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- (73) Patenthaver: **Institut National De La Recherche Agronomique (INRA), 145, Rue de l'Université, 75007 Paris, Frankrig**
Centre De Biotechnologie De Sfax, Route Sidi Mansour Km 6.5 BP "K", Sfax 3018, Tunesien
- (72) Opfinder: **Rhimi, Moez, Cité Hajaj Ibn Youssef-Djilma, 9110 Sidi Bouzid, Tunesien**
CHOUAYEKH, Hichem, chez Adel Chouayekh boucher à Sakiet, Ezzit route de Tunis Km 7, 3021 Sfax, Tunesien
MAGUIN, Emmanuelle, 63 avenue de l'Yvette, F-91440 Bures Sur Yvette, Frankrig
Bejar, Samir, Route de Sokra Km 4, Lotissement Faiza, 3058 Sfax, Tunesien
- (74) Fuldmægtig i Danmark: **Zacco Denmark A/S, Arne Jacobsens Allé 15, 2300 København S, Danmark**
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DESCRIPTION

[0001] The instant invention relates to the use of a L-arabinose isomerase for converting D-galactose into D-tagatose in a dairy product which contains D-galactose, wherein said dairy product containing D-galactose is selected from the group consisting of milk, milk supplemented with D-galactose, mild undergoing lactic acid fermentation, a fermented mild, and milk in which lactose was enzymatically processed, to a method of converting D-galactose into D-tagatose during lactic acid fermentation of milk, to a method of producing a sweetened fermented dairy product, and to a sweetening milk product additive which comprises a L-arabinose isomerase and/or a L-arabinose isomerase-expressing microorganism acceptable for food consumption. The invention also relates to a microorganism acceptable for food consumption expressing an exogenous L-arabinose isomerase, and to a mutated L-arabinose isomerase US100 from *Bacillus stearothermophilus*, in particular to a mutant with enhanced acidotolerance and stability at acid pH or to a mutant with lower optimal temperature.

[0002] Lactic acid bacteria (LAB) constitute a commercially important group of microorganisms extensively utilized in the food industry. Among them, the dairy starters *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) and *Streptococcus thermophilus* are universally used in cooperation in the fermentation of milk into yoghurt (Tamime and Robinson, 1999, Yoghurt Science and Technology, 2nd edn. Cambridge: Woodhead). During their growth, lactose the unique sugar found in milk is used as the primary energy source.

[0003] In LAB, two systems for transport and metabolism of lactose are known: (i) a phosphoenolpyruvate (PEP) lactose phosphotransferase system (PTS) with a phospho- β -galactosidase system (Postma et al., 1993, Microbiol Rev 57:543-94) and (ii) a lactose antiport permease (LacS) system with a β -galactosidase (LacZ). The PEP-PTS system is found in many species including lactococci (Reizer et al., 1988. Crit Rev Microbiol, 15:297-338; Thompson, 1987, FEMS Microb. Lett, 46:221-23). *L. bulgaricus* as well as *S. thermophilus* utilize lactose via the second pathway where the lactose is brought into cell as the free sugar and cleaved by β -galactosidase into glucose and galactose (Foucaud and Poolman, 1992, J Biol Chem, 267:22087-94; Leong-Morgenthaler et al., 1991, J Bacteriol, 173:1951-7). The glucose moiety of the generated monosaccharide is further metabolized to pyruvate which is used to produce lactate, whereas the galactose moiety is excreted from most strains of these species into the medium in equimolar amounts with the lactose uptake despite the fact that these starters contain genes of the Leloir pathway which is the most ubiquitous route for galactose catabolism in these bacteria (de Vin et al., 2005. Appl Environ Microbiol 71:3659-67; Hickey, et al., 1986. Appl Environ Microbiol 51:825-831; Hutkins and Ponne, 1991. Appl Environ Microbiol 57:941-944). Currently, the galactose negative phenotype has been mainly ascribed to a defect in the induction mechanism of enzymes of the Leloir pathway during growth on lactose and to the preferred mode of action antiport of the permease LacS *in vivo* (de Vin et al., 2005. Appl Environ Microbiol 71:3659-67; Turner and Martley. 1983. Appl Environ Microbiol 45:1932-1934).

[0004] Recently milk with lower lactose concentration was also proposed for lactose-intolerant persons. Lactose hydrolysis can achieve this decrease in lactose concentration leading to glucose and galactose. The Inventors showed that L-AI can be used to convert the galactose resulting from lactose hydrolysis in the low-calorie, sweetening D-tagatose.

[0005] In yoghurt manufacture, especially in the case of fruit/flavored products, sweetening compounds with high caloric value like sucrose are normally added to tone down the acidity of the product. The fact that nowadays we assist to an increasing of consumer demand for low caloric food products especially in the case of diabetes and low caloric diet persons, the actual challenge is to try to convert the unconsumed D-galactose during yoghurt production to low caloric sweetener like D-tagatose which could minimize the habitual adding of high caloric sugars. This natural D-galactose isomer, is a rare ketohexose with a sweetening power equivalent to sucrose but a much lower caloric value because it is poorly degraded by the human body, making it an interesting anti-hyperglycemic agent.

[0006] There is no biological source available for the rentable extraction of tagatose. Two processes have been described for the biological production of this sweetener. The first one relies on the ability of many micro-organisms, such as *Arthrobacter*, *Lactobacillus*, *Mycobacterium*, *Klebsiella* and *Gluconobacter*, to convert D-galactitol into D-tagatose (Muniruzzaman et al., J. ferment. Bioeng. 78 (1994) 145-148.; Shimonishi et al., J. Ferment. Bioeng. 79 (1995) 620-622.; Manzoni et al., Pro. Biochem. 36 (2001) 917-977; Rollini and Manzoni, Proc. Biochem. 40 (2005) 437-444). However, it is not of industrial use because the D-galactitol is an expensive substrate which has a low potential for industrial application. The second one is an *in vitro* enzymatic isomerisation of D-galactose using an L-arabinose isomerase (L-AI) activity (Haltrich et al., 1998. Ann N Y Acad Sci 864:295-9; Izumori et al., 1978. J Bacteriol 133:413-4; Jorgensen et al., 2004. Appl Microbiol Biotechnol 64:816-22; Manzoni et al., 2001. Process biochemistry 36:971-977). This enzyme is involved, *in vivo*, in the isomerisation of L-arabinose to L-ribulose.

[0007] The L-arabinose isomerase derived from *B. stearothermophilus* US100 (L-AI US100) has been characterized (international patent application WO 2006/071203; Rhimi and Bejar. 2006. Biochim Biophys Acta 1760:191-9). L-AI US100 is

optimally active at pH 7.5 and 80°C and, by contrast with the L-AI previously characterized, exhibits a low requirement for metal ions.

[0008] The inventors have demonstrated that L-AI US100 produced by lactic acid bacteria is active *in vivo* since it conferred an efficient bioconversion of D-galactose to D-tagatose which is secreted from the microbial cells into culture medium. In addition, purified L-AI US100 enzyme was also found to convert efficiently D-galactose into D-tagatose in milk, the industrial medium used for yoghurt production and also several probiotic products. These results provides for the exploitation of L-AI activity to produce new low caloric milk products containing D-tagatose produced by enzymatic isomerisation.

[0009] Isomerization of D-galactose by L-arabinose isomerases at high temperature increases the reaction rate and allows the shift of equilibrium between D-galactose and D-tagatose toward the latter. For this reason, many thermostable L-AIs were isolated and characterized including those from *Thermus*, *Thermotoga* and *Geobacillus* genera. Nevertheless, the majority of these enzymes have a high optimum pH which is a major drawback with respect to an application during lactic acid fermentation, wherein milk pH will be remote from the optimum pH of the enzyme.

[0010] Protein engineering of new acidotolerant L-arabinose isomerase was previously explored in order to found L-AIs active at acid pH. In fact, Kim et al 2006 reported that V408R and N475Q substitutions allow the shift of the *Geobacillus stearothermophilus* T6 L-AI optimal pH from 8.5 to 7.5 (J Appl Microbiol 101 (2006) 213). Moreover, Lee et al. described for the first time the characterisation of a new acidotolerant L-AI from the acidophilic *Alicyclobacillus acidocaldarius* (Appl Environ Microbiol 71 (2005) 7888). However, while this mutant L-AI is acidolerant, it requires divalent ions both for its activity and thermal stability (1 mM Co²⁺, 1 mM Mn²⁺ and 1 mM Mg²⁺) and has a relative lower optimal temperature evaluated to 65°C. Moreover, no structural explanation was proposed to explain the impact of these substitutions on the enzyme acidotolerance behaviour.

[0011] New L-AI acting at higher or lower temperature, low pH and displaying a very feeble requirement for metallic ions for their activity and thermal stability were looked for. A new acidotolerant L-AI US100 Q268K mutant, a mesophile L-AI US100 N175H mutant and an acidotolerant and mesophile double mutant L-AI US100 Q268K N175H were constructed, purified, and characterised, in particular as regards D-tagatose production.

[0012] The invention is defined by the claims.

L-arabinose isomerases

[0013] L-arabinose isomerases (EC 5.3.1.4) are intracellular enzymes that catalyse the conversion of L-arabinose to L-ribulose in biological systems. They are also referred to as D-galactose isomerases due to their ability, *in vitro*, to isomerize D-galactose into D-tagatose (Cheetam and Wootton, Enzyme and Microbial Technology 15 (1993) 105).

[0014] In the context of the invention, the term "L-arabinose isomerase" or "L-AI" generally denotes any enzyme which catalyses the conversion of L-arabinose to L-ribulose, and preferably also isomerizes D-galactose into D-tagatose. A L-arabinose isomerase may be a native (or wild-type) L-AI, or a mutant of a native L-AI which was genetically engineered to alter one or more phenotypic features of the native L-AI, in particular temperature optimum, pH optimum, ion requirement, substrate affinity, thermal stability and/or pH tolerance, provided said mutant retains the capacity to isomerize D-galactose into D-tagatose, and optionally to convert L arabinose into L ribulose.

[0015] The capacity of an enzyme to generate L-ribulose or D-tagatose may be readily determined by the skilled in the art with the cysteine carbazole sulfuric-acid method which was described by Dische and Borenfreund (J. Biol. Chem. 192 (1951) 583), with absorbance measured at 560 nm. The L-ribulose or D-tagatose production may also be detected by high-performance ionic chromatography (HPLC).

[0016] L-arabinose isomerases are expressed by a wide number of organisms, including plants such as Japanese rice, and microorganisms such as yeasts or bacteria.

[0017] In particular, numerous bacterial L-arabinose isomerases (L-AIs) have been isolated and described, and some have been deposited in sequence databases. For instance, L-arabinose isomerases (L-AIs) may derive from :

- a bacterium of the genus *Thermus* (*Thermoanaerobacter mathranii*), of the genus *Thermotoga* (*Thermotoga maritima* (UniProtKB/Swiss-Prot entry Q9WYB3), *Thermotoga neapolitana*),

- a bacterium of the genus *Geobacillus* (*Geobacillus stearothermophilus*, *Geobacillus thermodenitrificans*, *Geobacillus kaustophilus* UniProtKB/Swiss-Prot entry Q5KYP7),
- *Alicyclobacillus acidocaldarius*,
- a bacterium of the genus *Bacillus* (*Bacillus stearothermophilus* (UniProtKB/Swiss-Prot entry Q9S467), *Bacillus licheniformis* (UniProtKB/Swiss-Prot entry Q65GC0 Q65J10), *Bacillus clausii* (UniProtKB/Swiss-Prot entry Q5WL05), *Bacillus halodurans* (UniProtKB/Swiss-Prot entry Q9KBQ2), *Bacillus subtilis* (UniProtKB/Swiss-Prot entry P94523),
- *Clostridium acetobutylicum*,
- *E. coli* (UniProtKB/Swiss-Prot entry P58538, P08202, Q0TLS8, Q1 RGD7, Q8FL89),
- *Mycobacterium smegmatis* (UniProtKB/Swiss-Prot entry Q9RHG2),
- a bacterium of the genus *Salmonella* (*Salmonella paratyphi-a* (UniProtKB/Swiss-Prot entry Q5PDF2), *Salmonella Typhi* (UniProtKB/Swiss-Prot entry P58539), *Salmonella Choleraesuis* (UniProtKB/Swiss-Prot entry Q57TF9), *Salmonella typhimurium* (UniProtKB/Swiss-Prot entry P06189), *Shigella boydii* (UniProtKB/Swiss-Prot entry Q326H3),
- a bacterium of the genus *Shigella* (*Shigella sonnei* (UniProtKB/Swiss-Prot entry Q3Z5U9), *Shigella dysenteriae* (UniProtKB/Swiss-Prot entry Q32K31), *Shigella flexneri* (UniProtKB/Swiss-Prot entry Q7UDT4),
- a bacterium of the genus *Yersinia* (*Yersinia pestis* (UniProtKB/Swiss-Prot entry P58540, Q1CIX9, Q1C7J3), *Yersinia pseudotuberculosis* (UniProtKB/Swiss-Prot entry Q66AF8),
- *Acidobacteria bacterium* (UniProtKB/Swiss-Prot entry Q1IUW9),
- *Klebsiella pneumoniae* (UniProtKB/Swiss-Prot entry Q48433),
- *Oceanobacillus iheyensis* (UniProtKB/Swiss-Prot entry Q8EMP4),
- *Erwinia carotovora* (UniProtKB/Swiss-Prot entry Q6D4W5), or
- *Mannheimia succiniciproducens* (UniProtKB/Swiss-Prot entry Q65WJ5).

