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(73) Patenthaver: **DSM IP Assets B.V., Het Overloon 1, 6411 TE Heerlen, Holland**

(72) Opfinder: **JONG, DE, René Marcel, P.O. Box 4, NL-6100 AA Echt, Holland**
MÜLLER, Ulrike Maria, P.O.Box 4, NL-6100 AA Echt, Holland
DEKKER, Petrus Jacobus Theodorus, P.O. Box 4, NL-6100 AA Echt, Holland

(74) Fuldmægtig i Danmark: **RWS Group, Europa House, Chiltern Park, Chiltern Hill, Chalfont St Peter, Bucks SL9 9FG, Storbritannien**

(54) Benævnelse: **FORBEDREDE ENZYMVARIANTER AF LACTASE FRA KLUYVEROMYCES LACTIS**

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DESCRIPTION

Field of the invention

[0001] The invention relates to a variant polypeptide having lactase activity. The invention also relates to a nucleic acid sequence encoding such a variant polypeptide, to a nucleic acid construct comprising said nucleic acid sequence, to a recombinant expression vector comprising said nucleic acid construct and to a recombinant host cell comprising said expression vector. Further, the invention relates to a method for producing a lactase variant via use of such a host cell. Also, the invention relates to a method of producing a lactase polypeptide variant. The invention further relates to a composition comprising a lactase variant, to use of such a lactase variant or to the use of a lactase variant-containing composition in the preparation of a dairy product and to a process for the production of a dairy product.

Background of the invention

[0002] This invention relates to lactase. Lactase or beta-galactosidase (E.C: 3.2.1.23) is an enzyme, which catalyzes the hydrolysis of lactose (a disaccharide) into its component monosaccharides glucose and galactose. Lactose is present in dairy products and more specifically in milk, skimmed milk, cream, ice cream, fermented milk products such as yogurt, many young cheeses and other dairy products. The breakdown of lactose occurs in the intestinal wall of juvenile mammals (among which are humans) by the natural presence of lactase. Only a small part of the adult population has not lost this property and can still digest lactose. The nutritional and functional problems caused by lactose in most adults are caused by a lack of lactase and are well known and described. Members of such populations cannot hydrolyze lactose, which in such cases passes into the large intestine where it results in dehydration, poor calcium absorption, flatulence, belching and cramps, and, in severe cases, even watery explosive diarrhea.

[0003] An important industrial application of lactase is in the production of lactose-hydrolyzed milk products for lactose intolerant individuals. Such hydrolyzed milk products include pasteurized milk, UHT-milk and milk reconstituted from all or part of its original constituents with or without intermediate processing steps such as protein hydrolysis. Treatment with lactase may be done prior to or after the heat-treatment of the milk. The lactase treatment may be done by adding the enzyme to the milk or to one of its lactose-containing constituents.

[0004] The solubility properties of lactose are such that it may lead to its crystallization when present at high concentration, leading to a sandy or gritty texture in dairy products such as condensed milk, evaporated milk, dry milk, frozen milk, ice cream, and in confectionary products with a high content of milk. Full or partial hydrolysis of lactose by lactase eliminates this problem, providing products with a homogeneous texture and as a result a higher

consumer acceptance.

[0005] Another industrial application of lactase is to increase sweet taste in lactose containing products like milk or yoghurt. The hydrolysis of lactose in such products results in increased sweet taste as a result of the production of glucose, while the caloric value of the product does not increase. Conversely, the use of lactase may also decrease sugar addition in sweetened dairy products, without compromising the sweet taste.

[0006] Another industrial application of lactase is the hydrolysis of lactose products containing dairy components such as bread. Lactose is added in such products to enhance flavour, retain moisture, provide browning and improve toasting properties. Hydrolyzed lactose syrups are promising in terms of e.g. enhancing crust-colour development, improving flavour and aroma, modifying texture, extending shelf life and strengthening loaf structure.

[0007] Lactose hydrolysis by lactase in fermented milk products such as yoghurt will increase sweet taste. Also, when the lactase is added prior to the beginning of the fermentative process, it may increase the rate of acid development and thus reduce processing times. The lactase treatment of milk or milk-derived products such as whey makes such products suitable for application in animal feed and pet food for lactose intolerant animals such as cats. The lactose hydrolysis allows the manufacture of higher concentrated whey and at the same time prevents gut problems, similar to those described earlier for lactose-deficient patients. Lactose hydrolyzed whey is concentrated to produce a syrup containing 70-75% solids and is used as a food ingredient in ice cream, bakery and confectionary products.

[0008] Lactases have been described and isolated from a large variety or organisms, including micro-organisms. Lactase is often an intracellular component of micro-organisms like *Kluyveromyces* and *Bacillus*. *Kluyveromyces* and especially *K. fragilis* and *K.lactis,* and other yeasts such as those of the genera *Candida, Torula* and *Torulopsis* are a common source of yeast lactases, whereas *B. coagulans, B. circulans* or lactic acid bacteria are well known sources of bacterial lactases. Several commercial lactase preparations, derived from these organisms are available such as Maxilact® (from *K. lactis,* produced by DSM, Delft, the Netherlands). These lactases are so called neutral lactases since they have a pH optimum between pH = 6 and pH = 8.

[0009] Although yeast neutral lactases are often used in industry to produce lactose-free or lactose-reduced dairy products, the cost-in-use for the enzyme treatment is often high. Main reasons for the relative high cost-in-use of the enzyme are:

- In order to maintain hygienic conditions in the production plant the incubation is performed at low temperature. At this temperature the industrially used lactases are not very active and should be added at relative high dosage.
- The currently used lactases are inhibited by its products, especially galactose, at later stages of the incubation with lactase. When products with a low residual lactose concentration are required, extra enzyme has to be added to compensate for reduction

in activity due to galactose accumulation.
- The currently used lactase has a relative low specific activity in milk which requires the use of a high enzyme dosage in application.

Consequently, enzyme dosage and costs for producing lactose-reduced and lactose-free products are relative high.
It is evident that there is a need for one or multiple lactase variant(s) capable of overcoming at least one of the above-mentioned disadvantages.

[0010] Database Uniprot [Online] 11 July 2012, retrieved from EBI accession no. UNIPROT:I2JQJ6, describes a predicted beta-galactosidase of 495 amino acids.

[0011] R. Mahoney et al (Thermostability of yeast lactase (Kluyveromyces marxianus) in milk, Journal of Dairy Research, 1988, pages 423-433) describe the thermal stability of yeast lactase in milk and various synthetic media.

[0012] S. Bansal et al (Production of beta-galactosidase by Kluyveromyces marxianus MTCC 1388 using whey and effect of four different methods of enzyme extraction on beta-galactosidase activity, Indian Journal of Microbiology, 2008, pages 337-341) evaluate different methods for beta-galactosidase extraction from Kluyveromyces marxianus.

[0013] N. Lertwattanasakul et al (Utilization capability of sucrose, raffinose and inulin and its less-sensitiveness to glucose repression in thermotolerant yeast Kluyveromyces marxianus DMKU 3-1042, AMB Express, 2011, pages 1:20) describe the K. marxianus fermentation capabilities of its substrates, sucrose, raffinose and inulin, in the presence and absence of glucose at different temperatures.

[0014] N. Rodrussamee et al (Growth and ethanol fermentation ability on hexose and pentose sugars and glucose effect under various conditions in thermotolerant yeast Kluyveromyces marxianus, Applied Microbiology and Biotechnology, 2011, pages 1573-1586) describe the potential of K. marxianus for utilization of and ethanol production from sugars present in hemicellulose hydrolysate under different conditions.

Summary of the invention

[0015] The invention describes a variant polypeptide having lactase activity, i.e. a lactase variant. A lactase variant may have one or more improved properties in comparison with a reference polypeptide, the reference polypeptide having lactase activity. A reference polypeptide may be a wild-type lactase, for example the lactase from *K. lactis*. Variant polypeptides may be referred to as "lactase variant", an "improved lactase" and the like. Variants of *Kluyveromyces* neutral lactase were generated that have properties that lead to a reduction of the cost-in-use of such lactases in the production of lactose-reduced or lactose-free dairy products. A lactase variant with an improved property relevant for dairy production

may demonstrate:

- a higher specific activity on ONPG;
- a higher specific activity on lactose;
- a higher activity on lactose in milk at low temperature;
- a reduction in galactose inhibition; and/or
- a higher GOS production in milk.

[0016] Each of these improvements may be determined as compared with a reference polypeptide. Moreover, a variant polypeptide may have at least 2 or 3 or 4 improved properties in comparison with a reference polypeptide. Table 1 provides examples of combinations of improved properties.

[0017] According to the invention, there is thus provided a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution of an amino acid residue corresponding to any of amino acids

[0018] 233, 257, 258, 263, 274, 284, 297, 415, 440, 483, 619, 621, 622, 633, 862 or 1004 said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having lactase activity and wherein said variant has at least 80% sequence identity with SEQ ID NO:2.

[0019] The invention also provides:

- a nucleic acid sequence encoding a variant of the invention;
- a nucleic acid construct comprising such a nucleic acid sequence operably linked to one or more control sequences capable of directing the expression of a lactase in a suitable expression host;
- a recombinant expression vector comprising such a nucleic acid construct; and
- a recombinant host cell comprising such an expression vector.

[0020] The invention also relates to a method for producing a lactase comprising cultivating the host cell of the invention under conditions conducive to production of the lactase and recovering the lactase.

[0021] Also, the invention relates to a method of producing a lactase polypeptide variant, which method comprises:

1. a) selecting a polypeptide having lactase activity;
2. b) substituting at least one amino acid residue corresponding to any of amino acids

[0022] 233, 257, 258, 263, 274, 284, 297, 415, 440, 483, 619, 621, 622, 633, 862 or 1004 said positions being defined with reference to SEQ ID NO: 2;

c) optionally substituting one or more further amino acids as defined in b);

d) preparing the variant resulting from steps a)-c);

e) determining a property of the variant; and

f) selecting a variant having an altered property in comparison to the lactase comprising the sequence set out in SEQ ID NO: 2 and selecting a variant having at least 80% sequence identity with SEQ ID NO:2, thereby to produce a lactase polypeptide variant.

