGAGTATTCCAGCCTGACGGAATATCAGTTT ATTGCTGAGTAATGTTACGCCCTCGTCGCGGCCGCCCCGGCGCGCCGCAT CGATGCGCCCGGGGCGCTGCAGGGGGGACTCCTGTTGATAGATCCAGTAA TGACCTCAGAACTCCATCTGGATTTGTTTCAGAACGCTCGGTTGCCGCCGGG CGTTTTTTATTGGTGAGAATAGTACTAATTTGCTTTATTAAAGAGGAGAAA TTAATTAATGCCCCGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGGAA GTTCTTATACTTTCTAGAGAATAGGAACTTCGCATGCACGCAGCATATGCA AAAGGCTGGATAAAGTGTTACCCCTCTATCCAGCTCATCCATAAATACCTT ACCCTTTATCGTTTGGATTTCCAGTTCTCTGTGGCATAATAAACGAGTAG ATGCTCATTCTATCTTATGTTTCGCCTTAGTGCCTCATAAACTCCGGAAT GACGCAGAGCCGTTTACGGTGCTTATCGTCCACTGACAGATGTCGCTTATG CCTCATCAGACACCATGGACACAACGTTGAGTGAAGCACCCTTGTGTGTC ATACAGACCTGTTTTAACGCCTGCTCCGTAATAAGAGCAGGCGTTTTTTTA TGTATCAGAAAGTCCCGGAGGTGCTTGCCCTCCGGGTGAGAAAGAACTACT GTGGCGGGTTGTTCTGCAACGTTAACATCAAACCGTCGCGGCGCATCGCTG CAGCTTCTTCTGGTTTGTGCAGTCTGTCCAGCGCGTCGGCAAGCCATGCGT AATCGTAGGCGTCCGGACGTTGTTTCAGCGCCGCGCGGAAGGCGAGCGATG CTTCTGCCATTGCGCGTGCTTCATCAGCGACTGACCCAGTGTGCTCCACA ACAACGGGCGATCGCCACGTTTTTTGATCTGTTGGCGCAGCACTTTTTCCA GCTGCTCCGGTTATTGGTTTTTCAGACGCGGGATCGGCAGCAGTAGGCGAT CGTCATACTGGCGTTTCAGGCCGTGATGATAATTTGCTGGGCAGTATCAT GATCGTCACATTCAATAAGATGTTCCGCCATTGCCACCTGCAACGCTACCT GATGACGCGTTTTCCGGCTTTGGTTTTTCCACCAGTTACGCAAGCCTTCGC TACCGTTATCGGCACGCGCCTGATCCATCAGGCCAATCCATGCCTGTTGTT CCAGCATTGACGATGTTCTTCATCGCCAACGTGGGCTTTTCGC
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Table 5 : Genetic constructions to code for galactose consumption pathway

Example 7: Production of recombinant casein in a 1L fermenter with lactose and galactose consuming strains

Productions of casein using BL21 strains able to consume lactose and galactose were tested, using lactoserum as a feedstock, in conjunction with glucose. Acidic lactoserum was generated as described in Example 4 and then centrifuged for 20min at 12000rpm and 4°C. The supernatant was then filtered with a 0.22 uM filter.

Cultures of each recombinant strains described in Table 6 were used to inoculate 1L fermenters (Multifors 2 Microbila 1,4L TV, Infors), with medium containing lactoserum and glucose as sole carbohydrate source.

	Strains description	Ability to degrade galactose	Selection marker
SO_gal0	E. coli BL21(DE3) No galactose consumption pathway	-	Ampicillin
SO_gal1	E. coli BL21(DE3) Galactose consumption pathway coding on Plasmid-pSO149	+	Ampicillin and Spectinomycin
SO_gal2	E. coli BL21(DE3) Galactose consumption pathway coding on Plasmid-pSO148	+	Ampicillin and Spectinomycin
SO_gal3	E. coli BL21(DE3) SS9::pgal-galETKM Galactose consumption pathway coding on Chromosome	+	Ampicillin

Table 6: Strains used for this experiment.