[0018] A L-arabinose isomerase (L-AI) according to the invention may derive from any organism or microorganism, in particular any of the above bacteria, and more generally from any human or animal commensal micro-organism. For instance, said L-AI may derive from a thermophilic bacterium, in particular a bacterium of the genus *Thermus* (*Thermoanaerobacter mathranii*), of the genus *Thermotoga* (*Thermotoga maritima*, *Thermotoga neapolitana*), of the genus *Geobacillus* (*Geobacillus stearothermophilus*, *Geobacillus thermodenitrificans*), from *Alicyclobacillus acidocaldarius*, and from the genus *Bacillus* (*Bacillus stearothermophilus*). Said L-AI may also derive from a lactic acid bacterium, i.e. a bacterium belonging to the genera *Aerococcus*, *Alloicoccus*, *Atopobium*, *Bifidobacterium*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Melissococcus*, *Microbacterium*, *Oenococcus*, *Pediococcus*, *Propionibacterium*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*.

[0019] As used herein, a L-arabinose isomerase "deriving" from e.g a bacterium, is meant for a L-AI isolated from a bacterium (native or wild-type L-AI), or mutants thereof that retain the capacity to convert D-galactose into D-tagatose and optionally to isomerize L-arabinose into L-ribulose.

[0020] The capacity to convert D-galactose into D-tagatose and optionally to isomerize L-arabinose into L-ribulose may be detected as described in the following examples. Briefly, a conversion or isomerization assay may be performed at the optimal temperature for the enzyme, e.g. 80°C, for 1 min for L-arabinose or 10 min for D-galactose, in a reaction mixture containing 0.2 mM CoCl₂, 1 mM MnSO₄, 50 µl of crude enzyme extract at a suitable dilution, 5 mM of L-arabinose (or D-galactose) and MOPS buffer 100 mM (pH 7.5) up to a final volume of 1 ml. The reaction mixture is incubated. Then, conversion/isomerase activity may be measured by determining the amount of produced L-ribulose or D-tagatose by the cysteine carbazole sulfuric-acid method (Dische and Borenfreund. 1951. J Biol Chem 192:583-7), and measurement of absorbance at 560 nm. However, any other suitable method known by the skilled in the art, such as chromatography, may be used.

[0021] The L-arabinose isomerase may derive from *Alicyclobacillus acidocaldarius*. More specifically, said L-arabinose isomerase may be the *Alicyclobacillus acidocaldarius* L-AI cloned by Lee et al. (2005, Appl. Environ. Microbiol. 71 (12), 7888-7896; "AAA", GenPept accession number AAY68209), or its K269E mutant (AAAI-K269E).

[0022] The L-arabinose isomerase may also derive from *Bacillus halodurans*. More specifically, said L-arabinose isomerase may be the *Bacillus halodurans* L-AI described in Lee et al. (2005, Appl. Environ. Microbiol. 71 (12), 7888-7896; "BHA" UniProtKB/Swiss-Prot entry Q9KBQ2), or its E268K mutant (BHAI-E268K).

[0023] The L-arabinose isomerase may also derive from the *Bacillus stearothermophilus* strain US100. Said L-arabinose isomerase may be L-AI US100 as was described by Rhimi and Bejar (Biochim Biophys Acta. 2006 Feb;1760(2):191-9; international patent application WO 2006/071203). The polynucleotide sequence of L-AI US100 is shown in SEQ ID NO:1

(GenBank accession number AJ866972), and its amino acid sequence is shown in SEQ ID NO:2.

[0024] Said L-arabinose isomerase may also be a Q268K mutant of L-AI US100, i.e. a mutated L-AI US100 comprising a substitution Q to K at position 268 of SEQ ID NO:2. Indeed, as described herein, a mutant Q268K of L-AI US100 has been engineered. The biochemical and kinetic characterizations showed that this mutant is more acidotolerant and more stable at acid pH than wild-type L-AI US100, and that this mutant does not require addition of metal ions to operate production of D-tagatose.

[0025] Furthermore, a mutant N175H of L-AI US100 was developed which was found to show improved mesophily as compared with wild-type L-AI US100. The double mutant L-AI US100 Q268K N175H was further found to cumulate both improved acidotolerance and improved mesophily.

[0026] Thus, the invention relates also to a mutated L-AI US100 comprising (i) a substitution Q to K at position 268 of SEQ ID NO:2, or (ii) a substitution N to H at position 175 of SEQ ID NO:2, or (iii) a substitution N to H at position 175 of SEQ ID NO:2 and a substitution Q to K at position 268 of SEQ ID NO:2 and their coding sequences.

[0027] Accordingly the invention provides an isolated polypeptide comprising, or consisting of, the amino acid sequence shown in SEQ ID NO:2 wherein (i) Q268 is substituted by K (Q268K), or (ii) N175 is substituted by H (N175H), or (iii) Q268 is substituted by K and N175 is substituted by H (Q268K N175H).

[0028] The invention also relates to an isolated nucleic acid comprising, or consisting of, a sequence encoding the amino acid sequence shown in SEQ ID NO:2 wherein (i) Q268 is substituted by K, or (ii) N175 is substituted by H, or (iii) Q268 is substituted by K and N175 is substituted by H. In particular, said isolated nucleic acid may comprise, or consist of, the sequence SEQ ID NO:1 wherein (i) C at position 802 is substituted by A (802C>A), or (ii) A at position 523 is substituted by C (523A>C), or (iii) C at position 802 is substituted by A and A at position 523 is substituted by C (802C>A, 523A>C).

[0029] Pursuant to the nomenclature of Dunnen and Antonarakis (Hum Mutation. 2000; 15 :7-12), amino acid substitutions are designated for instant by "Q268K", which means that Gln in position 268 is replaced by Lys. At the nucleic acid level, substitutions are designated by ">", e.g. "802C>A" denotes that at nucleotide 802 of the reference sequence a C is changed to a A.

Microorganisms expressing L-arabinose isomerase

[0030] The application also discloses a microorganism which expresses a L-arabinose isomerase (L-AI). The microorganism is preferably a bacterium or a yeast.

[0031] Preferably, said microorganism is acceptable for food consumption. As used herein, a microorganism, in particular a bacterium or yeast, "acceptable for food consumption" denotes a microorganism which may be added into a milk intended for the manufacture of dairy products (in particular fermented dairy products) for human consumption.

[0032] The invention relates to a bacterium acceptable for food consumption which expresses an exogenous L-arabinose isomerase.

[0033] A bacterium acceptable for food consumption may be a lactic acid bacterium (LAB).

[0034] As used herein, "lactic acid bacteria" denote lactic acid-producing Gram-positive bacteria belonging to the genera *Aerococcus*, *Alloicoccus*, *Atopobium*, *Bifidobacterium*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Melissococcus*, *Microbacterium*, *Oenococcus*, *Pediococcus*, *Propionibacterium*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*. In the context of the invention, the LAB will preferably be a bacterium Generally Recognised As Safe (GRAS).

[0035] Industrially, the most useful lactic acid bacteria are species of the genus *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Leuconostoc*, *Bifidobacterium* and *Pediococcus*. Preferably, said lactic acid bacterium is selected from the group consisting of *Bifidobacterium bifidus*, *Bifidobacterium longum*; *Leuconostoc mesenteroides*, *Pediococcus cerevisiae*, *Lactococcus lactis*, *Lactobacillus bulgaricus*, *Lactobacillus sakei*, *Lactobacillus fermentum* and *Streptococcus thermophilus*. Some lactic acid bacteria are considered as "probiotics" (i.e. as providing benefit to health): for instance *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus johnsonii*, *Lactobacillus reuteri*, *Lactobacillus gasseri*, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, and *Bifidobacterium infantis*.

[0036] The microorganism, in particular the microorganism acceptable for food consumption, may also be a non-lactic acid bacterium, for instance a non-lactic acid probiotic bacterium, or more generally a probiotic microorganism, such as a yeast.

[0037] The L-AI-expressing microorganism, especially the L-AI-expressing bacterium, may naturally (i.e. intrinsically) express the L-AI. In such as case said L-AI is said to be endogenous to the microorganism. This is the case for instance of *Lactobacillus sakei*, and *Lactobacillus plantarum*.

[0038] The L-arabinose isomerase may also be exogenous, i.e. foreign or extrinsic, to the microorganism. A microorganism, such as a bacterium or a yeast, expressing an exogenous L-arabinose isomerase may be readily prepared by the skilled in the art by conventional genetic engineering methods. In particular, the bacterium or yeast may be transformed with a vector suitable for transformation of a bacterium or yeast, as appropriate, which vector comprises a nucleic acid sequence encoding a L-arabinose isomerase. Where the microorganism is a bacterium, any other suitable method may be used to introduce a sequence encoding a L-arabinose isomerase in the bacterium, for instance bacterial conjugation, of phage transfer.

[0039] As used herein, the term "transformation" generally means the introduction of a foreign gene, DNA or RNA sequence into a host cell, so that the host cell will express the introduced gene or sequence to produce a desired substance, typically a protein or enzyme coded by the introduced gene or sequence. As used herein, a host cell which has been "transformed" with a nucleic acid sequence encoding a L-arabinose isomerase denotes a cell that receives and expresses an introduced DNA or RNA encoding a L-AI, whatever the method used to introduce said DNA or RNA (e.g. transformation with a vector, bacterial conjugation, via phage transfer, transposition, homologous recombination).

[0040] The terms "vector", "cloning vector" and "expression vector" mean the vehicle by which a DNA or RNA sequence (e.g. a foreign gene) can be introduced into a host cell, so as to transform the host and promote expression of the introduced sequence. A common type of vector is a "plasmid", which generally is a self-contained molecule of doublestranded DNA, usually of bacterial origin, that can readily accept additional (foreign) DNA and which can readily be introduced into a suitable host cell. A plasmid vector often contains coding DNA and promoter DNA and has one or more restriction sites suitable for inserting foreign DNA. A large number of vectors have been described for replication and/or expression in a variety of prokaryotic hosts. Non-limiting examples include pKK plasmids (Clonetech), pUC plasmids, pET plasmids (Novagen, Inc., Madison, WI), pRSET or pREP plasmids (Invitrogen, San Diego, CA), or pMAL plasmids (New England Biolabs, Beverly, MA).

[0041] Accordingly, the application further discloses a vector suitable for transformation of a microorganism, in particular a bacterium or yeast acceptable for food consumption, which comprises a nucleic acid sequence encoding a L-arabinose isomerase.

[0042] In said vector, the nucleic acid sequence encoding a L-arabinose isomerase may be placed under the control of a strong and constitutive promoter, for instance the promoter of *L. bulgaricus* ATCC 11842 *hIbA* gene. Inducible promoters may also be used.

[0043] The sequence encoding a L-arabinose isomerase may also be fused with a secretion signal directing secretion of the encoded polypeptide.

[0044] The encoded L-arabinose isomerase may be any L-AI, as described above. In particular, the L-AI may derive from a bacterium selected from the group consisting of a bacterium of the genus *Thermus* (*Thermoanaerobacter mathranii*), of the genus *Thermotoga* (*Thermotoga maritima*, *Thermotoga neapolitana*), of the genus *Geobacillus* (*Geobacillus stearothermophilus*, *Geobacillus thermodenitrificans*), from *Alicyclobacillus acidocaldarius*, from of the genus *Lactobacillus* (in particular *Lactobacillus sakei*, *Lactobacillus plantarum*), from the genus *Bacillus* (*Bacillus stearothermophilus*) and from a lactic acid bacterium.

[0045] The vector according to the invention comprises a nucleic acid sequence encoding an L-arabinose isomerase selected from the group consisting of AAI, AAI-K269E, BAI, BAI-E268K, L-AI US 100, and L-AI US 100 mutants L-AI US100-Q268K, L-AI US100-N175H and L-AI US100- (Q268K, N175H).

[0046] The invention also encompasses the bacterium acceptable for food consumption, transformed with (i.e. which comprises) one of the vectors as described above, or which comprises a nucleic acid sequence encoding a L-arabinose isomerase as defined above.