[0023] Further the invention relates to:

- a composition comprising the variant of the invention or obtainable by a method of the invention;
- use of a variant lactase according to the invention or of a composition of the invention in the preparation of a dairy product; and
- a process for the production of a dairy product, which method comprises comprising adding an effective amount of a variant lactase according to the invention or of a composition of the invention to milk and carrying out appropriate further dairy product manufacturing steps.

Brief description of the sequence listing

[0024] SEQ ID NO 1 sets out the nucleic acid sequence of the wild type lactase gene sequence from *K. lactis*

[0025] SEQ ID NO: 2 sets out the amino acid sequence of the lactase sequence from *K. lactis.*

Detailed description of the invention

[0026] Throughout the present specification and the accompanying claims, the words "comprise", "include" and "having" and variations such as "comprises", "comprising", "includes" and "including" are to be interpreted inclusively. That is, these words are intended to convey the possible inclusion of other elements or integers not specifically recited, where the context allows.

[0027] The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to one or at least one) of the grammatical object of the article. By way of example, "an element" may mean one element or more than one element.

[0028] Herein, "lactase" or beta-galactosidase (E.C. 3.2.1.23) is an enzyme, which catalyzes the hydrolysis of lactose (a disaccharide) into its component monosaccharides glucose and galactose. Galacto-oligosaccharides (GOS) may be formed during this reaction due to the transferase activity of the lactase enzyme.

[0029] Lactase is found in the intestine of young mammals, in plants, fungi, yeasts and bacteria.

[0030] The lactase may be a neutral or an acidic lactase. Preferably, the variant polypeptide has neutral lactase activity, i.e. it has its pH optimum between pH=6 and pH=8.

[0031] The lactase may be an intracellular or an extracellular produced lactase. Preferably, the lactase is intracellular produced lactase.

[0032] A gene or cDNA coding for lactase, for example a variant of the invention, may be cloned and over-expressed in a host organism. Well known host organisms that may be used for lactase over-expression include *Aspergillus, Kluyveromyces, Trichoderma, Escherichia coli, Pichia, Saccharomyces, Yarrowia, Neurospora, Lactococcus* or *Bacillus.*

[0033] Herein, positions which may be substituted to achieve a variant of the invention are defined with reference to SEQ ID NO: 2 which is the *K. lactis* lactase.

[0034] The invention concerns a variant polypeptide having lactase activity as compared with a reference polypeptide having lactase activity. The reference polypeptide may typically be a wild-type polypeptide having lactase activity, such as the lactase of SEQ ID NO: 2 or a related sequence. The reference polypeptide may also be referred to as a parent polypeptide or comparison polypeptide.

[0035] More concretely, the invention relates to a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution of an amino acid residue corresponding to any of amino acids

[0036] 233, 257, 258, 263, 274, 284, 297, 415, 440, 483, 619, 621, 622, 633, 862 or 1004 said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having lactase activity and wherein said variant has at least 80% sequence identity with SEQ ID NO:2.

[0037] A wild type reference polypeptide may be obtained from any suitable organism.

Typically, a wild type reference polypeptide may be obtained from a microorganism, preferably one in which lactase is produced naturally.

[0038] Such microorganism includes yeast such as *Kluyveromyces.* A reference polypeptide may be a *K. lactis* wild type sequence.

[0039] Preferably, the reference polypeptide is the lactase set out in SEQ ID NO: 2.

[0040] A variant polypeptide as described herein is typically a non-naturally occurring polypeptide.

[0041] According to the invention, there is thus provided a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution of an amino acid residue corresponding to any of amino acids

[0042] 233, 257, 258, 263, 274, 284, 297, 415, 440, 483, 619, 621, 622, 633, 862 or 1004 said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2.

[0043] A variant polypeptide will have at least one improved property as compared to a reference polypeptide, in particular with respect to a property relevant to the use of the variant polypeptide in a process for preparing a dairy product.

[0044] In particular, the improved property may relate to activity or specific activity or to a reduction in galactose inhibition or to a higher GOS production in milk.

[0045] Table 1 sets out positions that influence specific properties of the variant lactases of the inventions.

Table 1: Preferred substitutions defined in relation to SEQ ID NO:2. Different properties like specific activity on ONPG or lactose as substrate, activity in milk at low temperature, reduction of inhibition of the lactase activity by galactose, and a higher galacto-oligosaccharide production in milk, is indicated

	preferred variant	most preferred	spec. act. ONPG	spec. act. Lactose	act. Milk	reduction gal inh	high GOS prod
T633	all AA	G	x		x		

Y440	all AA	F		x	x		
A483	all AA	S	x	x			
A1004	all AA	P			x		
A258	all AA	T			x		
D233	all AA	V			x		
N263	all AA	S			x		
K274	all AA	E			x		
N284	all AA	S			x		
D257	all AA	G			x		
E297	all AA	G			x		
L862	all AA	V			x		
V619	all AA	I	x		x	x	x
T415	all AA	C, A	x	x			
M622	all AA	L	x			x	x
I621	all AA	V	x			x	

[0046] A variant polypeptide of the invention may demonstrate higher specific activity on ONPG.

[0047] The invention thus provides a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution of an amino acid residue corresponding to any of amino acids

[0048] 233, 257, 258, 263, 274, 284, 297, 415, 440, 483, 619, 621, 622, 633, 862 or 1004
said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has higher specific activity on ONPG as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2.

[0049] Preferably, the invention provides a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution of an amino acid residue corresponding to any of amino acids

[0050] 415, 483, 619, 621, 622 or 633
said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has higher specific activity on ONPG as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2. Preferred is at least one substitution of an amino acid residue corresponding to any of amino acids 415 and/or 619 said position being defined with reference to SEQ ID NO: 2.

[0051] More preferably, the invention provides a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution selected

from

[0052] T415C, T415A, A483S, V619I, I621V, M622L or T633G,
said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has higher specific activity on ONPG as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2. Preferred is substitution T415C and/or V619I said position being defined with reference to SEQ ID NO: 2.

[0053] Another variant polypeptide of the invention may demonstrate higher specific activity on lactose. Since lactose is the natural substrate in dairy products of lactase, a higher specific activity of the variant polypeptide can lead to a reduction of the required dosage of the enzyme and therefore may lead to a lower cost of the treatment. By reducing the enzyme dosage in the application also the amount of side activities added is reduced, and therefore a higher quality of the final dairy product is to be expected.

[0054] The invention thus provides a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution of an amino acid residue corresponding to any of amino acids

[0055] 233, 257, 258, 263, 274, 284, 297, 415, 440, 483, 619, 621, 622, 633, 862 or 1004
said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has higher specific activity on lactose as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2.

[0056] Preferably, the invention provides a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution of an amino acid residue corresponding to any of amino acids

[0057] 415, 440 or 483
said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has higher specific activity on lactose as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2. Preferred is at least one substitution of an amino acid residue corresponding to any of amino acids 415 and/or 483 (this preference is based on analysis of lactase variants comprising a combination of substitutions) said position being defined with reference to SEQ ID NO: 2.

[0058] More preferably, the invention provides a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution selected

from

[0059] T415C, T415A, Y440F or A483S,
said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has higher specific activity on lactose as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2. Preferred is substitution T415A, T415C and/or A483S (this preference is based on analysis of lactase variants comprising a combination of substitutions) said position being defined with reference to SEQ ID NO: 2.

[0060] Even more preferably, the invention provides a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least two, three, four or five substitutions of an amino acid residue corresponding to any of amino acids

[0061] 233, 257, 258, 263, 274, 284, 297, 415, 440, 483, 619, 621, 622, 633, 862 or 1004
said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has higher specific activity on lactose as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2.

[0062] Examples of such mutants are mutants 16, 17, 18, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35 or 36 as described in Table 5.

[0063] Yet another variant polypeptide of the invention may demonstrate higher activity on lactose in milk, preferably at low temperatures (preferably said low temperatures are in the range of 4-12 °C). Since often lactases are used in milk at a low temperature, an increased activity of the variant polypeptide in this specific application may lead to the reduction of the enzyme dosage, and hence reduce the costs. Additionally, a higher activity of the variant polypeptide may lead to a reduction in the processing time of milk and therefore reduce the risk for possible microbial spoilage.

[0064] The invention thus provides a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution of an amino acid residue corresponding to any of amino acids

[0065] 233, 257, 258, 263, 274, 284, 297, 415, 440, 483, 619, 621, 622, 633, 862 or 1004
said positions being defined with reference to SEQ ID NO: 2 and wherein the variant demonstrates increased activity on lactose in milk at a low temperature as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2.

[0066] Preferably, the invention provides a variant polypeptide having lactase activity, wherein

the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution of an amino acid residue corresponding to any of amino acids

[0067] 233, 257, 258,263, 274, 284, 297, 440, 619, 633, 862 or 1004
said positions being defined with reference to SEQ ID NO: 2 and wherein the variant demonstrates increased activity on lactose in milk at a low temperature as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2. Preferred is a substitution of (at least) an amino acid residue corresponding to amino acid 440 said position being defined with reference to SEQ ID NO: 2 (this preference is based on analysis of lactase variants comprising a combination of substitutions). A preferred combination of substitutions is a substitution at position 440 and 619.

[0068] More preferably, the invention provides a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution selected from

[0069] D233V, D257G, A258T, N263S, K274E, N284S, E297G, Y440F, V619I, T633G, L862V or A1004P
said positions being defined with reference to SEQ ID NO: 2 and wherein the variant demonstrates increased activity on lactose in milk at a low temperature as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2. Preferred is a substitution of (at least) Y440F said position being defined with reference to SEQ ID NO: 2 (this preference is based on analysis of lactase variants comprising a combination of substitutions). A preferred combination of substitutions is Y440F+V619I.