10 A medium (MSA) containing 50% of lactoserum, supplemented with 0.71g/L Na₂SO₄, 11,3 g/L (NH₄)₂SO₄, 0.85g/L KH₂PO₄, 1,11 g/L Na₂HPO₄, 4 g/L MgSO₄(7H₂O), 1,6 mL/L of 5% antifoam solution (Struktol), and trace elements was used. The medium was sterilized by filtration with a 0.22 uM filter and supplemented with 100 µg/ml ampicillin and 33 g/L of glucose. Spectinomycin, at
15 a final concentration of 100 µg/ml of culture, was added in the cultures with

SO_gal1 et SO_gal2. In this medium, lactose concentration was about 20 g/L. For the culture with SO_gal3, a minimal medium (0.71g/L Na₂SO₄, 11,3 g/L (NH₄)₂SO₄, 0.85g/L KH₂PO₄, 1,11 g/L Na₂HPO₄, 4 g/L MgSO₄(7H₂O), 1,6 mL/L of 5% antifoam solution (Struktol), and trace elements with 20 g/L of lactose was used. The conditions are summarized in Table 7.

	BR1	BR2	BR3	BR4	BR5
Strain	SO_gal0	SO_gal1	SO_gal2	SO_gal3	SO_gal3
Medium	MLS	MLS	MLS	Minimum medium + 20 g/L of lactose	MLS

Table 7: Fermentations Conditions

0,5L of medium was inoculated, with a preculture of the recombinant clone, to reach an initial OD_{600nm} of 0.25. The fermentation was conducted in a fed batch mode, at pH=7.2; T=37°C; pO₂=15%, with the culture being fed with a solution containing 600 g/L of D-Glucose. Ampicillin was added after 12,5 hours (100 µg/ml of culture).

During the growth phase (t=0 to t=10hours), glucose represses lactose uptake, by carbon catabolic repression and only glucose is consumed. In our culture conditions, glucose was totally consumed after 10 to 12 hours, and no additional glucose was added for at least 30 min to enable the metabolic switch to lactose consumption and casein expression could be induced. The switch is correlated with the spike in the pO₂ concentration in the bioreactor. The culture was stopped at T=17.8 h, with OD₆₀₀ ranging from 94 to 107.

For analysis, an aliquot of cells was centrifuged and resuspended in 1:1 volume of lysis buffer (Tris HCl, pH 8.5). 2 mL samples of this cell suspension were treated by heating at 95°C for 60 minutes, as described in Example 4. The soluble and insoluble fractions were separated by centrifugation. The insoluble fraction was resuspended in 1 mL of a 2% SDS solution and 10 µL aliquots of both soluble and insoluble fractions were analyzed by SDS-PAGE. As shown on Figures 7 and 8, casein expression was efficiently induced in all conditions. Recombinant casein production was estimated to be in the range of 2.5 to 6 g/L of bacterial culture.

Lactose, glucose, and galactose consumption were measured for all samples. Results are shown in Figure 8. As shown in these graphics, lactose was

completely hydrolyzed and both added glucose and glucose resulting from lactose hydrolysis were efficiently consumed during the fermentation runs. Galactose resulting from lactose hydrolysis was consumed in BR2,3,4 and 5 and accumulated in BR1. As expected, the strains SO_gal1, SO_gal2 and SO gal3 could consume
5 the galactose in contrary to SO_gal0.