Conversion of D-galactose into D-tagatose in milk

[0047] Conceptually, conversion of D-galactose into D-tagatose by L-arabinose isomerase directly in the milk, in particular during lactic acid fermentation, could have been hampered by several parameters:

- milk is a fat, complex medium, and it was unpredictable whether milk components could inactivate L-arabinose isomerase, where the enzyme is directly added into the milk;
- lactic acid bacteria, in particular *L. bulgaricus* and *S. thermophilus*, are galactose negative bacteria that excrete galactose into their culture medium during lactic acid fermentation of milk. Since L-arabinose isomerases are intracellular enzymes, it was unknown whether bioconversion of extracellular D-galactose into D-tagatose by a L-AI expressing bacterium would be feasible, due to a possible lack of accessibility of the enzyme to its substrate;
- during lactic acid fermentation, pH of milk switches from 6.5-6.8 to about 4.5 to 5.5. As the optimal pH of L-AIs, and in particular of the model enzyme used by the inventors (L-AI of *Bacillus stearothermophilus* US 100), is remote from the pH reached in the course of lactic acid fermentation, it was questionable whether the rate of D-galactose conversion into D-tagatose could be significant.

[0048] However, the inventors demonstrated that L-AI can successfully ensure conversion of D-galactose into D-tagatose in milk, especially during lactic acid fermentation of milk.

[0049] Accordingly, the invention relates to the use of a L-arabinose isomerase (L-AI) for converting D-galactose into D-tagatose in a dairy product which contains D-galactose, wherein said dairy product containing D-galactose is selected from the group consisting of milk, milk supplemented with D-galactose, milk undergoing lactic acid fermentation, a fermented milk, and milk in which lactose was enzymatically processed.

[0050] A dairy product generally denotes a foodstuff produced from milk and includes milk (e.g. milk optionally partly or totally skimmed and/or homogenized and/or pasteurized). A dairy product which contains D-galactose may include milk supplemented with D-galactose, milk undergoing lactic acid fermentation, fermented milk, or milk in which lactose was processed into glucose and galactose (e.g. milk treated with a lactase, such as a β -galactosidase in order to hydrolyse enzymatically lactose).

[0051] In particular, the invention may relate to the use of a L-arabinose isomerase (L-AI) for converting D-galactose into D-tagatose during lactic acid fermentation of milk or in a fermented milk.

[0052] The L-arabinose isomerase may be any L-arabinose isomerase, as described above.

[0053] Conversion of D-galactose into D-tagatose may be conducted by adding a L-AI, or L-AI-expressing microorganism, directly into the dairy product containing D-galactose.

[0054] Conversion may also be conducted by contacting the dairy product containing D-galactose with a L-AI or L-AI-expressing microorganism. According to this embodiment, the L-AI or microorganism may be recovered from the end product, hence is absent from the end product. Contacting may be performed, for instance, by adding into the dairy product a L-AI or L-AI expressing microorganism trapped in a solid structure, for instance in alginate beads, and by removing the solid structure, preferably when D-galactose conversion is sufficient to obtain the desired property. Contacting may also be performed by contacting the dairy product with a L-AI or a L-AI-expressing microorganism, immobilised on a solid support such as a column or a reactor.

[0055] According to an embodiment, at least one L-arabinose isomerase is added into the dairy product. Where the dairy product is milk to be fermented, addition of the L-arabinose isomerase into the milk may be performed before beginning of lactic acid fermentation, in the course or at the end of lactic acid fermentation.

[0056] The skilled person may readily determine the quantity of L-arabinose isomerase which may be added into the dairy product, in particular in a milk intended for lactic acid fermentation. For instance, the quantity of L-AI added may be comprised between 0.1-20 mg/ml (mg L-AI/ml milk), preferably 0.5-10 mg/ml, more preferably 1-5 mg/ml, still preferably 2-3 mg/ml.

[0057] According to another embodiment, at least one L-arabinose isomerase-expressing microorganism is added into the dairy product. Said microorganism is preferably a bacterium, in particular a bacterium acceptable for food consumption. Where the dairy product is milk to be fermented, addition of the L-AI expressing microorganism into the milk may be performed before

beginning of lactic acid fermentation, in the course or at the end of lactic acid fermentation.

[0058] L-arabinose isomerase-expressing microorganisms have been defined above. It may be a microorganism which was transformed to express L-arabinose isomerase. Alternatively, the L-arabinose isomerase-expressing microorganism may naturally express L-arabinose isomerase.

[0059] The quantity of microorganism which may be added in the dairy product may be readily determined by the skilled person. In particular, the level of L-AI activity of the microorganism in the dairy product may be assessed by using the cysteine carbazole sulfuric-acid method according to Dische and Borenfreund (J. Biol. Chem. 192 (1951) 583), and then, it may be determined what quantities of bacteria are required to provide the desired L-AI activity in the dairy product.

[0060] The lactic acid fermentation of milk may be intended for the production of any fermented dairy product, for instance yoghurt (stirred style yoghurt, set style yoghurt or yoghurt beverages), cultured buttermilk (skim or whole milk fermented most frequently with *Lactococcus lactis*), Acidophilus milk (i.e. a milk fermented with *Lactobacillus acidophilus*), sour cream (cream inoculated most frequently with *Lactococcus lactis*), and local beverages or products such as kefir, koumiss, beverages based on lactobacillus or bifidus strains, labneh.

[0061] In particular, yoghurt is produced by inoculating milk with a starter culture which is generally a symbiotic blend of *Streptococcus thermophilus* (ST) and *Lactobacillus bulgaricus* (LB).

Method of producing dairy products containing D-tagatose, and edulcorating milk additive

[0062] The application discloses a method of converting D-galactose into D-tagatose in a dairy product containing D-galactose, which method comprises the step of adding at least one L-arabinose isomerase and/or one L-arabinose isomerase-expressing microorganism acceptable for food consumption into said dairy product containing D-galactose, whereby D-galactose present in the dairy product is converted into D-tagatose.

[0063] The application also discloses a method of converting D-galactose into D-tagatose in a dairy product containing D-galactose, which method comprises the step of contacting said dairy product containing D-galactose with at least one L-arabinose isomerase and/or one L-arabinose isomerase-expressing microorganism, whereby D-galactose present in the dairy product is converted into D-tagatose.

[0064] According to the invention the dairy product containing D-galactose is a fermented milk or a milk undergoing lactic acid fermentation and the above methods then consist of methods of converting D-galactose produced by lactic acid fermentation of milk into D-tagatose.

[0065] Said method of converting D-galactose produced by lactic acid fermentation of milk into D-tagatose may comprise the steps of :

1. a) adding into the milk at least one L-arabinose isomerase and/or one L-arabinose isomerase-expressing microorganism acceptable for food consumption;
2. b) optionally inoculating the milk with one or more lactic acid bacteria; and
3. c) operating lactic acid fermentation of milk;

whereby D-galactose produced in the milk by lactic acid fermentation is converted into D-tagatose.

[0066] The invention further provides a method of converting D-galactose produced by lactic acid fermentation of milk into D-tagatose, which method comprises the steps of:

1. a) contacting the milk with at least one L-arabinose isomerase and/or one L-arabinose isomerase-expressing microorganism;
2. b) optionally inoculating the milk with one or more lactic acid bacteria;
3. c) operating lactic acid fermentation of milk; and
4. d) optionally recovering said at least one L-arabinose isomerase and/or one L-arabinose isomerase-expressing microorganism from the fermented milk obtained from step c);

whereby D-galactose produced in the milk during lactic acid fermentation is converted into D-tagatose.

[0067] Step a) of the above methods of converting D-galactose produced by lactic acid fermentation of milk into D-tagatose may be carried out before, simultaneously or after step c).

[0068] Where the method involves addition of, or contacting with, a L-arabinose isomerase-expressing microorganism, if said microorganism is not a lactic acid bacteria, then the method comprises a step of inoculating the milk with at least one lactic acid bacterium so that lactic acid fermentation of milk can be carried out.

[0069] Indeed, the presence of lactic acid bacteria in the milk is compulsory in order to operate lactic acid fermentation. If the L-arabinose isomerase-expressing microorganism is a lactic acid bacterium, addition of one or more lactic acid bacteria as recited in step b) is optional.

[0070] In the method wherein the L-AI or L-AI expressing microorganism is contacted with the milk to be fermented or undergoing fermentation, step d) is preferably carried out once lactic acid fermentation of milk is completed.

[0071] Contacting may be performed as described above, for instance by adding into the dairy product a L-AI or L-AI expressing microorganism trapped in a solid structure, for instance in alginate beads, and by removing the solid structure when D-galactose conversion is essentially complete. Contacting may also be performed by contacting the dairy product with a L-AI or a L-AI-expressing microorganism, immobilised on a solid support such as a column or a reactor.

[0072] The milk to be fermented may be e.g. raw milk, milk homogenized and/or pasteurised, skim milk, a milk supplemented with D-galactose, or milk in which lactose was enzymatically processed into glucose and galactose. In the course of the above method of converting D-galactose into D-tagatose during lactic acid fermentation of milk, a fermented dairy product is obtained which contains D-tagatose. As D-tagatose is an edulcorant (sweetener), the method of converting D-galactose into D-tagatose also results in the production of a sweetened fermented dairy product.

[0073] The invention thus further provides a method of producing a sweetened fermented dairy product which comprises the steps of :

1. a) adding at least one L-arabinose isomerase and/or one L-arabinose isomerase-expressing microorganism acceptable for food consumption into said fermented dairy product; or
2. b) contacting said dairy product containing D-galactose with at least one L-arabinose isomerase and/or one L-arabinose isomerase-expressing microorganism;

whereby a sweetened fermented dairy product is obtained.

[0074] Accordingly, the invention further provides a method of producing a sweetened fermented dairy product which comprises the steps of :

1. a) adding into a milk at least one L-arabinose isomerase and/or one L-arabinose isomerase-expressing microorganism acceptable in milk fermentation;
2. b) optionally inoculating the milk with one or more lactic acid bacteria;
3. c) operating lactic acid fermentation of milk;

whereby a sweetened fermented dairy product is obtained.

[0075] Another method of producing a sweetened fermented dairy product may comprise the steps of :

1. a) contacting milk with at least one L-arabinose isomerase and/or one L-arabinose isomerase-expressing microorganism;
2. b) optionally inoculating milk with one or more lactic acid bacteria;
3. c) operating lactic acid fermentation of milk; and
4. d) optionally recovering said at least one L-arabinose isomerase and/or one L-arabinose isomerase-expressing microorganism from the fermented milk obtained from step c);

whereby a sweetened fermented dairy product is obtained.

[0076] Step a) of the above methods of producing a sweetened fermented dairy product may be carried out before, simultaneously or after step c).

[0077] Where the method involves addition of, or contacting with, a L-arabinose isomerase-expressing microorganism, if said

microorganism is not a lactic acid bacteria, then the method comprises a step of inoculating the milk with at least one lactic acid bacterium so that lactic acid fermentation of milk can be carried out.

[0078] In the method wherein the L-AI or L-AI expressing microorganism is contacted with the milk to be fermented or undergoing fermentation, step d) is preferably carried out once lactic acid fermentation of milk is completed.

[0079] In the above methods, said microorganism is preferably a bacterium acceptable for food consumption.

[0080] The lactic acid bacteria (either expressing L-AI or not) to be used in the methods of converting D-galactose into D-tagatose, or of producing a sweetened fermented dairy product, may be any of the lactic acid bacteria which have been defined above. Furthermore, the L-arabinose isomerase and/or one L-arabinose isomerase-expressing microorganism to be added into the milk are as described above.

[0081] The step c) of operating lactic acid fermentation of milk is simply performed by culturing the lactic acid bacteria under conditions and for a time sufficient for lactic acid fermentation to take place. The conditions (temperature, etc) and time of culture will be chosen depending on the type of fermented milk product which is intended to be prepared. D-galactose is produced by lactic acid bacteria during lactic acid fermentation and converted into D-tagatose by L-AI activity.

[0082] As described above, the fermented dairy product which is produced may be selected from the group consisting of yoghurt, cultured buttermilk, Acidophilus milk, sour cream, kefir, koumiss, and labneh.

[0083] In the end, the L-arabinose isomerase or L-arabinose isomerase-expressing microorganism (in particular a bacterium, and preferably a bacterium acceptable for food consumption) which is added into the milk acts as a sweetening additive during lactic acid fermentation of milk. Accordingly, the invention also relates to a sweetening milk additive which comprises a L-arabinose isomerase and/or a L-arabinose isomerase-expressing microorganism as defined herein. Said additive is in particular intended to be used for the manufacture of fermented dairy products.

[0084] The invention will be further described in view of the following Figures and Examples.