[0070] Although, the presence of at least one substitution of an amino acid residue corresponding to any of amino acids

[0071] 233, 257, 258, 263, 274, 284, 297, 440, 619, 633, 862 or 1004
said positions being defined with reference to SEQ ID NO: 2
is sufficient to obtain a variant polypeptide having lactase activity and further showing increased activity on lactose in milk at a low temperature, it is herein shown that also double or triple mutated polypeptide variants demonstrate increased activity on lactose in milk at a low temperature.

[0072] As a result the invention also provides a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, which comprises an amino acid sequence which, when aligned with the sequence set out in SEQ ID NO:2, comprises at least two substitutions selected from 263, 274 or 284 (more preferably N263S, K274E or N284S) said

positions being defined with reference to SEQ ID NO: 2 and wherein the variant demonstrates increased activity on lactose in milk at a low temperature as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2.

[0073] The invention further provides a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, which comprises an amino acid sequence which, when aligned with the sequence set out in SEQ ID NO:2, comprises substitutions at positions 263, 274 and 284 (preferably said substitutions are N263S, K274E and N284S)
said positions being defined with reference to SEQ ID NO: 2 and wherein the variant demonstrates increased activity on lactose in milk at a low temperature as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2.

[0074] The invention further provides a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, which comprises an amino acid sequence which, when aligned with the sequence set out in SEQ ID NO:2, comprises comprises an amino acid sequence which, when aligned with the sequence set out in SEQ ID NO:2, comprises substitutions at positions 257 and 297 (preferably said substitutions are D257G and E297G),
said positions being defined with reference to SEQ ID NO: 2 and wherein the variant demonstrates increased activity on lactose in milk at a low temperature as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2.

[0075] The invention also provides a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least three, four of five substitutions of an amino acid residue corresponding to any of amino acids

[0076] 233, 257, 258, 263, 274, 284, 297, 415, 440, 483, 619, 621, 622, 633, 862 or 1004
said positions being defined with reference to SEQ ID NO: 2 and wherein the variant demonstrates increased activity on lactose in milk at a low temperature as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2.

[0077] Examples of such mutants are mutants 15, 17, 18, 19, 20, 21 or 22 as described in Table 5.

[0078] Yet a further variant polypeptide of the invention may demonstrate a reduction in galactose inhibition. Galactose inhibition leads to slow hydrolysis of lactose at later time points, when the lactose concentration is low, and the galactose concentration is high. It would thus be desirable to have a lactase enzyme which has reduced galactose inhibition, especially when

one wishes to produce a dairy product where the lactose concentration is lower than 0.5 g/L.

[0079] The invention thus provides a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution of an amino acid residue corresponding to any of amino acids

[0080] 233, 257, 258, 263, 274, 284, 297, 415, 440, 483, 619, 621, 622, 633, 862 or 1004
said positions being defined with reference to SEQ ID NO: 2 and wherein the variant demonstrates decreased galactose inhibition as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2.

[0081] Preferably, the invention provides a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution of an amino acid residue corresponding to any of amino acids
619, 621 or 622
said positions being defined with reference to SEQ ID NO: 2 and wherein the variant demonstrates decreased galactose inhibition as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2.

[0082] More preferably, the invention provides a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution selected from
V619I, I621V or M622L
said positions being defined with reference to SEQ ID NO: 2 and wherein the variant demonstrates decreased galactose inhibition as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2.

[0083] Yet a further variant polypeptide of the invention may demonstrate increased GOS production in milk. GOS (galacto-oligosaccharides) are prebiotics which are defined as non-digestible food ingredients that beneficially affect the host by stimulating the growth and/or activity of beneficial bacteria in the colon. Not all lactases are equally well suited for preparing GOS. It would be desired to have another lactase enzyme which is capable of accumulation of GOS at the low lactose concentration that is present in milk (< 50 g/L).

[0084] The invention thus provides a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution of an amino acid residue corresponding to any of amino acids

[0085] 233, 257, 258, 263, 274, 284, 297, 415, 440, 483, 619, 621, 622, 633, 862 or 1004
said positions being defined with reference to SEQ ID NO: 2 and wherein the variant demonstrates increased GOS production in milk as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2.

[0086] Preferably, the invention provides a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution of an amino acid residue corresponding to any of amino acids
619 or 622
said positions being defined with reference to SEQ ID NO: 2 and wherein the variant demonstrates increased GOS production in milk as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2.

[0087] More preferably, the invention provides a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution selected from
V619I or M622L
said positions being defined with reference to SEQ ID NO: 2 and wherein the variant demonstrates increased GOS production in milk as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2.

[0088] A variant lactase of the invention may also comprise additional modifications in comparison to the parent at positions other than those specified above, for example, one or more additional substitutions, additions or deletions. A variant of the invention may comprise a combination of different types of modification of this sort. A variant may comprise one, two, three, four, at least 5, at least 10, at least 15, at least 20, at least 25, at least 30 or more such modifications (which may all be of the same type or may be different types of modification). Typically, the additional modifications may be substitutions. The invention thus also provides a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution of an amino acid residue corresponding to any of amino acids

[0089] 233, 257, 258, 263, 274, 284, 297, 415, 440, 483, 619, 621, 622, 633, 862 or 1004
said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having lactase activity and wherein said variant polypeptide comprises additional substitutions other than those defined and wherein said variant has at least 80% sequence identity with SEQ ID NO:2.

[0090] A variant according to the invention (for example a variant having one or more substitution as set out in Table 1 or Table 2) has at least 80% homology/identity with SEQ ID NO: 2, for example at least about 85% homology with the parent polypeptide, such as least about 90% homology with the parent polypeptide, at least 95% homology with the parent polypeptide, at least about 98% homology with the parent polypeptide or at least about 99% homology with the parent polypeptide. Such a variant will typically have one or more substitution or sets of substitutions as set out in Table 1 or Table 2.

[0091] A variant of the invention will retain lactase activity. That is to say, a variant of the invention will be capable of converting lactose to glucose and galactose or a variant of the invention will be capable of converting lactose to glucose and galactose and capable of forming GOS. A variant of the invention is one which is capable of performing an enzymatic conversion of lactose and which may be used in the preparation of a dairy product, such as a milk or yoghurt.

[0092] A variant of the invention will exhibit improved properties in comparison with the reference lactase polypeptide from which it is derived. Such an improved property will typically be one which is relevant if the variant were to be used as set out below, for example in a method for preparing a dairy product.

[0093] A polypeptide variant which exhibits a property which is improved in relation to the reference lactase is one which demonstrates a measurable reduction or increase in the relevant property, typically such that the variant is more suited to use as set out below, for example in a method for the production of a dairy product.

[0094] The property may thus be decreased by at least 10%, at least 20%, at least 30%, at least 40% at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99%. Alternatively, the property may be increased by at least 10%, at least 25%, at least 50%, at least 100%, at least, 200%, at least 500% or at least 1000%. The percentage decrease or increase in this context represents the percentage decrease or increase in comparison to the reference lactase polypeptide. It is well known to the skilled person how such percentage changes may be measured - it is a comparison of the activity of the reference lactase and the variant lactase.

[0095] The variants described herein are collectively comprised in the terms "a polypeptide according to the invention" or "a variant according to the invention".

[0096] The terms "peptide" and "oligopeptide" are considered synonymous (as is commonly recognized) and each term can be used interchangeably as the context requires to indicate a chain of at least two amino acids coupled by peptidyl linkages. The word "polypeptide" is used herein for chains containing more than about seven amino acid residues. All oligopeptide and polypeptide formulas or sequences herein are written from left to right and in the direction from amino terminus to carboxy terminus. The one-letter code of amino acids used herein is commonly known in the art and can be found in Sambrook, et al. (Molecular Cloning: A

Laboratory Manual, 2nd, ed. Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

[0097] A polypeptide of the invention may be in isolated form, such as substantially isolated form. By "isolated" polypeptide or protein is intended a polypeptide or protein removed from its native environment. For example, recombinantly produced polypeptides and proteins expressed in host cells are considered isolated for the purpose of the invention as are recombinant polypeptides which have been substantially purified by any suitable technique. A polypeptide variant according to the invention can be recovered and purified from recombinant cell cultures by methods known in the art.

[0098] Polypeptides of the present invention include products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, fungal, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

[0099] The invention also describes biologically active fragments of the polypeptide variants according to the invention. Such fragments are considered to be encompassed within the term "a variant of the invention".

[0100] Biologically active fragments of a polypeptide variant of the invention include polypeptides comprising amino acid sequences sufficiently identical to or derived from the amino acid sequence of a variant protein of the invention which include fewer amino acids than the full length protein but which exhibit at least one biological activity of the corresponding full-length protein. Typically, biologically active fragments comprise a domain or motif with at least one activity of a variant protein of the inventionMoreover, other biologically active portions, in which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one or more of the biological activities of the native form of a polypeptide of the invention.

[0101] A protein fragment will comprise one or more of the substitutions defined herein.

[0102] The present invention also provides a nucleic acid sequence encoding the variant polypeptides of the invention. The invention thus also provides a nucleic acid sequence encoding a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution of an amino acid residue corresponding to any of amino acids

[0103] 233, 257, 258, 263, 274, 284, 297, 415, 440, 483, 619, 621, 622, 633, 862 or 1004 said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one

or more altered properties as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2.

[0104] As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules which include an open reading frame encoding a variant as described herein. A gene may include coding sequences, non-coding sequences, introns and regulatory sequences. That is to say, a "gene", as used herein, may refer to an isolated nucleic acid molecule as defined herein. Accordingly, the term "gene", in the context of the present application, does not refer only to naturally-occurring sequences.

[0105] A nucleic acid molecule of the present invention can be generated using standard molecular biology techniques well known to those skilled in the art taken in combination with the sequence information provided herein.