CLAIMS

1. A method for obtaining a casein composition, comprising:
 - i. providing microorganisms that have been transformed with at least one
5 nucleic acid coding for a casein,
 - ii. culturing said microorganisms, using a medium comprising lactoserum
so as to express and produce casein,
 - iii. thereby providing a microorganism composition wherein the pH of the
composition is equal or above 6.5 and preferably below 9
 - 10 iv. heating the microorganism composition so as to reduce the amount of
the other proteins in soluble fraction of the composition, wherein
heating is performed at a temperature equal or above 75°C,
 - v. recovering the soluble fraction from the heated cell composition of iv),
thereby obtaining a casein composition in the soluble fraction.
- 15 2. The method of claim 1, wherein lactoserum is present in the culture medium at
the beginning of the cultivation.
3. The method of claim 1 or 2, wherein lactoserum is added in the medium during
20 exponential phase or stationary phase.
4. The method of any one of claims 1 to 3, wherein another source of carbon is
present in addition to lactoserum in the culture medium in ii, wherein this other
source of medium is preferably a sugar, notably glucose, fructose, saccharose,
25 possibly present in molasse.
5. The method of any one of claims 1 to 4, wherein the microorganisms are
bacteria.
- 30 6. The method of claim 5, wherein the bacteria are *E. coli*, in particular *gal⁺ E. coli*.
7. The method of any one of claims 1 to 6, wherein expression of the at least one
nucleic acid is induced by the presence of lactose.

8. The method of claim 7, wherein the at least one nucleic acid is under the control of a promoter fused with the *laco* operator region of the lactose operon, and wherein the microorganisms express the *lacI* repressor of said operon.
- 5 9. The method of any one of claims 1 to 8, wherein culture of the microorganisms is initiated in presence of a sugar other than lactose, and wherein consumption of lactose present in the lactoserum is initiated at during exponential growth phase, in particular around or after the middle of the exponential phase or at the beginning of the stationary growth phase of the microorganisms.
- 10 10. The method of any one of claims 1 to 9, wherein lactoserum is added at a to reach a lactose concentration of at least 1% (w/v).
- 15 11. The method of any one of claims 1 to 10, wherein the lactoserum is a lactoserum permeate.
- 20 12. The method of any one of claims 1 to 11, wherein lactoserum is acid lactoserum, and wherein pH of culture medium is adjusted around pH=7, during microorganism culture.
- 25 13. The method of any one of claims 1 to 12, wherein lactose represents at least 2% (w/w, wet weight) of the lactoserum.
- 30 14. The method of any one of claims 1 to 13, further comprising washing the microorganism composition to remove the culture medium before heating in step iv) of said method for obtaining a casein composition.
15. The method of any one of claims 1 to 14, further comprising lysing the microorganism composition before iv).
16. The method of any one claims 1 to 14, wherein the microorganisms in the microorganism composition are not lysed before iv) and heating in step iv) of said method for obtaining a casein composition lyses the microorganisms.

17. The method of any one of claims 1 to 16, comprising further processing the soluble fraction by adding at least one step chosen among addition of activated charcoal, membrane filtration, chromatography or casein precipitation, thereby obtaining a casein composition that is purified.
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18. The method of any one of claims 1 to 17, wherein heating in in step iv) of said method for obtaining a casein composition is performed at a temperature equal or higher than 95°C.
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19. The method of any one of claims 1 to 18, wherein the casein is selected from beta casein, alpha-S1 casein, alpha-S2 casein and mixture of these caseins, in particular wherein the casein is selected from beta casein, alpha-S1 casein and mixture of these caseins.
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20. The method of any one of claims 1 to 19, wherein the microorganisms are transformed with a nucleic acid coding for alpha-casein and another nucleic acid coding for beta casein, wherein expression of the two nucleic acids is induced by lactose.
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21. The method of any one of claims 1 to 20, wherein the microorganisms are not transformed with a nucleic acid coding for kappa-casein.
22. The method of any one of claims 1 to 21, wherein the caseins represent at least 60 %, preferably at least 62 %, preferably at least 65 %, preferably at least 67
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- %, preferably at least 70 % of the total proteins of the soluble fraction.
23. The method of any one of claims 1 to 22, wherein the ratio of casein as compared to the total proteins is increased in the soluble fraction as compared to the ratio in the microorganism composition.
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24. A method for culturing microorganisms, comprising:
- providing microorganisms that have been transformed with at least one nucleic acid coding for a casein, wherein expression of said nucleic acid is inducible by lactose,