FIGURES

[0085]

Figure 1: Acidification and production of D-tagatose during the growth of MRS9 in MRS supplemented with galactose. Growth was carried out at 37°C. (A): pH profile, (◆):D-tagatose production with MRS9 strain.

Figure 2: Acidification and production of D-tagatose during the growth MRS12 in M17 supplemented with galactose. Growth was carried out at 37°C. (A): pH profile, (■):D-tagatose production with MRS12 strain.

Figure 3: Production of D-tagatose during the growth at pH 6.4 of MRS9 in MRS supplemented with galactose and MRS12 in M17 supplemented with galactose. Growth was carried out at 37°C. (A): pH profile, (◆):D-tagatose production with MRS9 strain, (■):D-tagatose production with MRS12 strain.

Figure 4: Kinetic of the D-galactose conversion into D-tagatose in milk with D-galactose (10 g/L) and the purified L-AI US100 (3 mg/ml). Incubation was performed at 42 °C.

Figure 5: Temperature activity profile of the purified L-AI US100 and its derivatives. Assays were carried out under standard conditions in presence of 5 mM L-arabinose. Activities at the optimal temperature were defined as 100%.

Figure 6: pH activity profiles of the purified L-AI US100 and its derivatives. Assays were carried out under standard conditions in presence of 5 mM L-arabinose. Activities at the optimal pH were defined as 100%.

Figure 7: Appearance of D-tagatose using beads containing cells expressing the wild type L-AI US100 in milk enriched in D-galactose by lactose hydrolysis.

Figure 8: Appearance of D-tagatose using beads containing cells expressing the mutated L-AI N175H Q268K in milk enriched in D-galactose by lactose hydrolysis.

EXAMPLES**Example 1: Bioconversion of D-galactose to D-tagatose in milk, using micro-organisms expressing an L-arabinose isomerase****1.1 Material and Methods****Bacterial strains and growth conditions**

[0086] *L. bulgaricus* and *S. thermophilus* strains were grown under anaerobic conditions at 42°C or 37°C in MRS medium (Difco) (De Man, Rogosa, and Sharpe. 1960. J. Appl Bacteriol 23:130-135) and M17 lactose medium, respectively (Terzaghi, and Sandine. 1975. Appl Microbiol 29:807-813). These starters were also cultured in skim milk (Nilac Low Heat Milk powder, NIZO) heated for 10 min at 95°C (Courtin, Monnet, and Rul. 2002. Microbiology 148:3413-21). Stock cultures of each strain of *S. thermophilus* and *L. bulgaricus* were prepared after growth at 37°C in skim milk supplemented with antibiotic when required. The cultures were stopped at pH of 5.5, and bacterial numbers were estimated by plating appropriate dilutions of the culture on agar medium: M17Lactose was used for *S. thermophilus* cells, and MRS-Lactose pH 5.2 for *L. bulgaricus* cells. At the end of culture, bacteria were directly frozen in liquid nitrogen and kept at -80°C.

[0087] Mixed cultures of wild-type and recombinant *S. thermophilus* and *L. bulgaricus* strains were performed at 42 and 37°C respectively, by inoculating skim milk with 10^6 c.f.u.ml⁻¹ of stock cultures of each strain. Periodically, the pH of the culture was measured, and bacteria were enumerated as described above. When required, antibiotics were used at the following concentrations: for *E. coli*, ampicillin (100 µg/ml) or erythromycin (160 µg/ml); for *L. bulgaricus* and *S. thermophilus*, erythromycin (5 µg/ml). For liquid culture, the recombinant LAB strains were inoculated from an overnight culture at DO_{600 nm} = 0.1 and stopped at DO_{600 nm} = 1.

DNA manipulations and sequencing

[0088] Classical molecular biology techniques and isolation of plasmid DNA from *E. coli* and *S. thermophilus* were performed as described by Sambrook et al. (1989). Plasmid DNA from *L. bulgaricus* was prepared as previously described by Serror et al. (2002. Appl Environ Microbiol 68:46-52). DNA restriction and modifications enzymes were used as recommended by the suppliers. The oligonucleotides used in this work were purchased from PROLIGO (Japan) and MWG (France). When required, DNA fragments were isolated from agarose gels by using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Bioscience). PCR amplifications, using *Pfu* polymerase (Appligene), were performed with a Gene Amp® PCR System 2700 (Applied Biosystems). Plasmid constructions were transferred from *E. coli* to *L. bulgaricus* VI104 and *S. thermophilus* LMD9 by electroporation (Serror et al. 2002. Appl Environ Microbiol 68:46-52; Somkuti, G. A., and D. H. Steinberg. 1988. Biochimie 70:579-85).

[0089] DNA sequencing was performed using an automated 3100 Genetic Analyser (Applied Biosystems).

Plasmids

[0090] All plasmids and strains used in this work are listed in Table 1. The pMR2 plasmid corresponds to pVI165 (an *E. coli*-lactic acid bacteria shuttle, constructed in the laboratory) carrying a 1577 bp SacII-SacI DNA fragment, harboring the *araA* US100 gene encoding an L-arabinose isomerase referred to as L-AI US100 without promoter (Rhim and Bejar. 2006. Biochim Biophys Acta 1760:191-9.). The plasmid pMR3 corresponds to pVI165 carrying a 147 bp fragment containing p_{HLbA}, a strong constitutive promoter from *L. bulgaricus* ATCC11842 (Serror & Chouayekh unpublished, van de Guchte et al, 2006. PNAS). The plasmid pMR4 corresponds to pMR3 carrying the *araA* US100 gene isolated from pMR1 (SacII - SacI fragment) downstream of the p_{HLbA} promoter.

Table 1: Bacterial strains and plasmids

Strains	Characteristics	Source/reference
<i>Streptococcus thermophilus</i> LMD9	Type strain	Bolotin et al. Nat Biotechnol. 2004; 22(12):1554-8.
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> ATCC 11842	Type strain	van de Guchte et al. Proc Natl Acad Sci USA. 2006 103(24):9274-9.
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> VI104		Serror et al.. Appl Environ Microbiol. 2002. 68(1):46-52
Plasmids		
pVI165	pVI1055 (Serror et al. Microbiology. 2003.149:1503-11) containing a cluster of 3 transcription terminators (from pVI152)	(Chouayekh & van de Guchte, unpublished).
pMR2	<i>araA</i> US100 (Rhimmi & Bejar 2006. BBA 1760:191-9) cloned in pVI165	This work
pMR3	<i>p_{hlbA}</i> promoter cloned in pVI165	This work
pMR4	<i>araA</i> US100 from pMR1 cloned in pMR3 downstream the <i>hlbA</i> promoter	This work

Preparation of crude enzyme extracts

[0091] For lactic acid bacteria, cultures done in the appropriate media were centrifuged and the pellets were suspended in 100 mM MOPS Buffer (pH 7.5). However, in skim milk cultures, corresponding to the separate or co-culture of *L. bulgaricus*/pMR4 and *S. thermophilus*/pMR4 strains, before cell centrifugation 0.3 volume of 1 M Tris-sodium citrate was added to the culture. Cells were then centrifuged and cells pellets were washed three times with extraction Buffer (20 mM Sodium phosphate, pH 6.4). Then, recovered pellet was suspended in 100 mM MOPS Buffer (pH 7.5).

[0092] Cell disruption was performed using a mini-bead beater 8TM cell disrupter, and cell debris were removed by centrifugation at 15000 rpm for 30 min at 4°C.

Protein quantification, electrophoresis and identification by mass spectrometry

[0093] Protein concentration was determined by the Bradford method (Bradford. 1976. Anal. Biochem 72:248-254). The protein samples were separated in 12 % SDS-PAGE gel electrophoresis according to the method of Laemmli (Laemmli. 1970. Nature 227:680-5). Protein bands were visualized by Coomassie blue Biosafe (BioRad) staining.

[0094] Extracted proteins, corresponding to each recombinant strain, were separated on SDS-PAGE electrophoresis. After coloration with Coomassie blue Biosafe staining (BioRad), bands having the expected molecular weight were excised from the gel and washed in acetonitrile/50 mmol.l⁻¹ ammonium hydrogenocarbonate (v/v). Then, samples were stirred for 15 min, vacuum-dried for 30 min, and 0.5 mg trypsin (Promega) in 25 ml ammonium hydrogenocarbonate (50 mmol.l⁻¹) was added. Samples were digested for 16 h at 37°C. For Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) analyses, 2 µl TFA 5 % was added to stop the trypsinolysis reaction. Peptides mixtures were analyzed using a cyano-4-hydroxycinamic matrix prepared at 5 mg/ml in 50 % acetonitrile containing 0.1 % trifluoroacetic acid. The trypsin autolytic peptides (842.5 and 2211.1 Da) were used as internal calibrants. Peptides were selected in the mass range 700-4000 Da.

[0095] Peptides were analysed using a Voyager DE STR MALDI-TOF mass spectrometer (Applied Biosystems). Recorded MS/MS spectra were compared to theoretical fragmentations of a trypsinolysed *araA* US100 protein (Accession number: CAI29261), via the Sequest algorithm incorporated in the BioWorks software (version 2, Thermo Finnigan).

Assay for L-arabinose isomerase activity

[0096] L-arabinose isomerase activity was measured by the determination of the amount of the produced L-ribulose or D-tagatose. In standard conditions, the reaction mixture contained 0.2 mM CoCl₂, 1 mM MnSO₄, 50 μ l of crude enzyme extract at a suitable dilution, 5 mM of L-arabinose (D-galactose) and MOPS buffer 100 mM (pH 7.5) to bring the final volume to 1 ml. The reaction mixture was incubated at 80°C for 1 min and 10 min for L-arabinose and D-galactose respectively. Then, samples were immediately chilled on ice to stop the reactions. The generated L-ribulose (or D-tagatose) was determined by the cysteine carbazole sulfuric-acid method, and the absorbance was measured at 560 nm (Dische and Borenfreund, 1951. J Biol Chem 192:583-7). The D-tagatose production was also checked with high-performance ionic chromatography (HPLC) using Polypore CA column (250 \times 4.6 mm). The products were separated by isocratic elution with water at a flow rate of 0.3 ml/min and detected with a refractive index detector (SHIMADZU, Refractive Index Detector RID-10A). A 10 g/l solution of lactose, D-galactose and D-tagatose were used as standards. One unit of L-arabinose isomerase activity was defined as the amount of enzyme catalyzing the formation of 1 μ mol keto-sugar per min under the above specified conditions.

Detection of in vivo D-tagatose production conferred by L-AI US100 expression

[0097] The *L. bulgaricus* and *S. thermophilus* strains containing pMR4 were cultured in MRS and M17 media containing D-galactose at final concentration of 10 g/l, respectively. Using an overnight culture, the medium was inoculated at DO₆₀₀ nm of 0.1. Samples were collected each hour, until 12 hours, followed by the monitoring of the L-AI US100 activity by cysteine carbazole sulfuric-acid method in the supernatant. The D-tagatose production was also checked HPLC.

Detection of the in vitro D-tagatose production in milk using L-AI US100

[0098] The purified L-AI US100 (3 mg/ml) was added in milk supplemented with D-galactose (final concentration of 10 g/l). Alternatively, the purified enzyme was added to the co-culture of the wild-type and recombinant *L. bulgaricus* and *S. thermophilus* strains performed in milk. The study of the kinetic bioconversion of D-galactose was investigated until 20 hours. Milk proteins were removed by defecation step which consists of the addition of one volume of 30 % Zinc acetate and 15 % potassium Ferrocyanure (w/v) solutions respectively, followed by a centrifugation at 15000 during 20 min. Then, sugars were recovered in the aqueous phase. The D-tagatose production, in this supernatant fraction, was proved via cysteine carbazole sulfuric-acid method and confirmed by HPLC.

1.2 Results

Production of active L-AI US 100 in/from yoghurt starters

[0099] The plasmid pMR4 containing p_{HlbA}:araA US100 fusion and the two negative controls pMR2 (containing the araA gene without promoter) and pMR3 (containing p_{HlbA} alone) were introduced in the *L. bulgaricus* (strain V1104) and *S. thermophilus* (strain LMD9) yoghurt starters, which do not metabolize galactose during lactose fermentation. Expression of L-AI activity was checked in crude extracts of the resulting strains grown in synthetic and milk media using cysteine carbazole sulfuric-acid method (Material and methods).

[0100] After growth in synthetic media, the L-AI activity was only detected in the crude cellular extracts prepared from the *L. bulgaricus* and *S. thermophilus* strains containing pMR4 (p_{HlbA}:araA US100), i.e. MRS9 and MRS12, respectively. The activities measured were 25 \pm 0.32 and 15 \pm 0.7 U/mg for *L. bulgaricus* and *S. thermophilus*, respectively (Table 2).