[0106] For example, using standard synthetic techniques, the required nucleic acid molecule may be synthesized *de novo*. Such a synthetic process will typically be an automated process.

[0107] Alternatively, a nucleic acid molecule of the invention may be generated by use of site-directed mutagenesis of an existing nucleic acid molecule, for example a wild-type nucleic acid molecule. Site-directed mutagenesis may be carried out using a number of techniques well known to those skilled in the art.

[0108] An "isolated polynucleotide" or "isolated nucleic acid" is a DNA or RNA that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. Thus an isolated nucleic acid may include some or all of the 5' non-coding (e.g., promotor) sequences that are immediately contiguous to the coding sequence. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA that is part of a hybrid gene encoding an additional polypeptide that is substantially free of cellular material, viral material, or culture medium (when produced by recombinant DNA techniques), or chemical precursors or other chemicals (when chemically synthesized). Moreover, an "isolated nucleic acid fragment" is a nucleic acid fragment that is not naturally occurring as a fragment and would not be found in the natural state.

[0109] As used herein, the terms "polynucleotide" or "nucleic acid molecule" are intended to include DNA molecules (e.g., cDNA or genomic DNA) and RNA molecules (e.g., mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA. The nucleic acid may be synthesized using oligonucleotide analogs or derivatives (e.g., inosine or

phosphorothioate nucleotides). Such oligonucleotides can be used, for example, to prepare nucleic acids that have altered base-pairing abilities or increased resistance to nucleases.

[0110] The terms "homology" or "percent identity" are used interchangeably herein. For the purpose of this invention, it is defined here that in order to determine the percent identity of two amino acid sequences or two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid for optimal alignment with a second amino or nucleic acid sequence). The amino acid or nucleotide residues at corresponding amino acid or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid or nucleotide residue as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = number of identical positions/total number of positions (i.e. overlapping positions) x 100). Preferably, the two sequences are the same length.

[0111] A sequence comparison may be carried out over the entire lengths of the two sequences being compared or over fragment of the two sequences. Typically, the comparison will be carried out over the full length of the two sequences being compared. However, sequence identity may be carried out over a region of, for example, twenty, fifty, one hundred or more contiguous amino acid residues.

[0112] The skilled person will be aware of the fact that several different computer programs are available to determine the homology between two sequences. For instance, a comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid or nucleic acid sequences is determined using the Needleman and Wunsch (J. Mol. Biol. (48): 444-453 (1970)) algorithm which has been incorporated into the GAP program in the Accelrys GCG software package (available at http://www.accelrys.com/products/gcg/), using either a Blosum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. The skilled person will appreciate that all these different parameters will yield slightly different results but that the overall percentage identity of two sequences is not significantly altered when using different algorithms.

[0113] The protein sequences or nucleic acid sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the BLASTN and BLASTP programs (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403-10. BLAST protein searches can be performed with the BLASTP program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25(17): 3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective

programs (e.g., BLASTP and BLASTN) can be used. See the homepage of the National Center for Biotechnology Information at http://www.ncbi.nlm.nih.gov/.

[0114] The invention further provides a nucleic acid construct comprising a nucleic acid sequence encoding a variant polypeptide having lactase activity, wherein the variant has an amino acid sequence which, when aligned with the lactase comprising the sequence set out in SEQ ID NO: 2, comprises at least one substitution of an amino acid residue corresponding to any of amino acids

[0115] 233, 257, 258, 263, 274, 284, 297, 415, 440, 483, 619, 621, 622, 633, 862 or 1004 said positions being defined with reference to SEQ ID NO: 2 and wherein the variant has one or more altered properties as compared with a reference polypeptide having lactase activity (such as the polypeptide of SEQ ID NO: 2) and wherein said variant has at least 80% sequence identity with SEQ ID NO:2, wherein said nucleic acid sequence is operably linked to one or more control sequences capable of directing the expression of a lactase in a suitable expression host cell.

[0116] Another aspect of the invention pertains to vectors, preferably expression vectors, containing a nucleic acid encoding a variant lactase polypeptide of the invention.

[0117] As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. The terms "plasmid" and "vector" can be used interchangeably herein as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

[0118] The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vector includes one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operatively linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (e.g., in an in

vitro transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (e.g., polyadenylation signal). Such regulatory sequences are described, for example, in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cells and those which direct expression of the nucleotide sequence only in a certain host cell (e.g. tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, encoded by nucleic acids as described herein (e.g. a lactase variant of SEQ ID NO: 2, for example a functional equivalent or fragment, or a fusion protein comprising one or more of such variants).

[0119] The recombinant expression vectors of the invention can be designed for expression of variant proteins of the invention in prokaryotic or eukaryotic cells.

[0120] Expression vectors useful in the present invention include chromosomal-, episomal- and virus-derived vectors e.g., vectors derived from bacterial plasmids, bacteriophage, yeast episome, yeast chromosomal elements, viruses such as baculoviruses, papova viruses, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids.

[0121] The DNA insert should be operatively linked to an appropriate promoter. Promoters that are capable of directing a high expression level of lactase in filamentous fungi are preferred. Such promoters are known in the art. The expression constructs may contain sites for transcription initiation, termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG at the beginning and a termination codon appropriately positioned at the end of the polypeptide to be translated.

[0122] Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell.

[0123] Vectors preferred for use in bacteria are for example disclosed in WO-A1-2004/074468. Other suitable vectors will be readily apparent to the skilled artisan.

[0124] Known bacterial promotors suitable for use in the present invention include the promoters disclosed in WO-A1-2004/074468.

[0125] A variant of the invention may be expressed in form such that it may include additional

heterologous functional regions, for example secretion signals. A variant of the invention may also comprise, for example, a region of additional amino acids, particularly charged amino acids, added to the N-terminus of the polypeptide for instance to improve stability and persistence in the host cell, during purification or during subsequent handling and storage. Also, peptide moieties may be added to a variant of the invention to facilitate purification, for example by the addition of histidine residues or a T7 tag.

[0126] The variants of the invention, such as proteins of the present invention or functional equivalents thereof, e.g., biologically active portions and fragments thereof, can be operatively linked to a non-variant polypeptide (e.g., heterologous amino acid sequences) to form fusion proteins. A "non-variant polypeptide" in this context refers to a polypeptide having an amino acid sequence corresponding to a protein which is not substantially homologous to a variant lactase of the invention.

[0127] Within a fusion protein, the variant of the invention can correspond to a full length sequence or a biologically active fragment of a polypeptide of the invention. A fusion protein may comprise at least two biologically active portions. Within the fusion protein, the term "operatively linked" is intended to indicate that the variant polypeptide and the non-variant polypeptide are fused in-frame to each other. The non-variant polypeptide can be fused to the N-terminus or C-terminus of the variant polypeptide.

[0128] Expression and secretion of a variant lactase may be enhanced by expressing the variant in the form of a fusion protein. In this context, a nucleic acid sequence may encode for a fusion protein comprising lactase. More specifically, the fusion partner may be glucoamylase or a fragment thereof. The lactase, or a fusion protein thereof, may be secreted over the host cell membrane.

[0129] A signal sequence can be used to facilitate secretion and isolation of a variant of the invention. A fusion protein may be produced by standard recombinant DNA techniques.

[0130] The skilled person will recognise that changes can be introduced by mutation into the nucleotide sequences according to the invention thereby leading to changes in the amino acid sequence of the resulting protein without substantially altering the function of such a protein.

[0131] Accordingly, a lactase variant of the invention is preferably a protein which comprises an amino acid sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous to SEQ ID NO: 2, and typically also retains at least one functional activity of the reference polypeptide. Variants of the invention can also be identified e.g. by screening combinatorial libraries of mutants, e.g. truncation mutants, of the protein of the invention for lactase activity. A variegated library of variants can be generated by combinatorial mutagenesis at the nucleic acid level. A variegated library of variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential protein sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g. for phage display). There are a variety of

methods that can be used to produce libraries of potential variants of the polypeptides of the invention from a degenerate oligonucleotide sequence. Methods for synthesizing degenerate oligonucleotides are known in the art (see, e.g., Narang (1983) Tetrahedron 39:3; Itakura et al. (1984) Annu. Rev. Biochem. 53:323; Itakura et al. (1984) Science 198:1056; Ike et al. (1983) Nucleic Acid Res. 11:477).

[0132] Variants of a given reference lactase enzyme can be obtained by the following standard procedure:

- Mutagenesis (error-prone, doped oligo, spiked oligo) or synthesis of variants
- Transformation in, for example *E.coli* or *K. lactis*
- Cultivation of transformants, selection of transformants
- Expression
- Optional purification and concentration
- Primary Screening
- Identification of an improved variant (for example in relation to specific activity)

[0133] In one embodiment the invention relates to a method of producing a lactase polypeptide variant according to the invention, which method comprises:

1. a) selecting a reference lactase polypeptide;
2. b) substituting at least one amino acid residue corresponding to any of 233, 257, 258, 263, 274, 284, 297, 415, 440, 483, 619, 621, 622, 633, 862 or 1004

said positions being defined with reference to SEQ ID NO: 2;

c) optionally substituting one or more further amino acids as defined in b);

d) preparing the variant resulting from steps a)-c);

e) determining a property of the variant, for example as set out in the Examples; and

f) selecting a variant having an altered property in comparison to the lactase comprising the sequence set out in SEQ ID NO: 2 and selecting a variant having at least 80% sequence identity with SEQ ID NO:2, thereby to produce a lactase polypeptide variant.

[0134] In a preferred embodiment in the method of producing a lactase polypeptide variant according to the invention, the reference lactase polypeptide has the sequence set out in SEQ ID NO: 2.

[0135] More preferably in step b) of the method according to the invention at least one amino acid residue corresponding to any of

[0136] 233, 257, 258, 263, 274, 284, 297, 415, 440, 483, 619, 621, 622, 633, 862 or 1004 is substituted, said positions being defined with reference to SEQ ID NO: 2. The reference polypeptide may have at least about 80 % homology with SEQ ID NO: 2.