- ii. culturing said microorganisms, in a culture medium comprising glucose as a source of carbon, wherein expression of the nucleic acid is not induced
 - iii. depleting the culture medium of glucose when the microorganisms are in the exponential phase growth, wherein lactoserum is present in the culture medium at the time of said depletion, thereby providing the lactose present in the lactoserum as a source of carbon, and wherein expression of the nucleic acid is induced by the lactose present in the lactoserum
 - iv. further culturing the microorganisms in the presence of a source of carbon.
25. The method of claim 24, wherein glucose is not present as a source of carbon in iv.
26. The method of claim 24, wherein lactoserum is not present as a source of carbon in iv.
27. The method of claim 24, wherein both glucose and lactoserum are sources of carbon in iv.
28. The method of any one of claims 24 to 27, wherein the microorganisms also metabolize galactose as a source of carbon.
29. The method of any one of claims 24 to 28, wherein the nucleic acid coding for a casein is under the control of a promoter fused with the *laco* operator region of the lactose operon, and wherein the microorganisms express the *lacI* repressor of said operon.
30. The method of any one of claims 24 to 29, wherein glucose and lactoserum are present in the culture medium of ii.
31. The method of any one of claims 24 to 29, wherein lactoserum is not present in the culture medium of ii and is added in iii.
32. The method of any one of claims 24 to 31, wherein depletion of glucose in iii is performed by consumption of the glucose by the microorganisms, in the absence of refeed of glucose.

33. A method for reducing environmental impact of a casein composition prepared by fermentation of transgenic microorganisms, comprising
- i. providing microorganisms that have been transformed with at least one nucleic acid coding for a casein,
 - ii. culturing said microorganisms, using a medium comprising lactoserum so as to express and produce casein,
 - iii. thereby providing a microorganism composition wherein the pH of the composition is equal or above 6.5 and preferably below 9
 - iv. heating the microorganism composition so as to reduce the amount of the other proteins in soluble fraction of the composition, wherein heating is performed at a temperature at a temperature equal or above 75°C,
 - v. recovering the soluble fraction from the heated cell composition of iv),
- thereby obtaining a casein composition in the soluble fraction, wherein the environmental impact is reduced as compared to the environmental impact of a casein composition obtained by the same steps, except that glucose or sucrose or molasses are used in place of lactoserum in b.
34. The method of claim 33, the reduction in environmental impact is observed for at least one criteria selected from (i) Climate change, (ii) acidification, (iii) Ecotoxicity, (iv) Use of non-renewable energy resources, (v) Eutrophication of freshwater, (vi) Eutrophication of marine water, (vii) Eutrophication of terrestrial land, (viii) Human toxicity, carcinogenic, (ix) Human toxicity, non-carcinogenic, (x) Ionizing radiation, impact on human health, (xi) Land use, (xii) Use of material resources, (xiii) Ozone depletion potential, (xiv) Particulate matter formation, (xv) Photochemical oxidant formation, and (xvi) Water use, preferably (i) climate change.
35. A method for obtaining a dairy substitute, comprising performing the method according to any one of claims 1 to 23, mixing the casein composition with at least one other ingredient comprising at least one component selected from the group consisting of proteins, water, calcium, lipids, and carbohydrate to obtain

a liquid pre-curd composition (LpCC), and further processing the liquid pre-curd composition (LpCC) to obtain a dairy substitute.

36. The method of claim 35, wherein processing of the LpCC comprises

- 5 vi. adding at least one curdling agent to the liquid composition so as to
 obtain a curd, and
- vii. further processing said curd in order to obtain a dairy substitute,
 wherein the dairy substitute is a cheese substitute or a yoghurt
 substitute.

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37. The method of claim 36, wherein the further step in vi) comprises acidic precipitation of casein, at room temperature or by heating at 90°C at pH=4.6.

38. A dairy substitute susceptible to be obtained by a method according to any one
15 of claims 35 to 37.

39. The dairy substitute of claim 38, wherein the environmental impact is reduced
for at least one of the criteria listed in claim 34.

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