Table 2: L-AI US100 activity expressed by the *L. bulgaricus* and *S. thermophilus* strains grown in synthetic or milk media.

Strain description	Strain designation	L-AI US100 activity (U/mg) Laboratory medium	L-AI US100 activity (U/mg) Milk
V1104/pMR2	MRS7	ND	ND
V1104/pMR3	MRS8	ND	ND
V1104/pMR4	MRS9	25 \pm 0.32	21 \pm 0.14
LMD9/pMR2	MRS10	ND	ND

Strain description	Strain designation	L-AI US100 activity (U/mg) Laboratory medium	L-AI US100 activity (U/mg) Milk
LMD9/pMR3	MRS11	ND	ND
LMD9/pMR4	MRS12	15±0.7	12±0.18
VI104/pMR4 & LMD9/pMR4	MRS9 and MRS12	ND	32±0.54
ND: Not Detected			

[0101] In addition to the measurement of the L-AI activity *in vitro*, crude cellular extracts prepared from *L. bulgaricus* and *S. thermophilus* harboring the various plasmids pMR2, pMR3 and pMR4 were run on SDS-PAGE and stained with Coomassie blue. At the molecular weight expected for the L-AI US100 monomer (56 kDa), new bands were detected in the samples corresponding to the *L. bulgaricus* and *S. thermophilus* strains carrying pMR4. These bands as well as their weaker counterparts in the control samples were excised and proteins were analyzed by mass spectrometry (MS). L-AI US100 was unambiguously identified in the bands originating from the strains containing pMR4 whereas it was not detected in the thinner bands obtained with negative controls.

[0102] Subsequently, the expression and functionality of L-AI US100 were evaluated in milk, the usual growth condition of yoghurt starters. *L. bulgaricus* and *S. thermophilus* strains carrying pMR4 (*p_{hIbA} araA*) were cultivated separately or in association in milk and the LAI activity was measured in crude cellular extracts. In single species cultures, the *in vitro* L-AI specific activities were 21±0.14 and 12±0.18 U/mg for *L. bulgaricus* and *S. thermophilus* strains carrying pMR4, respectively. In the co-culture, the L-AI specific activity reproducibly reached 32±0.54 U/mg (Table 2). An analysis using SDS-Page electrophoresis and mass spectrometry confirmed the presence of L-AI US100 only in the extracts derived from the milk cultures of strains containing pMR4.

[0103] These observations were in good agreement with the *in vitro* L-AI activity measurements that clearly proved the efficient expression of L-AI US100 in both yoghurt starter species under the control of the *p_{hIbA}* promoter.

Bioconversion of D-galactose into D-tagatose by strains expressing the L-AI US100 in medium enriched with D-galactose

[0104] To test the D-galactose isomerisation by the *in vivo* expressed L-AI US100, the *L. bulgaricus* and *S. thermophilus* recombinant strains were grown in laboratory media enriched with 10 g/l of D-galactose. Kinetic studies during the growth of *L. bulgaricus* and *S. thermophilus* carrying pMR4 revealed that a new ketohehexose was formed in the culture medium (Figures 1-2). This ketohehexose identified as D-tagatose by HPLC, accumulated up to 20% of the overall ketohehexose (i.e. about 2 g/L) after 12 hours of fermentation.

Bioconversion of D-galactose into D-tagatose during milk fermentation

[0105] The *in vivo* bioconversion of the D-galactose produced during milk fermentation by means of the LAB starters expressing *araA* (MRS9 and MRS12) was then tested. A sample fermented by the parental LAB starters which did not exhibit any L-AI activity was also used as a control. The sugar composition of the growth medium was determined by HPLC at various incubation times at 42°C (Table 3). D-galactose accumulated in all cultures but D-tagatose was only identified in the cultures supplemented with L-AI via its expression from the LAB starters.

Table 3: Analysis of sugar composition in milk fermented by various *L. bulgaricus* and *S. thermophilus* starters.

	Time (h)	VI104 & LMD9	MRS9 & MRS12
D-galactose (g/l)	0	0	0
	10	6.6	3.9
	20	10.2	6.2
D- tagatose (g/l)	0	0	0
	10	0	0.33
	20	0	0.72

[0106] Considering that the optimal pH for L-AI activity is about pH 7.5-8, it was wondered whether the acidification of the medium resulting from the production of lactic acid by the LAB starters (Figures 1-2) could impair the efficiency of the expressed L-AI and the kinetic of D-tagatose conversion. In order to investigate the effect of the medium acidification on the yield of D-tagatose production, MRS9 and MRS12 strains were grown in a bioreactor allowing the neutralization of lactic acid by the addition of NaOH (5N) (Figure 3). In this system, the final bioconversion yield was similar but the kinetic of apparition of D-tagatose was much faster: the maximal amount of D-tagatose was reached after 9 h instead of 12 h.

[0107] All these results, established that the L-AI US100 enzyme produced by the LAB strains is active and allows the conversion of D-galactose in medium enriched with D-galactose and during milk fermentation although the enzyme is in the bacterial cytoplasm.

[0108] *Conversion of D-galactose by supernatant of LAB secreting the L-AI US100* The L: strain MG1363 containing the plasmid *pSEC:araUS100* was used to expressed the *ara* gene fused to a sequence encoding the peptide signal of Usp45, the main secreted protein of *L. lactis*. The control strain is an isogenous strain containing the same plasmid deleted of the secretion signal. The L-AI activity was detected in the supernatant of the culture MG1363/*pSec:araUS100* indicating that the enzyme is secreted when associated to a functional peptide signal. The supernatant was added to synthetic growth medium and milk supplemented with D-galactose (10 g/L). The bio-conversion of galactose in D-tagatose was observed in both media albeit at low efficiency; after 10 h of incubation, 0.4 g/L of D-tagatose was measured in each of the samples. This observation established for the first time that the L-AI enzyme can be secreted into the medium when its gene is fused to a peptide signal encoding sequence and is secreted in its active form in the medium. This mode of enzyme production may be interesting for industrial preparation of the enzyme after improvement of the efficiency of expression-secretion.

Example 2: Production of D-tagatose by a L-arabinose isomerase used as an additive in milk enriched in D-galactose

2.1 Material and Methods

Bacterial strains and growth conditions

[0109] The bacterial strain used to purify the L-AI US100 for this work is HB101 containing pMR6, a pTRC99A derivative carrying the *araA* gene of *B. stearotheophilus* US100. This *E. coli* strain was grown in aerated Luria-Bertani medium (Difco) at 37°C (Sambrook et al., 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA). *L. bulgaricus* and *S. thermophilus* strains were cultured in skim milk (Nilac Low Heat Milk powder, NIZO) heated for 10 min at 95°C (Courtin, Monnet, and Rul 2002. Microbiology 148:3413-21). Stock cultures of each strain of *S. thermophilus* and *L. bulgaricus* were prepared after growth at 37°C in skim milk supplemented with antibiotic when required. The cultures were stopped at pH of 5.5, and bacterial numbers were estimated by plating appropriate dilutions of the culture on agar medium: M17Lactose was used for *S. thermophilus* cells, and MRS-Lactose pH 5.2 for *L. bulgaricus* cells. At the end of culture, bacteria were directly frozen in liquid nitrogen and kept at -80°C. Mixed cultures of *S. thermophilus* and *L. bulgaricus* strains were performed at 42°C by inoculating skim milk with 10^6 c.f.u/ml⁻¹ of stock cultures of each strain. Periodically, the pH of the culture was measured, and bacteria were enumerated as described above.

Preparation of crude extracts and protein purification

[0110] Cells were harvested by centrifugation at (7500 × g, for 10 min) and the pellets were suspended in 100 mM HEPES buffer supplemented, where indicated, with 1 mM MnCl₂ and 0.2 mM CoCl₂. Then, cell suspensions were incubated for two hours on ice in the presence of 5 mg/ml lysozyme, 100 mM PMSF (phenylmethane-sulfonyl fluoride) and 2 µg/ml pepstatin A. Cell disruption was carried out by sonication at 4°C for 6 min (pulsations of 3 s, amplify 90) using a Vibra-Cell™ 72405 Sonicator and cell debris were removed by centrifugation (30 000×g, for 30 min at 4°C).

For protein purification, crude cell extract from each strain was heated (70°C for 30 min) followed by centrifugation at 30 00×g, for 30 min at 4°C. Proteins were precipitated between 60 and 80 % ammonium sulphate saturation, suspended in 100 mM HEPES buffer pH 7.5, concentrated and desalted in centrifugal micro-concentrators (Amicon, Inc) with a 30 kDa membrane cut off. Purification to homogeneity was achieved by fast-performance liquid chromatography (FPLC-Amersham-Bioscience) using a

UNO-Q anion-exchange column equilibrated with 100 mM HEPES buffer (pH 7.5). The proteins were eluted at a flow rate of 3 ml/min using a linear NaCl gradient ranging from 0 to 1 M in the same HEPES buffer. Pooled fractions displaying L-AI activity were desalted and concentrated.

Assay for L-arabinose isomerase activity

[0111] L-arabinose isomerase activity was measured by the determination of the amount of the produced L-ribulose or D-tagatose. In standard conditions, the reaction mixture contained 0.2 mM CoCl₂, 1 mM MnSO₄, 50 µl of crude enzyme extract at a suitable dilution, 5 mM of L-arabinose (D-galactose) and MOPS buffer 100 mM (pH 7.5) to bring the final volume to 1 ml. The reaction mixture was incubated at 80°C for 1 min and 10 min for L-arabinose and D-galactose respectively. Then, samples were immediately chilled on ice to stop the reactions. The generated L-ribulose (or D-tagatose) was determined by the cysteine carbazole sulfuric-acid method, and the absorbance was measured at 560 nm (Dische and Borenfreund. 1951. J Biol Chem 192:583-7). The D-tagatose production was also checked with high-performance ionic chromatography (HPLC) using Polypore CA column (250 × 4.6 mm). The products were separated by isocratic elution with water at a flow rate of 0.3 ml/min and detected with a refractive index detector (SHIMADZU, Refractive Index Detector RID-10A). A 10 g/l solution of lactose, D-galactose and D-tagatose were used as standards. One unit of L-arabinose isomerase activity was defined as the amount of enzyme catalyzing the formation of 1 µmol keto-sugar per min under the above specified conditions.

Detection of the *in vitro* D-tagatose production in milk using L-AI US100

[0112] The purified L-AI US100 (3 mg/ml) was added in milk supplemented with D-galactose (final concentration of 10 g/l). Alternatively, the purified enzyme was added to the co-culture of the wild-type and recombinant *L. bulgaricus* and *S. thermophilus* strains done in milk. The study of the kinetic bioconversion of D-galactose was investigated until 20 hours. Milk proteins were removed by defecation step which consists on the addition of one volume of 30 % Zinc acetate and 15 % potassium Ferrocyanure (w/v) solutions respectively, followed by a centrifugation at 15000 during 20 min. Then, sugars were recovered in the aqueous phase. The D-tagatose production, in this supernatant fraction, was proved via cysteine carbazole sulfuric-acid method and confirmed by HPLC.

2.2 Results

Isomerisation of D-galactose to D-tagatose by the addition of L-AI US100 in milk enriched with D-galactose

[0113] The possibility to add the L-AI US100 enzyme directly in milk to promote the isomerisation of D-galactose into D-tagatose was tested. As a first step, the purified enzyme was added to milk supplemented with D-galactose and incubated at 42°C. The kinetic of D-tagatose production was followed using the cysteine carbazole sulfuric-acid method. The bioconversion of D-galactose increased proportionally with the incubation period and a maximal conversion rate of 24 % was reached after nearly 8 hours of incubation (Figure 4). The conversion of D-galactose into D-tagatose was confirmed by HPLC analysis, which showed a decrease in D-galactose concentration concomitant with an increase in D-tagatose production. These results and comparison with the activity measured in a standard reaction performed at 42°C and pH 6.5 (not shown) evidenced that L-AI US100 enzyme was fully active when used as an additive in milk.

Isomerisation of D-galactose produced during milk fermentation by the addition of L-AI US100

[0114] The *in vivo* bioconversion of the D-galactose produced during milk fermentation by means of the L-AI US100 enzyme was then tested. The milk was fermented by LAB starters in presence of 1 mg/ mL of purified L-AI enzyme used as an additive. Analysis of sugar composition of the fermentation medium showed an accumulation of D-tagatose. The sugar composition of the growth medium was determined by HPLC at various time of the fermentation (Table 4). D-galactose accumulated in all cultures but D-tagatose was only identified in the cultures supplemented with L-AI by the addition of purified enzyme.

Table 4: Analysis of sugar composition in milk fermented by LAB with L-AI US100. LAB starter is *L. bulgaricus* V1104 and *S. thermophilus* LMD9.