[0137] In another embodiment, the invention features cells, e.g., transformed host cells or recombinant host cells that contain a nucleic acid encompassed by the invention. A "transformed cell" or "recombinant cell" is a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a nucleic acid according to the invention. Both prokaryotic and eukaryotic cells are included, e.g., bacteria, fungi, yeast, and the like, especially preferred are cells from yeasts, for example, *K. lactis*. Host cells also include, but are not limited to, mammalian cell lines such as CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and choroid plexus cell lines.

[0138] Examples of suitable bacterial host organisms are gram positive bacterial species such as *Bacillaceae* including *Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans, Bacillus lautus, Bacillus megaterium* and *Bacillus thuringiensis, Streptomyces* species such as *Streptomyces murinus,* lactic acid bacterial species including *Lactococcus* spp. such as *Lactococcus lactis, Lactobacillus* spp. including *Lactobacillus reuteri, Leuconostoc* spp. and *Streptococcus* spp. Alternatively, strains of a gram negative bacterial species such as a species belonging to Enterobacteriaceae, including *E. coli* or to Pseudomonadaceae may be selected as the host organism.

[0139] A suitable yeast host organism may advantageously be selected from a species of *Saccharomyces* including *Saccharomyces cerevisiae* or a species belonging to *Schizosaccharomyces*. Further useful yeast host organisms include *Pichia* spp. such as methylotrophic species hereof, including *Pichia pastoris*, and *Kluyveromyces* spp. including *Kluyveromyces lactis*.

[0140] Suitable host organisms among filamentous fungi include species of *Acremonium, Aspergillus, Fusarium, Humicola, Mucor, Myceliophtora, Neurospora, Penicillium, Thielavia, Tolypocladium* or *Trichoderma,* such as e. g. *Aspergillus aculeatus, Aspergillus awamori, Aspergillus foetidus, Aspergillus japonicus, Aspergillus oryzae, Aspergillus nidulans* or *Aspergillus niger,* including *Aspergillus nigervar. awamori, Fusarium bactridioides, Fusarium cereals, Fusarium crookwellense, Fusarium culmorum, Fusarium graminearum, Fusarium graminum, Fusarium heterosporum, Fusarium negundi, Fusarium oxysporum, Fusarium reticulatum, Fusarium roseum, Fusarium sambucinum, Fusarium sarcochroum, Fusarium sporotrichiodes, Fusarium sulphureum, Fusarium torulosum, Fusarium trichothecioides, Fusarium venenatum, Humicola insolens, Humicola langinosa, Mucor miehei, Myceliophtora thermophila, Neurospora crassa, Penicillium chrysogenum, Penicillium camenbertii, Penicillium purpurogenum, Rhizomucor miehei, Thielavia terestris, Trichoderma harzianum, Trichoderma koningii, Trichoderma longibrachiatum, Trichoderma reesii* or *Trochoderma viride.*

[0141] A host cell can be chosen that modulates the expression of the inserted sequences, or

modifies and processes the product encoded by the incorporated nucleic acid sequence in a specific, desired fashion. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may facilitate optimal functioning of the encoded protein.

[0142] Various host cells have characteristic and specific mechanisms for post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems familiar to those of skill in the art of molecular biology and/or microbiology can be chosen to ensure the desired and correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product can be used. Such host cells are well known in the art.

[0143] If desired, a stably transfected cell line can produce a variant according to the invention. A number of vectors suitable for stable transfection of mammalian cells are available to the public, methods for constructing such cell lines are also publicly known, e.g., in Ausubel et al. (supra).

[0144] The present invention further discloses a composition comprising the lactase variants according to the invention. The invention thus provides a composition comprising the variant polypeptide as described herein and at least one component selected from salt (like sodium or potassium chloride), preservative, a polyol (like glycerol), metal ions (like magnesium or manganese ions).

[0145] The composition may optionally comprise other ingredients such as e.g. other enzymes. Such a composition may comprise the variant polypeptide of the invention or one obtainable by a method of the invention for identifying a variant lactase.

[0146] In addition to the variant lactase, and one or more additional enzymes, if present, a composition according to the invention may comprise additives that are conventionally used in lactase preparations such as e. g. KCl or glycerol.

[0147] The invention further relates to use of a variant polypeptide of the invention or a composition of the invention in the preparation of a dairy product.

[0148] The invention also relates to a process for the production of a dairy product, which method comprises comprising adding an effective amount of a variant polypeptide or a composition of the invention to milk and allowing the variant polypeptide to exerts its enzymatic activity.
As used herein, a dairy product encompasses any composition that is produced from milk, for instance casein and/or whey protein. Examples are milk, milk-derived products, fermented milk products (e.g. yoghurt), condensed milk, UHT milk, evaporated milk, powdered milk, frozen milk, ice cream, cream, butter, butter milk, whey; and/or cheese. The product may also be a hydrolysate or a product obtained by fractionation of milk or whey, like caseinate, milk protein concentrate, whey protein concentrate (WPC), whey protein isolate (WPI), or (concentrated)

whey permeate and products made thereof

[0149] The milk is for example obtained from cow, buffalo, goat, sheep, camel, donkey, horse, reindeer, moose or yak.

[0150] A reference herein to a patent document or other matter which is given as prior art is not to be taken as an admission that that document or matter was known or that the information it contains was part of the common general knowledge as at the priority date of any of the claims.

[0151] The invention will now be elucidated with reference to the following examples without however being limited thereto.

Examples

General material and methods

Molecular and genetic techniques

[0152] Standard genetic and molecular biology techniques are known in the art (*e.g.* Maniatis et al. "Molecular cloning: a laboratory manual" (1982) Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.; Miller "Experiments in molecular genetics" (1972) Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.; Sambrook and Russell "Molecular cloning: a laboratory manual" (3rd edition)" (2001) Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press; Ausubel "Current protocols in molecular biology" (1987) Green Publishing and Wiley Interscience, New York).

Plasmids and Strains

[0153] pBAD/HisA was obtained from Invitrogen™ (LifeTechnologies Corporation, Carlsbad, CA, USA). The beta-galactosidase deficient strain *Escherichia coli* BW25113 (*Δ(araD-araB)567, ΔlacZ4787*(::rrnB-3), λ^-*, rph-1, Δ(rhaD-rhaB)568, hsdR514)* (Datsenko KA, Wanner BL (2000) Proc Natl Acad Sci USA 97: 6640-6645) was used for the expression of the *Kluyveromyces lactis* beta-galactosidase variants.

Media

[0154] 2xPY medium (16 g/l BD BBL™ Phytone™ Peptone, 10 g/l Yeast Extract, 5 g/l NaCl) was used for growth of *Escherichia coli.* Antibiotics (100 microgram/ml ampicillin) were supplemented to maintain plasmids. For induction of gene expression L-arabinose was used at 0.02 % final concentration.

Example 1: DNA constructs and transformation

[0155] Synthetic DNA constructs were designed to start with a *Bbs*I restriction site resulting in an *Nco*I compatible overhang and ending with a *Bbs*I restriction site after the stop codon resulting in an *Hind*III compatible overhang. Internal *Bbs*I restriction sites were removed in the design of the synthetic DNA construct. As an example, a DNA fragment encoding the wild type *K. lactis* beta-galactosidase sequence is listed as SEQ ID NO: 1. All variants were designed in a similar fashion and cloned as *Bbs*I fragments in the *Nco*I/*Hind*III sites of expression vector pBAD/HisA.

[0156] Amino acid changes that were introduced in the 14 variants that are depicted in Table 2. Position of the change is indicated in comparison with the wild type *K. lactis* beta-galactosidase amino acid sequence (SEQ ID NO: 2). Some variants have multiple changes introduced into the amino acid sequence of the beta-galactosidase protein, like variant #8, and #7. A wild-type gene encoding the unchanged beta-galactosidase protein was also used in gene cloning and transformation and was later used to compare with enzymes made with the variant genes.

Table 2: Amino acid changes introduced in the protein sequence of *K. lactis* beta-galactosidase
Aminoacids are depicted according to the single letter annotation

Variant#	***Mutations***		
1	T633G		
2	Y440F		
3	A483S		
4	A1004P		
5	A258T		
6	D233V		
7	*N263S*	*K274E*	*N284S*
8	*D257G*	*E297G*	
9	L862V		
10	V619I		
11	T415C		
12	T415A		
13	M622L		
14	I621 V		

[0157] Transformation of *E.coli* BW25113 was done using the Zymo Research Z-Competent™ *E.coli* transformation kit & buffer set (T3001). The transformed *E.coli* strains were plated on 2xPY agar plates containing 100 µg/ml ampicillin, 0.02 % L-arabinose, and 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and incubated at 30°C overnight.

[0158] X-gal is an analog of lactose, and hydrolyzed by the β-galactosidase enzyme. X-gal, when cleaved by β-galactosidase, yields galactose and 5-bromo-4-chloro-3-hydroxyindole. The latter then spontaneously dimerizes and is oxidized into 5,5'-dibromo-4,4'-dichloro-indigo, an intensely blue product which is insoluble. The plates were stored at 4°C for at least 24 hours to allow blue color formation upon hydrolysis of X-Gal. As the wild type *E. coli* strain BW25113 lacks β-galactosidase activity due to deletion of the lac operon, formation of blue color confirmed active expression of the chosen β-galactosidase variant. Of each construct three blue color forming transformants were tested for β-galactosidase production using small scale 24 well cultivation (example 2), and the best producing transformant was selected for further enzyme characterization.