	Time (h)	Starter strains only	Starter and enzyme (mg/ml)			
			1	2	3	4
D- tagatose (g/l)	0	0	0	0	0	0
	4	0	0.3	0.5	0.6	0.5
	6	0	0.6	0.8	0.9	0.7
	10	0	1.5	1.7	2.1	1.7
	20	0	2.3	2.3	2.7	2.3

Optimisation of the enzyme concentration needed for the D-tagatose production during milk fermentation.

[0115] D-tagatose production by adding the purified L-AI US100 was explored. In fact, the concentration of the added protein in culture was varied between 1 and 4 mg/ml. The results obtained indicate that the kinetic D-galactose conversion increased proportionally to the protein concentration until 3 mg/l. Above this concentration, the addition of higher enzyme concentrations did not lead to a noteworthy effect (Table 4).

[0116] Altogether, these results establish for the first time that an L-arabinose isomerase used as an additive during milk fermentation efficiently converts the D-galactose produced by the fermentation in D-galactose.

Example 3: Bioconversion of D-galactose to D-tagatose in milk by L-arabinose isomerase derivatives

3.1 Materials and Methods

Bacterial strains, media, plasmids and growth conditions

[0117] *Escherichia coli* HB101 (F *hsdS20 ara-1 recA13 proA12 lacY1 galK2 rpsL20 mtl-1 oxyI-5*) was used as an *araA*-deficient host strain for the purification of the L-arabinose isomerase and its derivatives. Culture of *E. coli* strains, harbouring wild-type and mutated gene, was done in Luria Bertani (LB) medium (Sambrook et al., 1989 supra) and supplemented, when necessary, with ampicillin (100 µg/ml) and IPTG (160 µg/ml). The plasmid pMR5 containing the wild-type *araA* US100 gene (Rhim and Bejar, Biochim Biophys Acta 1760 (2006) 191), was used as template and for the production of the wild-type L-AI US100 protein. Plasmid pMR37 carries the *araA* S71 P gene, pMR35 carries the *araA* N175H gene, pMR21 contains the *araA* Q268K gene and pMR36 carries the *araA* N175H Q268K gene.

PCR and DNA manipulation

[0118] Polymerase chain reactions were carried out in Gene Amp[®] PCR System 9700 (Applied Biosystems). The amplification reaction mixtures (50 µl) contained *pfu* (Turbo DNA polymerase) amplification buffer, 10 mM (NH₄)₂SO₄, 100 ng of each primer, 50 ng of DNA template, and 2 Units of *pfu* enzyme (Appligene). The cycling parameters were 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 60°C for 60 s, and 72°C for 120 s. Preparation of plasmid DNA, digestion with restriction endonucleases and separation of fragments by agarose gel electrophoresis were performed as described by Sambrook et al. (1989, supra).

Construction of L-arabinose isomerase derivatives

[0119] The L-arabinose isomerase derivatives were all generated by site-directed PCR mutagenesis (as described below for mutation N175H and Q268K and with the Quick change II XL-site directed mutagenesis Kit [Stratagene] for S71 P and the double mutant N175H Q268K) using the wild-type L-AI US100 coding sequence in plasmid pMR5 as template. Thus, two non-mutagenic external primers F-*araA* and R-*araA*, and for each derivatives two complementary internal primers containing the desired mutation

were designed (Table 5).

Table 5: Oligonucleotides used for site directed mutagenesis. Nucleotide sequences corresponding to the mutated amino-acids are underlined.

Designation	Primer set
FaraA	GTGAACGGGGAGGAGCAATG (SEQ ID NO :3)
RaraA	GAAATCTTACCGCCCCCGCC (SEQ ID NO :4)
S71P D	GAATGGAATCGCGATCCGGTGTTCGGTTCCCA (SEQ ID NO :7)
S71 P R	TGGGAACGAAACACCGGATCGCGATTCCATTC (SEQ ID NO :8)
N175H D	GCGTTTGCGGAAAGCCGCAACCTAAAAGTGGCTCGT (SEQ ID NO :9)
N175H R	ACGAGCCACTTTTAGGTTGCGGCTTTCGCAAACGC (SEQ ID NO :10)
Q268K D	GCCTTTTTGAAAGACGGGAAC (SEQ ID NO :5)
Q268K R	GTTCCCGTCTTTCAAAAAGGC (SEQ ID NO :6)

[0120] Then, two separate PCR reactions were done and the resulting fragments were amplified using pMR5 as template with the corresponding primers for each reaction. The resulting two PCR fragments were extracted separately, and then a third amplification was carried out on a mixture containing these fragments in the presence of the external primers. The PCR products were purified using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Bioscience), following the manufacturer's instructions.

[0121] The purified mutated DNA fragments were cloned into a pUT57 vector, previously linearized with the *SmaI* restriction enzyme, and transformed into the HB101 strain. Transformants were plated on LB medium supplemented with ampicillin (100 µg/ml) and IPTG (160 µg/ml). The obtained colonies were analysed by restriction and mutations were confirmed by DNA sequencing using an automated DNA sequencer (Applied Biosystems).

Preparation of crude extracts and protein purification

[0122] Cells were harvested by centrifugation at (7500 × g, for 10 min) and the pellets were suspended in 100 mM HEPES buffer supplemented, where indicated, with 1 mM MnCl₂ and 0.2 mM CoCl₂. Then, cell suspensions were incubated for two hours on ice in the presence of 5 mg/ml lysozyme, 100 mM PMSF (phenylmethane-sulfonyl fluoride) and 2 µg/ml pepstatin A. Cell disruption was carried out by sonication at 4°C for 6 min (pulsations of 3 s, amplify 90) using a Vibra-Cell™ 72405 Sonicator and cell debris were removed by centrifugation (30 000×g, for 30 min at 4°C).

[0123] For protein purification, crude cell extract from each strain was heated (70°C for 30 min) followed by centrifugation at 30 000×g, for 30 min at 4°C. Proteins were precipitated between 60 and 80 % ammonium sulphate saturation, suspended in 100 mM HEPES buffer pH 7.5, concentrated and desalted in centrifugal micro-concentrators (Amicon, Inc) with a 30 kDa membrane cut off. Purification to homogeneity was achieved by fast-performance liquid chromatography (FPLC-Amersham-Bioscience) using a UNO-Q anion-exchange column equilibrated with 100 mM HEPES buffer (pH 7.5). The proteins were eluted at a flow rate of 3 ml/min using a linear NaCl gradient ranging from 0 to 1 M in the same HEPES buffer. Pooled fractions displaying L-AI activity were desalted and concentrated.

Protein quantification and electrophoresis

[0124] Protein concentrations were determined using Bradford's method with bovine serum albumin as standard (Bradford. Anal Biochem 72 (1976) 248). The purified enzyme samples were migrated in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (Laemmli.1970. Nature 227:680). Protein bands were visualized by Coomassie brilliant blue R-250 (BioRad) staining.

Enzyme assays and determination of the temperature and pH effects

[0125] L-arabinose isomerase activity was measured by determining the amount of L-ribulose or D-tagatose formed in the

following reaction. The standard reaction mixture contained 0.2 mM CoCl₂, 1 mM MnCl₂, 50 µl of enzyme preparation at a suitable dilution, 5 mM of L-arabinose or D-galactose and MOPS buffer 100 mM (pH 7.5) to bring the final volume to 1 ml. The reaction mixture was incubated at 80°C for 1 min and 10 min for L-arabinose and D-galactose, respectively, followed by cooling of the samples on ice to stop the reaction. The generated L-ribulose (or D-tagatose) was determined by the cysteine carbazole sulfuric-acid method, and the absorbance was measured at 560 nm (Dische and Borenfreund, J Biol Chem 192 (1951) 583). The D-tagatose production was also confirmed by high-performance ionic chromatography (HPLC) using a Polypore CA column (250 × 4.6 mm). The products were separated by isocratic elution with water at a flow rate of 0.3 ml/min and detected with a refractive index detector (SHIMADZU, Refractive Index Detector RID-10A). Solutions 5 g/l of D-galactose and D-tagatose were used as standards. The thermoactivity profile was studied by performing the standard reaction (described above) at temperatures comprised between 30 and 90°C. The pH effect was measured by carrying out the standard reaction (described above) at pHs ranging from 4.0 to 9.0 (4 to 6 adjusted with acetate, 6.0 to 8.0 using MOPS buffer and 8.0 to 9.0 using Glycine buffer).

3.2. Results

Mutant design, purification and biochemical characterization

[0126] The optimal conditions for L-AI US100 activity (80°C and pH 7.5) may not be always adapted to the conditions of food processing e.g., in the process of milk fermentation by LAB starters (42°C to 44°C and pH between 6.5 and 4.5). Therefore, the biochemical properties of various L-AI derivatives were investigated. The catalytic residues implicated in the isomerisation reaction catalysed by L-AI US100, and those involved in the enzyme affinity were previously identified (Rhimi et al., J Bacteriol. 2007. 189(9):3556-63). In order to investigate the relationship between the structure and the function of the L-AI US100, several derivatives of this enzyme were generated by site-directed PCR mutagenesis. The activity of several of these L-AI derivatives was tested at various temperatures and pH.

[0127] The activity study at different temperatures showed that the L-AI US100 protein, the L-AI US100 S71 P and the L-AI US100 Q268K had similar profiles and were optimally active at 80°C. In contrast, L-AI US100 N175H and the double mutant L-AI US100 N175H Q268K exhibited different patterns with optimal temperatures of 60-70°C and 55-60°C, respectively (Figure 5). Regarding the optimal pH for activity, L-AI US100 and its derivatives L-AI US100 S71P and L-AI US100 N175H similarly displayed the highest activity at pH ranging between 7.5 and 8.0, whereas other derivatives such as the L-AI US100 Q268K and the double mutant L-AI US 100 N175H Q268K had an optimal pH of 6.0-6.5 and 7.0, respectively (Figure 6). In addition thereto, the relative activity values of the mutated L-AI N175H Q268K and L-AI Q268K were increased from pH 5.0 to 6.5 by more than 15% and by 25 % as compared to those of wild-type L-AI US100, respectively.

[0128] These results demonstrate that the N175H mutation shifted the enzyme optimal temperature from 80°C to 60-70°C without affecting the enzyme optimal pH while the Q268K mutation significantly shifted the optimal pH of the mutated enzyme from 7.5-8, as observed for the wild-type L-AI US100, to 6.0-6.5 while it did not change the enzyme optimal temperature. In other words, the N175H mutation improved the mesophily of the enzyme while the Q268K mutation increased its acidotolerance. The effect of these mutations cumulates in the L-AI US100 N175H Q268K derivative which is both more mesophile and more acidotolerant than the wild-type L-AI US100 enzyme. Although the mutation S71P had no effect on the two parameters tested above, it (as other L-AI mutations) may prove beneficial for other biochemical parameters and for given applications.

D-tagatose production by L-AI derivatives in milk enriched with D-galactose

[0129] The conversion of D-galactose (10 g/L) in milk was tested with four of the L-AI US100 derivatives (3 mg/mL). At various time during the incubation at 42°C, samples were harvested and the amount of D-tagatose produced was measured (Table 6). Although at 20h, the final conversion yield was similar for all enzymes, the production of D-tagatose was faster in the samples containing L-AI N175H Q268K or L-AI N175H than in samples with L-AI Q268K or L-AI US100. This observation is in complete agreement with the biochemical properties of the L-AI derivatives. The mesophily of L-AI N175H and L-AI N175H Q268K indeed allow these enzymes to be more active in the condition tested i.e., pH6.5 and 42°C than L-AI US100 and the acidotolerant L-AI Q268K.

Table 6: production of D-tagatose by L-AI US100 and four of its derivatives in milk enriched with D-galactose.

Time (h)	L-AI	S71P	N175H	Q268K	N175H Q268K
0	0	0	0	0	0

Time (h)	L-AI	S71P	N175H	Q268K	N175H Q268K
4	0.6	0.6	0.9	0.6	1.0
6	0.9	0.9	1.3	1.1	1.7
8	1.0	1.0	1.7	1.4	2.0
10	1.1	1.1	1.9	1.7	2.1
20	2.3	2.3	2.4	2.3	2.4

D-tagatose production by L-AI derivatives during milk fermentation by LAB

[0130] The conversion of D-galactose in D-tagatose by L-AI US100 and each of its four derivatives (S71P, Q268K, N175H, N175H Q268K) was then tested during milk fermentation by LAB starters, *L. bulgaricus* V1104 and *S. thermophilus* LMD9. At various fermentation times, samples were harvested to measure the amount of D-tagatose produced and the pH (Table 7). Although at 20 h, the final conversion yield was similar for all enzymes, the production of D-tagatose was faster in samples containing L-AI N175H, L-AI N175H Q268K or L-AI Q268K than in samples with L-AI US100 or L-AI S71P. The conversion of D-galactose appeared faster with L-AI N175H (more mesophile) than with L-AI Q268K (more acidotolerant) but the best conversion rate was observed with L-AI N175H Q268K, the L-AI double mutant which exhibited both a lower optimum temperature and a lower optimum pH for activity compared to L-AI US100. These data demonstrate that modifications of biochemical properties of at least three L-AI derivatives improved their performance during the specific process such as the production of yoghurt or fermented milk.

Table 7: production of D-tagatose (Tag., in g/L) by various L-AI during milk fermentation.

Time (h)	L-AI		S71P		N175H		Q268K		N175H Q268K	
	pH	Tag.	pH	Tag.	pH	Tag.	pH	Tag.	pH	Tag.
0	6.6	0	6.6	0	6.6	0	6.6	0	6.6	0
4	5.7	0.5	5.7	0.5	5.7	0.8	5.7	0.7	5.8	1.1
8	5.4	0.7	5.4	0.7	5.4	1.2	5.3	1.0	5.5	1.6
6	5	0.9	5	0.9	5	1.7	4.9	1.5	5.1	1.9
10	4.9	1.2	4.9	1.2	5	2.1	4.8	2.0	4.8	2.4
20	4.9	2.5	4.9	2.5	4.9	2.5	4.8	2.5	4.7	2.5

[0131] Altogether, construction, purification and use of new L-AI US100 derivatives are described herein. Simple biochemical characterization showed that some mutants have optimum pH and temperature that differs from that of the wild-type L-AI US100. In agreement with these observations, experiments in milk confirmed that these L-AI US100 derivatives can be more efficient for the production of D-tagatose than the genuine enzyme. This work is at the same time a first example, and a clear demonstration that engineering of the L-AI enzyme using random or targeted mutagenesis can allow generating useful L-AI derivatives better suited for the conditions of a given industrial process and therefore very promising for industrial application.

Example 4: Bioconversion by L-arabinose isomerase in milk enriched in galactose by enzymatic hydrolysis of lactose

4.1 Materials and Methods

Strains

[0132] In this example two *E. coli* strains were used. In the strain HB101 containing the pMR4:*araUS100* plasmid, the wild-type *araA US100* gene is under the control of the constitutive *phlBA* promoter. The other strain corresponds to HB101 containing a mutated *araA* gene encoding the double mutant N175H Q268K, under the control of an IPTG-inducible promoter (*plac*) in the pUT57 plasmid.

Immobilisation of the micro-organisms and of the enzymes.

[0133] Culture of the *E. coli* strains were grown until OD at 600 nm of 3. Cells were then centrifuged and the supernatant discarded. After a drying step the pellet was weighted and suspended in MOPS buffer (pH7.5, 100 mM). Alginate was then added to the cell suspension to obtain the final concentrations of 30 g/l for cells and of 3% for alginate. Cells-containing alginate beads were then formed as droplets.

Lactose hydrolysis in milk.

[0134] The natural lactose of a milk sample was hydrolysed by the addition of Beta-galactosidase originated from *Lactobacillus bulgaricus* ATCC11842. The kinetic of D-glucose apparition was monitored during time; at 25h of incubation, 25 g/l of D-glucose was obtained. As lactose cleavage lead to equimolar amounts of glucose and galactose, we deduced from this measure that the amount of D-galactose also equals to 25 g/L

4.2. Results**Conversion of D-galactose after the enzymatic hydrolysis of lactose in milk using micro-organisms expressing the arabinose isomerase trapped in alginate beads**

[0135] Milk was enriched in D-galactose by the enzymatic hydrolysis of lactose into D-glucose and D-galactose at one to one ratio. At 25 h, a concentration of D-glucose reached 25 g/L. At that time, the alginate beads containing the strains expressing the wild-type L-AI US100 or mutated L-AI N175H Q268K were added to the treated milk. The kinetics of bioconversion of D-Galactose in D-tagatose was then followed during the incubation at three temperatures: 60, 65 and 70°C (figures 7, 8). For the wild-type enzyme, a conversion rate of 48% was obtained at the highest tested temperature after 20 h of incubation whereas as expected for the double mutated L-AI which is less thermophile than the wild-type enzyme exhibited a lower maximal conversion rate at the same time point (38%).

[0136] Altogether, these data established for the first time that cells expressing L-AI and embedded in a matrix can be used efficiently for the production of D-tagatose in milk enriched in D-galactose by an enzymatic treatment. Moreover, this type of setting could allow repeated use of the matrix. Consequently, the overall enzyme stability may become a crucial factor in this case.

SEQUENCE LISTING

[0137]

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<130> BET 07P0458

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<170> PatentIn version 3.4

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1				5					10					15		
cac	ttg	tac	gga	gaa	gaa	gcg	tta	aaa	caa	gtc	gaa	gaa	cat	tca	aga	96
His	Leu	Tyr	Gly	Glu	Glu	Ala	Leu	Lys	Gln	Val	Glu	Glu	His	Ser	Arg	
			20					25					30			
atc	atg	gtc	aat	gaa	tgg	aat	cgc	gat	tcg	gtg	ttt	ccg	ttc	cca	ttc	144
Ile	Met	Val	Asn	Glu	Trp	Asn	Arg	Asp	Ser	Val	Phe	Pro	Phe	Pro	Phe	
			35				40					45				
gtt	ttc	aaa	tca	gtc	gtg	acg	acg	cca	gag	gaa	atc	cgg	cgc	gtt	tgc	192
Val	Phe	Lys	Ser	Val	Val	Thr	Thr	Pro	Glu	Glu	Ile	Arg	Arg	Val	Cys	
						55					60					
ctt	gag	gcg	aat	gcg	agc	gaa	caa	tgc	gct	ggg	gtc	atc	act	tgg	atg	240
Leu	Glu	Ala	Asn	Ala	Ser	Glu	Gln	Cys	Ala	Gly	Val	Ile	Thr	Trp	Met	
					70					75					80	
cat	aca	ttc	tcg	cca	gcg	aaa	atg	tgg	att	ggc	ggc	ctt	ttg	gag	ttg	288
His	Thr	Phe	Ser	Pro	Ala	Lys	Met	Trp	Ile	Gly	Gly	Leu	Leu	Glu	Leu	
				85					90					95		
aga	aaa	ccg	tta	ttg	cat	ctt	cac	acc	cag	ttt	aac	cgt	gat	att	ccg	336
Arg	Lys	Pro	Leu	Leu	His	Leu	His	Thr	Gln	Phe	Asn	Arg	Asp	Ile	Pro	
			100					105					110			
tgg	gac	agc	atc	gat	atg	gac	ttt	atg	aac	tta	aac	caa	tcg	gct	cac	384
Trp	Asp	Ser	Ile	Asp	Met	Asp	Phe	Met	Asn	Leu	Asn	Gln	Ser	Ala	His	
			115			120						125				
ggt	gac	cgg	gaa	tac	gga	ttt	atc	ggc	gcg	aga	atg	ggc	gtg	gcc	cgg	432
Gly	Asp	Arg	Glu	Tyr	Gly	Phe	Ile	Gly	Ala	Arg	Met	Gly	Val	Ala	Arg	
			130			135					140					
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Lys	Val	Val	Val	Gly	His	Trp	Glu	Asp	Pro	Glu	Val	Arg	Glu	Arg	Leu	
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Ala	Lys	Trp	Met	Arg	Thr	Ala	Val	Ala	Phe	Ala	Glu	Ser	Arg	Asn	Leu	
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 Gly Asp Lys Val Gly Ala Gln Ile Gln Phe Gly Trp Ser Val Asn Gly
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 Pro Ala Gly Arg Gln Glu Gly Pro Val Arg Glu Ser Ile Arg Glu Gln
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 Ala Arg Ile Glu Leu Gly Leu Lys Ala Phe Leu Gln Asp Gly Asn Phe
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 Gly Glu Gly Asp Trp Lys Thr Ala Ala Leu Val Arg Leu Met Lys Val
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 Phe Glu Leu Gly Asn Glu Leu Ile Leu Gly Ala His Met Leu Glu Val
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 Arg Asp Ser Ala Glu Ala Trp Ile Leu Ala Gly Gly Ala His His Thr
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Lys Val Asn Glu Leu Leu Asp Glu Tyr Glu Glu Leu Tyr Asp Ile Val
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Phe Glu Leu Gly Asn Glu Leu Ile Leu Gly Ala His Met Leu Glu Val
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Cys Pro Thr Ile Ala Ala Thr Arg Pro Arg Ile Glu Val His Pro Leu
 355 360 365

Ser Ile Gly Gly Lys Glu Asp Pro Ala Arg Leu Val Phe Asp Gly Gly
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Pro Lys Leu Pro Val Ala Arg Ile Leu Trp Lys Pro Arg Pro Ser Leu
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Arg Asp Ser Ala Glu Ala Trp Ile Leu Ala Gly Gly Ala His His Thr
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Cys Phe Ser Phe Ala Val Thr Ala Glu Gln Leu Gln Asp Phe Ala Glu
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36

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Patentkrav

- 5 **1.** Anvendelse af en L-arabinoseisomerase til direkte omdannelse af D-galactose til D-tagatose i et mejeriprodukt, som indeholder D-galactose, hvor mejeriproduktet, som indeholder D-galactose, er udvalgt fra gruppen bestående af mælk, mælk tilsat med D-galactose, mælk, der udsættes for en mælkesyrefermentering, en fermenteret mælk og mælk, hvori lactosen var enzymatisk behandlet.
- 10 **2.** Anvendelse ifølge krav 1, hvor L-arabinoseisomerasen stammer fra en bakterie, som er udvalgt fra gruppen bestående af en bakterie af slægten *Thermus*, *Thermotoga*, *Geobacillus*, *Lactobacillus* og *Bacillus*, af *Alicyclobacillus acidocaldarius* og af en mælkesyrebakterie.
- 15 **3.** Anvendelse ifølge et af kravene 1 til 2, hvor L-arabinoseisomerasen er udvalgt fra gruppen bestående af AAI, AAI-K269E, BAI, BAI-E268K, L-AI US 100, L-AI US 100 omfattende en substitution Q til K ved position 268 af SEQ ID NO:2, L-AI US 100 omfattende en substitution N til H ved position 175 af SEQ ID NO:2 og L-AI US 100 omfattende en substitution N til H ved position 175 og Q til K ved position 268 af SEQ ID NO:2.
- 20 **4.** Anvendelse ifølge et af kravene 1 til 3, hvor mindst en L-arabinoseisomerase-eksprimerende mikroorganisme er tilsat eller i kontakt med mejeriproduktet, som indeholder D-galactose.
- 25 **5.** Anvendelse ifølge krav 4, hvor den L-arabinoseisomerase-eksprimerende mikroorganisme blev transformeret til at eksprimere L-arabinoseisomerasen eller naturligt eksprimerer L-arabinoseisomerasen.
- 30 **6.** Anvendelse ifølge krav 4 eller 5, hvor den L-arabinoseisomerase-eksprimerende mikroorganisme er en mælkesyrebakterie.
- 35 **7.** Fremgangsmåde til omdannelse af D-galactose, som er fremstillet ved mælkesyrefermentering af mælk, til D-tagatose, hvilken fremgangsmåde omfatter trinnene:

a) til mælken tilsættes der mindst en L-arabinoseisomerase og/eller en L-arabinoseisomerase-eksprimerende mikroorganisme, som er tilladt til fødevarerforbrug; og

5 b) der gennemføres en mælkesyrefermentering af mælk; hvorved D-galactosen, der er fremstillet i mælken ved mælkesyrefermentering, omdannes til D-tagatose.

10 **8.** Fremgangsmåde til omdannelse af D-galactose, som er fremstillet ved mælkesyrefermentering af mælk, til D-tagatose, hvilken fremgangsmåde omfatter trinnene:

a) mælken bringes i kontakt med mindst en L-arabinoseisomerase og/eller en L-arabinoseisomerase-eksprimerende mikroorganisme; og

15 b) der gennemføres en mælkesyrefermentering af mælk; hvorved D-galactosen, der er fremstillet i mælken ved mælkesyrefermentering, omdannes til D-tagatose.

20 **9.** Fremgangsmåde ifølge krav 7 eller 8, hvorved den L-arabinoseisomerase-eksprimerende mikroorganisme er en mælkesyrebakterie.

25 **10.** Fremgangsmåde ifølge et af kravene 7 til 9, hvorved L-arabinoseisomerasen udvælges fra gruppen bestående af AAAI, AAAI-K269E, BHAI, BHAI-E268K, L-AI US 100, L-AI US 100 omfattende en substitution Q til K ved position 268 af SEQ ID NO:2, L-AI US 100 omfattende en substitution N til H ved position 175 af SEQ ID NO:2 og L-AI US 100 omfattende en substitution N til H ved position 175 og Q til K ved position 268 af SEQ ID NO:2.