Example 2: Cultivation and preparation of beta-galactosidase enzyme samples

[0159] The *E.coli* BW25113 transformants expressing a variant beta-galactosidase gene were replicated from the agar plates into 96 wide well plates (NUNC 267334, NUNC A/S, Roskilde, Denmark) with 200 µl 2*PY and 100 µg/ml ampicillin followed by an overnight incubation at 30°C, 550 rpm and 80% humidity in an INFORS HT Microtron shaker (Infors AG, Bottmingen, Switzerland). 15 µl from these precultures was used to inoculate 24 wells plates (AXYGPDW10ML24CLIDS, Axygen™, Corning, NY 14831 USA) comprising 3 ml 2*PY with 100 µg/ml ampicillin. The 24 well plates were covered with a breathseal (6786051, greiner bio-one, Frickenhausen, Germany) and incubated at 30°C, 550 rpm and 80% humidity in an INFORS HT Microtron shaker until an optical density 600nm of 0.4-0.6 was reached. Then, L-arabinose was added to a final concentration of 0.02% and the 24 well plates were further incubated for 20-24 hours at 20°C, 750 rpm and 80% humidity in the INFORS HT Microtron shaker. The 24 well plates were centrifuged for 10 minutes at 2750 rpm and 4°C and supernatant was removed by decanting the plate. The cell pellets obtained were stored at -20°C for at least 24 hours. The frozen cell pellets were resuspended in 1 ml extraction buffer (50 mM Tris-HCl pH 7.5, 0.2 mM MgSO4, 2 mg/ml lysozym, 0.1 mg/ml DNAse I, 1x Complete Protease inhibitor cocktail (EDTA free, Roche)) by use of a vortex, incubated at room temperature for 30 minutes followed by centrifugation for 10 minutes at 2750 rpm and 4°C. The supernatant comprising the overexpressed beta-galactosidase (Cell Free Extract, CFE) was formulated by addition of 1 volume of glycerol and used in the different activity assays.

Example 3: Determination of the amount of lactase protein

[0160] The amount of lactase protein produced by E. coli was determined using HP-SEC (Thermo Scientific Dionex UltiMate 3000 Rapid Separation). For this 2 µl of the cell-free extracts (CFE) of Example 1 were loaded on a BEH200, sec 1.7 µm 4.6 X 150 mm column (Waters). The mobile phase consisted of 100 mM of potassium phosphate buffer (pH 7.32) and was kept at a flow of 0.1 mL / min. The column temperature kept at 25°C, while the flow was set at 0.1 mL / min. Elution of protein was followed by measuring the absorbance at 280 nm. Since the lactase protein is larger than most other proteins in the CFE it was the first protein peak that was eluted from the column under these conditions. The area under this peak was calculated and quantified by comparison to a bovine serum albumin (BSA) standard. Results of this quantification are used for the calculation of the (specific) activity of the proteins as described in Examples 4-7.

Table 3A: Results of the analysis of the (specific) activity of the variants in the various assays described in Examples 4-7. Values that are significantly ($p<0.05$) higher or lower (% inhibition) from the average of wild-type lactases (6 samples) are marked.

Variant	Mutation			Specific Activity_NLU/mg lactase Example 4	Specific activity LACU/mg lactase Example 5	Specific activity LACGU/mg lactase Example 6	inhibition% Examples 5 vs. 6	milk:mg glucose/mg lactase in 4 h Example 7	milk:mg/l GOS after 48 h Example 7
1	T633G			81	105	27	75	352	1209
2	Y440F			28	160	39	76	279	977
3	A483S			79	175	42	76	205	1494
4	A1004P			63	100	25	75	253	1062
5	A258T			66	98	29	71	305	1076
6	D233V			67	97	26	74	285	1026
7	*N263S*	*K274E*	*N284S*	60	91	25	72	290	964
8	*D257G*	*E297G*		71	99	28	72	256	1201
9	L862V			73	100	27	73	258	1269
10	V619I			79	75	23	69	300	2050
11	T415C			98	147	35	77	179	1436
12	T415A			78	169	37	78	164	1341
13	M622L			78	43	16	62	186	1818
14	I621V			76	40	19	52	99	616
average	wild-type			67	103	28	73	206	1277
st. dev.				4	10	1	2	12	164

Table 3B: Results of the analysis of the (specific) activity of the variants in the various assays described in Examples 4-7. The values of Table 3A are expressed as relative values compared to the values of the wild type enzyme in the same assay.

				: activity e Example 4	: activity se Example 5	: activity ase Example 6	on 6	in milk	oduction in milk

Variant	Mutation			Relative specific NLU/mg lactase	Relative specific LACU/mg lactase	Relative specific LACGU/mg lact	Relative inhibiti Examples 5 vs.	relative activity Example 7	relative GOS pr Example 7
1	T633G			120	102	97	102	171	95
2	Y440F			42	155	140	104	135	77
3	A483S			117	169	152	104	99	117
4	A1004P			94	97	92	102	122	83
5	A258T			98	95	104	97	148	84
6	D233V			100	94	93	101	138	80
7	*N263S*	*K274E*	*N284S*	90	88	92	99	141	75
8	*D257G*	*E297G*		106	96	100	99	124	94
9	L862V			109	96	98	100	125	99
10	V619I			117	72	84	95	145	161
11	T415C			146	143	125	105	86	112
12	T415A			115	164	132	107	79	105
13	M622L			117	41	59	85	90	142
14	I621V			113	39	70	71	48	48
average	wild-type			100	100	100	100	100	100
st. dev.				7	10	5	3	6	13

Example 4: Activity determination on ONPG as substrate

[0161] The activity determination using o-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate was essentially according to the procedure described in the Food Chemical Codex (FCC 8th edition, p1319-1320: Lactase (neutral) β-galactosidase activity).

[0162] The samples produced in Example 2 were diluted 200-fold until ~0.1 neutral lactase units (NLU) per mL using buffer A (100 mM potassium phosphate (pH6.5) containing 0.05 mM EDTA, 0.1 mM MgSO4 and 0.2 % (W/V) Triton X100). The same buffer, but without Triton X100, is used for the preparation of the substrate (50 mg o-nitrophenyl-β-D-galactopyranoside (Sigma-Aldrich) in 20 mL). After preheating the substrate the following is mixed together: 125 µL of substrate and 25 µL of sample. The reaction is allowed to proceed for 10 minutes at 37 °C, after which the reaction is stopped by the addition of 25 µL sodium carbonate (30 g / L) and 20 µL of ultrapure water. The resulting absorbance at 405 nm can be used and compared to the calibration curve made from o-nitrophenol (ONP). The measurements occurred on a Konelab clinical analyzer (Thermo Scientific Arena 30). Calculation of the activity was performed as described in the Food Chemical Codex and corrected for the difference in assay temperature. The correction factor was 1.25 and was established empirically. The specific activity of the different lactase variants was determined by dividing these values by the protein dosage (as determined in Example 3) in the assay and the result is depicted in Table 3.

Example 5: Activity determination on lactose as substrate

[0163] The samples were diluted to ~0.4 NLU/mL in buffer B (100 mM sodium phosphate

(pH6.5) containing 0.05 mM EDTA and 1 mM MgSO4). The substrate consisted of 4.8% lactose monohydrate dissolved in buffer B. The enzyme mix consisted of 780 units of horseradish peroxidase (Sigma Aldrich), 0.25 units of glucose oxidase (DSM) and 12,5 mg (+/- 1 mg) 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, Sigma Aldrich) in a total of 10 mL of buffer B. The assay is measured relatively against a serial dilution of neutral lactase (0.1-0.8 NLU / mL). For the reaction to occur the following is transferred to one well of a standard microtiter plate: 25 µL buffer B, 25 µL of sample or standard, 25 µL of enzyme mix. After preincubation for 10 minutes, 175 µL of substrate is added and the reaction is measured on a MTP reader (Tecan Infinity M1000) at 420 nm and 30°C for 30 minutes. The absorbance is measured every 30 seconds and the slope per five data points (2.5 minutes) was calculated. The maximum slope over the complete assay is used to calculate the activity. This maximum slope is expressed as µmol glucose produced by lactase per min under the conditions described here (LACU). The specific activity of the different lactase variants is calculated by dividing these values by the protein concentration in mg/ml (as determined in Example 3) in the assay. The specific activity in LACU/mg of these lactase variants is depicted in Table 3. A high specific activity on lactose may lead to a lower dosage of the enzyme in possible applications.

Example 6: Activity determination in the presence of galactose

[0164] The samples were diluted to ~0.4 NLU/mL in buffer B. The substrate consisted of 4.8% lactose monohydrate dissolved in buffer B. The enzyme mix consisted of 780 units of horse radish peroxidase (Sigma Aldrich), 0.25 units of glucose oxidase (DSM) and 12,5 mg (+/- 1 mg) 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, Sigma Aldrich) in a total of 10 mL of buffer B. The inhibition buffer C consisted of 1500 mM galactose (purity > 99.9%) in buffer B. The assay is measured relatively against a serial dilution of neutral lactase (0.1 - 0.8 NLU / mL). For the reaction to occur the following is transferred to one well of a standard microtiterplate: 25 µL buffer C (except for the standards), 25 µL of sample or standard, 25 µL of enzyme mix. After pre-incubating for 10 minutes, 175 µL of substrate is added and the reaction is measured on a MTP reader at 420 nm and 30 °C for 30 minutes. The absorbance is measured every 30 seconds and the slope per five data points was calculated. The maximum slope over the complete assay is used to calculate the activity. This maximum slope is expressed as µmol glucose produced by lactase per min under the conditions described here (LACGU). The specific activity in LACGU/mg of the different lactase variants is calculated by dividing these values by the protein concentration in mg/ml (as determined in Example 3) in the assay and the results are shown in Table 3.

[0165] The percentage inhibition by galactose is calculated using the formula

$$\% \text{ inhibition} = 100 * (x - y) / x$$

where x stands for the specific activity in LACU/mg lactase as described in Example 5, and y stands for the specific activity in LACGU/mg lactase as described in Example 6. The results of the calculation of the % inhibition are shown in Table 3. A low %inhibition may lead to a higher activity at conditions in the application where lactose concentration is low and galactose

concentration is high, when a low residual lactose concentration is required.

Example 7: Activity determination in milk at low temperature

[0166] 1 mL of commercial semi-skimmed UHT milk (Campina) was mixed with 0.2 mL enzyme (~20 NLU / mL) as produced in Example 2, in deep well microtiter plates. The samples were incubated under static conditions for 4, 24 or 48 hours at 6 °C. After this incubation the reactions were terminated by a heat treatment at 90 °C for 6 minutes, after which samples were directly placed in a freezer at -20 °C until analysis. Sample preparation for NMR was performed as follows: 48 µL 4.0 M HCl was added and the plates were sealed and mixed by tilting. Then, the plates were shaken for 20 minutes at 600 rpm and subsequently centrifuged for 10 minutes at 4750 rpm. From the clear supernatant 0.3 mL was transferred to a new plate and combined with 0.2 mL of a solution containing 20 g / L maleic acid (internal standard) and 40 g / L EDTA in D_2O. The plates were sealed, mixed by tilting and briefly centrifuged. After lyophilization, the residue was dissolved in 0.05 mL D_2O and lyophilization was repeated. The dried residue was dissolved in 0.7 mL D_2O, again lyophillized overnight and again dissolved in 0.7 mL D_2O. After careful mixing, the samples were centrifuged for 10 minutes at 4750 rpm and 0.6 mL was transferred to a NMR tube. The samples were measured on a Bruker Avance III spectrometer equipped with a cryoprobe operating at a proton frequency of 700 MHz at a probe temperature of 290K. The samples were measured in single fold using 8 scans and a delay of 30 seconds. From the NMR spectra the following compounds were quantified: Lactose (δ = 4.67 (d)), glucose δ = 4.64 (d), galactose δ = 4.58 (d) and galacto oligo saccharide (GOS, integral of the area from δ = 4.52 to approx. δ = 4.38). In Table 3 the amount of glucose detected after 4 hours incubation per mg added enzyme is indicated. Also the amount of GOS after 48 hours, when most lactose is hydrolysed and little residual lactose is left (<0.5 g/l), is depicted.

[0167] A high lactose hydrolysis activity of the enzyme in milk at a low temperature (4-12 °C) may lead to a reduced dosage of the enzyme in such an application relevant for the dairy industry, and therefore reduced cost. An increased GOS production may lead to a prebiotic effect of the produced milk.

Example 8: Combinations of lactase variants

[0168] Different combinations of mutations in the lactase gene were generated as described in Example 1, except that multiple amino acid changes were combined in the expression product of a gene construct. The different variants containing these combined amino acid changes are depicted in Table 4. Position of the change is indicated in comparison with the wild type *K. lactis* beta-galactosidase amino acid sequence (SEQ ID NO: 2).

Table 4: Amino acid changes introduced in the protein sequence of *K. lactis* beta-galactosidase. Amino acids are depicted according to the single letter annotation

Variant#	*Mutations*					
15	Y440F	V619I	T633G			
16	Y440F	V619I	A483S			
17	Y440F	V619I	T633G	A258T	A483S	
18	Y440F	V619I	T415A	A258T	A483S	
19	Y440F	V619I	T633G	L862V		
20	Y440F	V619I	T415A	L862V		
21	Y440F	V619I	T415C	L862V		
22	Y440F	V619I	A483S	L862V		
23	Y440F	T415A				
24	T415A	A483S				
25	T415C	A483S				
26	T633G	A483S				
27	Y440F	V619I	T415C			
28	V619I	A483S	T415A			
29	A258T	V619I	A483S	T415A		
30	A258T	V619I	T633G	T415A	A483S	
31	A258T	V619I	Y440F	E264V	A483S	
32	A258T	V619I	Y440F	L862V	E264V	A483S
33	L862V	V619I	T633G	T415A		
34	A483S	V619I	T415A	L862V		
35	A483S	V619I	T633G	L862V		
36	L862V	V619I	E264V	A483S		

[0169] Again, the modified lactase genes were expressed in *E. coli* and lactase protein was isolated as described in Example 2. The amount of lactase protein that was expressed was determined as described in Example 3. The activity of these enzyme samples on the hydrolysis of lactose was determined as described in Example 5 and compared to the activity of the wild type enzyme expressed and isolated exactly the same way. Also the activity of the enzyme samples on the hydrolysis of lactose in milk at low temperature after 4 hours was determined as described in Example 7.

[0170] The specific activity of the different lactase variants is calculated by dividing the measured values by the lactase protein concentration in mg/ml in the assay. The specific activity of these lactase variants in both assays was expressed as relative activity compared to the activity of the wild type enzyme obtained in the same assays. For this the specific activity of the wild type lactase was set at 100 in both assays, and the calculated specific activities of the

variants was related to this. The results of this analysis are depicted in Table 5.

Table 5: Results of the analysis of the (specific) activity of the variants in the various assays. Values are depicted as relative to the value found with wild type lactase. that are significantly ($p<0.05$) higher from the average of wild-type lactases are marked.

Variant	Mutation						Relative specific activity on lactose Example 8	Relative activity in milk Example 8
15	Y440F	V619I	T633G				47	139
16	Y440F	V619I	A483S				115	107
17	Y440F	V619I	T633G	A258T	A483S		144	162
18	Y440F	V619I	T415A	A258T	A483S		130	122
19	Y440F	V619I	T633G	L862V			46	118
20	Y440F	V619I	T415A	L862V			145	116
21	Y440F	V619I	T415C	L862V			135	114
22	Y440F	V619I	A483S	L862V			119	134
23	Y440F	T415A					138	74
24	T415A	A483S					167	33
25	T415C	A483S					142	61
26	T633G	A483S					133	65
27	Y440F	V619I	T415C				144	106
28	V619I	A483S	T415A				154	61
29	A258T	V619I	A483S	T415A			149	41
30	A258T	V619I	T633G	T415A	A483S		141	98
31	A258T	V619I	Y440F	E264V	A483S		135	100
32	A258T	V619I	Y440F	L862V	E264V	A483S	128	96
33	L862V	V619I	T633G	T415A			122	106
34	A483S	V619I	T415A	L862V			153	102
35	A483S	V619I	T633G	L862V			169	99
36	L862V	V619I	E264V	A483S			118	78
average	wild-type						100	100
st. dev.							6	6

[0171] From this analysis it can be deduced that several combination variants show advantageous lactose hydrolysis in both assays.

SEQUENCE LISTING

[0172]

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<211> 1025

<212> PRT

<213> Kluyveromyces lactis

<400> 2

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Glu Ser Leu Asn Gly Pro Trp Ala Phe Ala Leu Phe Asp Ala Pro Leu
        35                  40                  45
Asp Ala Pro Asp Ala Lys Asn Leu Asp Trp Glu Thr Ala Lys Lys Trp
    50                  55                  60
Ser Thr Ile Ser Val Pro Ser His Trp Glu Leu Gln Glu Asp Trp Lys
65                  70                  75                  80
Tyr Gly Lys Pro Ile Tyr Thr Asn Val Gln Tyr Pro Ile Pro Ile Asp
                85                  90                  95
Ile Pro Asn Pro Pro Thr Val Asn Pro Thr Gly Val Tyr Ala Arg Thr
            100                 105                 110
Phe Glu Leu Asp Ser Lys Ser Ile Glu Ser Phe Glu His Arg Leu Arg
        115                 120                 125
Phe Glu Gly Val Asp Asn Cys Tyr Glu Leu Tyr Val Asn Gly Gln Tyr
    130                 135                 140
Val Gly Phe Asn Lys Gly Ser Arg Asn Gly Ala Glu Phe Asp Ile Gln
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Lys Tyr Val Ser Glu Gly Glu Asn Leu Val Val Val Lys Val Phe Lys
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Trp Ser Asp Ser Thr Tyr Ile Glu Asp Gln Asp Gln Trp Trp Leu Ser
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Gly Ile Tyr Arg Asp Val Ser Leu Leu Lys Leu Pro Lys Lys Ala His
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225                 230                 235                 240
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Thr Lys Glu Phe Ile Ser Phe Ser Thr Lys Lys Asn Glu Glu Thr Ala
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Phe Lys Ile Asn Val Lys Ala Pro Glu His Trp Thr Ala Glu Asn Pro
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Thr Leu Tyr Lys Tyr Gln Leu Asp Leu Ile Gly Ser Asp Gly Ser Val
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Ile Gln Ser Ile Lys His His Val Gly Phe Arg Gln Val Glu Leu Lys
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Asp Gly Asn Ile Thr Val Asn Gly Lys Asp Ile Leu Phe Arg Gly Val
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Asn Arg His Asp His His Pro Arg Phe Gly Arg Ala Val Pro Leu Asp
        355                 360                 365
Phe Val Val Arg Asp Leu Ile Leu Met Lys Lys Phe Asn Ile Asn Ala