30 **11.** Fremgangsmåde ifølge et af kravene 7 til 10, hvorved den L-arabinoseisomerase-eksprimerende mikroorganisme blev transformeret til at eksprimere L-arabinoseisomerasen eller naturligt eksprimerer L-arabinoseisomerasen.

35 **12.** Fremgangsmåde til fremstilling af et sødet fermenteret mejeriprodukt, hvilken fremgangsmåde omfatter trinnene:

a) der tilsættes mindst en L-arabinoseisomerase og/eller en L-arabinoseisomerase-eksprimerende mikroorganisme, der er tilladt til fødevarerforbrug, til det fermenterede mejeriprodukt; eller

5 b) mejeriproduktet, som indeholder D-galactose, bringes i kontakt med mindst en L-arabinoseisomerase og/eller en L-arabinoseisomerase-eksprimerende mikroorganisme;

hvorved der opnås et sødet fermenteret mejeriprodukt.

10 **13.** Sødningsmælkeadditiv, som omfatter en L-arabinoseisomerase og/eller en L-arabinoseisomerase-eksprimerende mikroorganisme.

14. Bakterie, som egner sig til fødevarerforbrug, som eksprimerer en eksogen L-arabinoseisomerase.

15 **15.** Bakterie ifølge krav 15, som er blevet transformeret med en vektor, omfattende en nukleinsyresekvens, der koder for en L-arabinoseisomerase.

16. Bakterie ifølge krav 14 eller 15, som er en mælkesyrebakterie.

20 **17.** Bakterie ifølge et af kravene 14 til 16, hvor L-arabinoseisomerasen er udvalgt fra gruppen bestående af AAI, AAI-K269E, BAI, BAI-E268K, L-AI US 100, L-AI US 100 omfattende en substitution Q til K ved position 268 af SEQ ID NO:2, L-AI US 100 omfattende en substitution N til H ved position 175 af SEQ ID NO:2 og L-AI US 100 omfattende en substitution Q til K ved position 268 og N til H ved position 175 af SEQ ID NO:2.

25 **18.** Vektor, som er egnet til transformation af en bakterie, hvilken vektor omfatter en nukleinsyresekvens, der koder for en L-arabinoseisomerase, som er udvalgt fra L-arabinoseisomeraserne L-AI US 100 omfattende en substitution Q til K ved position 268 af SEQ ID NO:2, L-AI US 100 omfattende en substitution N til H ved position 175 af SEQ ID NO:2 og L-AI US 100 omfattende en substitution N til H ved position 175 og Q til K ved position 268 af SEQ ID NO:2.

30 **19.** Muteret L-arabinoseisomerase US100 af SEQ ID NO:2 omfattende en substitution Q til K ved position 268 af SEQ ID NO:2 og/eller en substitution

N til H ved position 175.

5 **20.** Isoleret nukleinsyre, som omfatter en sekvens, der koder for den muterede L-arabinoseisomerase US100 af SEQ ID NO:2 omfattende en substitution Q til K ved position 268 af SEQ ID NO:2 og/eller en substitution N til H ved position 175.

10

DRAWINGS

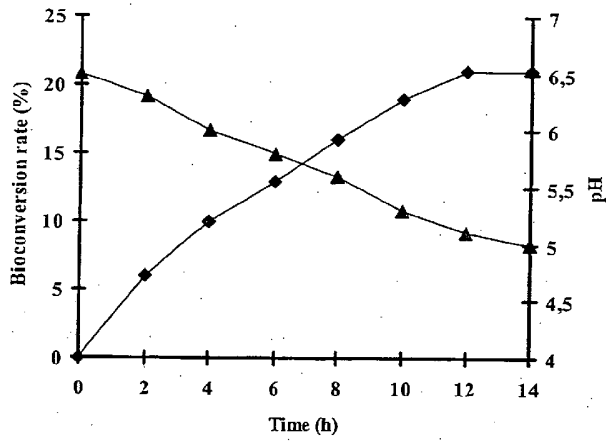


FIG.1

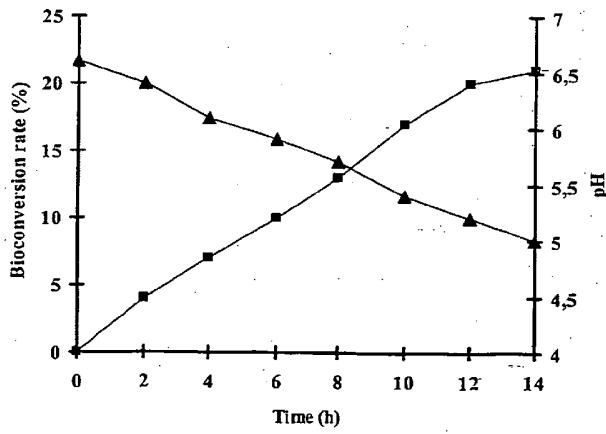


FIG.2

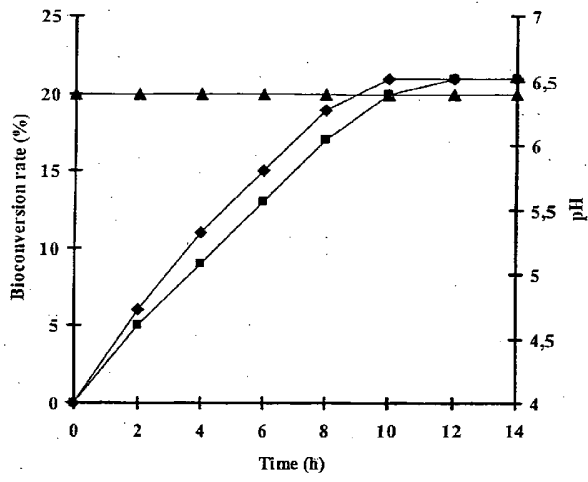


FIG.3

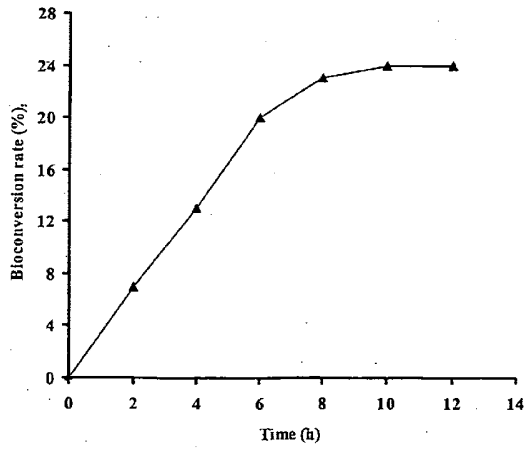


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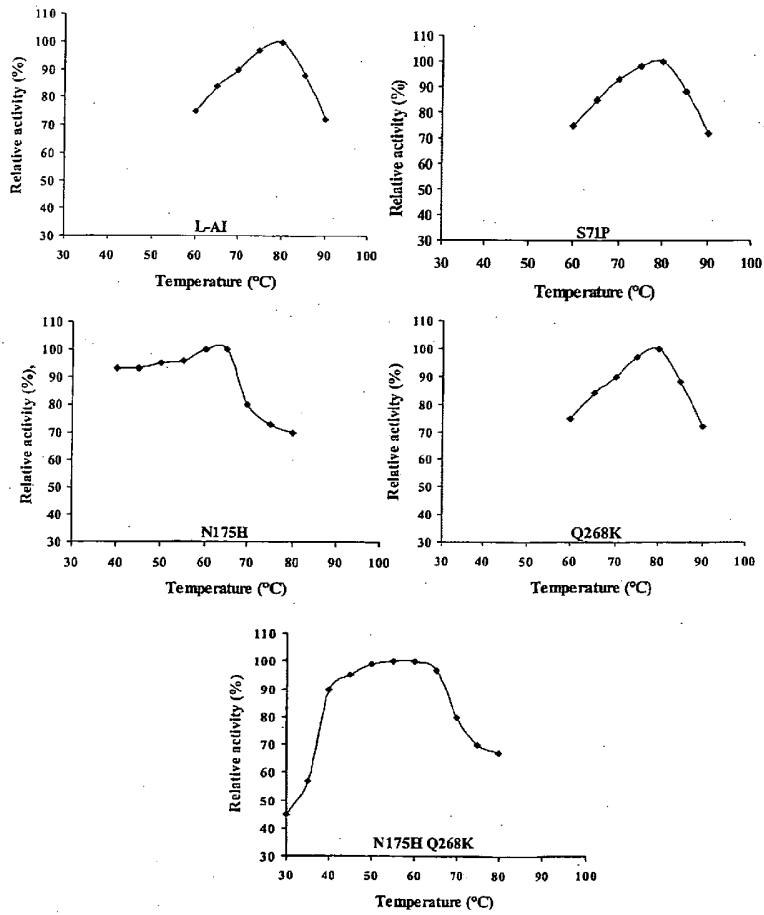


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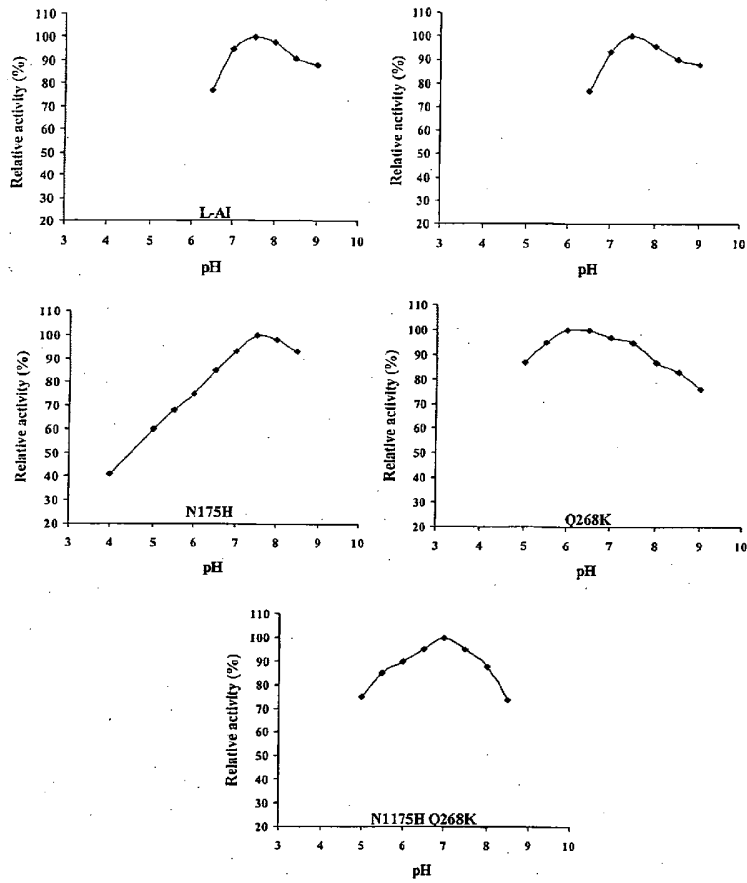


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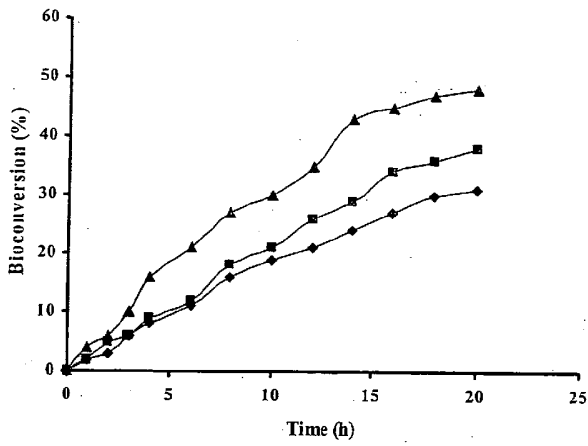


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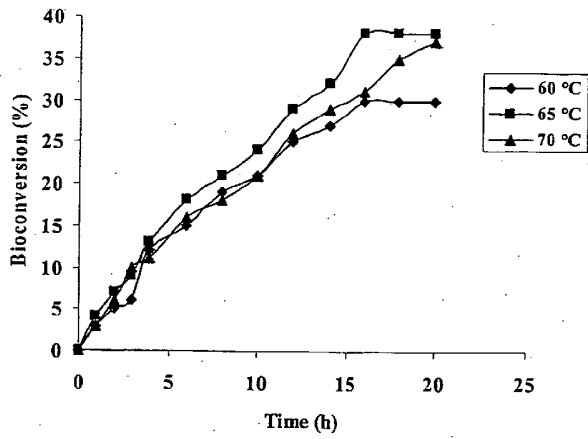


FIG.8