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385                 390                 395                 400
Asp Lys Leu Gly Phe Trp Val Ile Asp Glu Ala Asp Leu Glu Thr His
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Gly Val Gln Glu Pro Phe Asn Arg His Thr Asn Leu Glu Ala Glu Tyr
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Pro Asp Thr Lys Asn Lys Leu Tyr Asp Val Asn Ala His Tyr Leu Ser
        435                 440                 445
Asp Asn Pro Glu Tyr Glu Val Ala Tyr Leu Asp Arg Ala Ser Gln Leu
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Val Leu Arg Asp Val Asn His Pro Ser Ile Ile Ile Trp Ser Leu Gly
465                 470                 475                 480
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                485                 490                 495
Lys Gln Leu Asp Pro Thr Arg Leu Val His Tyr Glu Gly Asp Leu Asn
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Ala Leu Ser Ala Asp Ile Phe Ser Phe Met Tyr Pro Thr Phe Glu Ile
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Gln Gly Gly Phe Ile Trp Glu Trp Ala Asn His Gly Ile Glu Phe Glu
            580                 585                 590
Asp Val Ser Thr Ala Asp Gly Lys Leu His Lys Ala Tyr Ala Tyr Gly
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Gly Asp Phe Lys Glu Glu Val His Asp Gly Val Phe Ile Met Asp Gly
    610                 615                 620
Leu Cys Asn Ser Glu His Asn Pro Thr Pro Gly Leu Val Glu Tyr Lys
625                 630                 635                 640
Lys Val Ile Glu Pro Val His Ile Lys Ile Ala His Gly Ser Val Thr
                645                 650                 655
Ile Thr Asn Lys His Asp Phe Ile Thr Thr Asp His Leu Leu Phe Ile
            660                 665                 670
Asp Lys Asp Thr Gly Lys Thr Ile Asp Val Pro Ser Leu Lys Pro Glu
        675                 680                 685
Glu Ser Val Thr Ile Pro Ser Asp Thr Thr Tyr Val Val Ala Val Leu
    690                 695                 700
Lys Asp Asp Ala Gly Val Leu Lys Ala Gly His Glu Ile Ala Trp Gly
705                 710                 715                 720
Gln Ala Glu Leu Pro Leu Lys Val Pro Asp Phe Val Thr Glu Thr Ala
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Glu Lys Ala Ala Lys Ile Asn Asp Gly Lys Arg Tyr Val Ser Val Glu
            740                 745                 750
Ser Ser Gly Leu His Phe Ile Leu Asp Lys Leu Leu Gly Lys Ile Glu
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Ser Leu Lys Val Lys Gly Lys Glu Ile Ser Ser Lys Phe Glu Gly Ser
    770                 775                 780
Ser Ile Thr Phe Trp Arg Pro Pro Thr Asn Asn Asp Glu Pro Arg Asp
785                 790                 795                 800
Phe Lys Asn Trp Lys Lys Tyr Asn Ile Asp Leu Met Lys Gln Asn Ile
                805                 810                 815
His Gly Val Ser Val Glu Lys Gly Ser Asn Gly Ser Leu Ala Val Val
            820                 825                 830
Thr Val Asn Ser Arg Ile Ser Pro Val Val Phe Tyr Tyr Gly Phe Glu
        835                 840                 845
Thr Val Gln Lys Tyr Thr Ile Phe Ala Asn Lys Ile Asn Leu Asn Thr
    850                 855                 860
Ser Met Lys Leu Thr Gly Glu Tyr Gln Pro Pro Asp Phe Pro Arg Val
865                 870                 875                 880

Gly Tyr Glu Phe Trp Leu Gly Asp Ser Tyr Glu Ser Phe Glu Trp Leu
                885                 890                 895
Gly Arg Gly Pro Gly Glu Ser Tyr Pro Asp Lys Lys Glu Ser Gln Arg
            900                 905                 910
Phe Gly Leu Tyr Asp Ser Lys Asp Val Glu Glu Phe Val Tyr Asp Tyr
        915                 920                 925
Pro Gln Glu Asn Gly Asn His Thr Asp Thr His Phe Leu Asn Ile Lys
    930                 935                 940
Phe Glu Gly Ala Gly Lys Leu Ser Ile Phe Gln Lys Glu Lys Pro Phe
945                 950                 955                 960
Asn Phe Lys Ile Ser Asp Glu Tyr Gly Val Asp Glu Ala Ala His Ala
                965                 970                 975
Cys Asp Val Lys Arg Tyr Gly Arg His Tyr Leu Arg Leu Asp His Ala
            980                 985                 990
Ile His Gly Val Gly Ser Glu Ala Cys Gly Pro Ala Val Leu Asp Gln
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Tyr Arg Leu Lys Ala Gln Asp Phe Asn Phe Glu Phe Asp Leu Ala Phe
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Glu
1025
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REFERENCES CITED IN THE DESCRIPTION

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- Lactase (neutral) β-galactosidase activityFood Chemical Codex1319-1320 [0161]

Patentkrav

1. Variant polypeptid med lactaseaktivitet, hvor varianten har en aminosyresekvens, der, når den er rettet ind med lactasen, der omfatter sekvensen ifølge SEQ ID NO: 2, omfatter mindst én substitution af en aminosyrerest svarende til en hvilken som helst af aminosyrerne
233, 257, 258, 263, 274, 284, 297, 415, 440, 483, 619, 621, 622, 633, 862 eller 1004,
hvor positionerne er defineret ved henvisning til SEQ ID NO: 2, og hvor varianten har en eller flere ændrede egenskaber sammenlignet med et referencepolypeptid med lactaseaktivitet, og hvor varianten har mindst 80 % sekvensidentitet med SEQ ID NO: 2.

2. Variant polypeptid ifølge krav 1, hvor referencepolypeptidet er lactasen ifølge SEQ ID NO: 2.

3. Variant polypeptid ifølge krav 1 eller 2, hvor det variante polypeptid er et ikke-naturligt forekommende polypeptid.

4. Variant polypeptid ifølge et hvilket som helst af kravene 1 til 3, hvor varianten fremviser forøget specifik aktivitet på ONPG sammenlignet med et referencepolypeptid med lactaseaktivitet.

5. Variant polypeptid ifølge krav 4, der omfatter en aminosyresekvens, der, når den er rettet ind med sekvensen ifølge SEQ ID NO: 2, omfatter mindst én substitution af en aminosyrerest svarende til en hvilken som helst af aminosyrerne 415, 483, 619, 621, 622 eller 633.

6. Variant polypeptid ifølge krav 4 eller 5, der omfatter en aminosyresekvens, der, når den er rettet ind med sekvensen ifølge SEQ ID NO: 2, omfatter mindst én substitution valgt blandt T415C, T415A, A483S, V619I, I621V, M622L eller T633G.

7. Variant polypeptid ifølge et hvilket som helst af kravene 1 til 3, hvor varianten fremviser forøget specifik aktivitet på lactose sammenlignet med et referencepolypeptid med lactaseaktivitet.

8. Variant polypeptid ifølge krav 7, der omfatter en aminosyresekvens, der, når den er rettet ind med sekvensen ifølge SEQ ID NO: 2, omfatter mindst én substitution af en aminosyrerest svarende til en hvilken som helst af aminosyrerne 415, 440 eller 483.

9. Variant polypeptid ifølge krav 7 eller 8, der omfatter en aminosyresekvens, der, når den er rettet ind med sekvensen ifølge SEQ ID NO: 2, omfatter mindst én substitution valgt blandt T415C, T415A, Y440F eller A483S.

10. Variant polypeptid ifølge et hvilket som helst af kravene 1 til 3, hvor varianten fremviser forøget aktivitet på lactose i mælk sammenlignet med et referencepolypeptid med lactaseaktivitet.

11. Variant polypeptid ifølge krav 10, der omfatter en aminosyresekvens, der, når den er rettet ind med sekvensen ifølge SEQ ID NO: 2, omfatter mindst én substitution af en aminosyrerest svarende til en hvilken som helst af aminosyrerne 233, 257, 258,263, 274, 284, 297, 440, 619, 633, 862 eller 1004.

12. Variant polypeptid ifølge krav 10 eller 11, der omfatter en aminosyresekvens, der, når den er rettet ind med sekvensen ifølge SEQ ID NO: 2, omfatter mindst én substitution valgt blandt D233V, D257G, A258T, N263S, K274E, N284S, E297G, Y440F, V619I, T633G, L862V eller A1004P.

13. Variant polypeptid ifølge krav 10 eller 11 eller 12, der omfatter en aminosyresekvens, der, når den er rettet ind med sekvensen ifølge SEQ ID NO: 2, omfatter mindst to substitutioner valgt blandt N263S, K274E eller N284S.

14. Variant polypeptid ifølge krav 10 eller 11 eller 12, der omfatter en aminosyresekvens, der, når den er rettet ind med sekvensen ifølge SEQ ID NO: 2, mindst omfatter substitutionerne D257G og E297G.

15. Variant polypeptid ifølge et hvilket som helst af kravene 1 til 3, hvor varianten fremviser reduceret galactosehæmning sammenlignet med et referencepolypeptid med lactaseaktivitet.

16. Variant polypeptid ifølge krav 15, der omfatter en aminosyresekvens, der, når den er rettet ind med sekvensen ifølge SEQ ID NO: 2, omfatter mindst én substitution af en aminosyrerest svarende til en hvilken som helst af aminosyrerne 619, 621 eller 622.

17. Variant polypeptid ifølge krav 15 eller 16, der omfatter en aminosyresekvens, der, når den er rettet ind med sekvensen ifølge SEQ ID NO: 2, omfatter mindst én substitution valgt blandt V619I, I621V eller M622L.

18. Variant polypeptid ifølge et hvilket som helst af kravene 1 til 3, hvor varianten fremviser forøget GOS-produktion i mælk sammenlignet med et referencepolypeptid med lactaseaktivitet.

19. Variant polypeptid ifølge krav 18, der omfatter en aminosyresekvens, der, når den er rettet ind med sekvensen ifølge SEQ ID NO: 2, omfatter mindst én substitution af en aminosyrerest svarende til en hvilken som helst af aminosyrerne 619 eller 622.

20. Variant polypeptid ifølge krav 18 eller 19, der omfatter en aminosyresekvens, der, når den er rettet ind med sekvensen ifølge SEQ ID NO: 2, omfatter mindst én substitution valgt blandt V619I eller M622L.

21. Variant polypeptid ifølge et hvilket som helst af

ovennævnte krav, der omfatter andre yderligere substitutioner end de, der er defineret i krav 1.

22. Nukleinsyresekvens, der koder for et variant polypeptid ifølge et hvilket som helst af ovennævnte krav.

23. Nukleinsyrekonstruktion, der omfatter nukleinsyresekvensen ifølge krav 22, der er operabelt koblet til en eller flere kontrolsekvenser, der er i stand til at styre ekspressionen af en lactase i en egnet ekspressionsvært.

24. Rekombinant ekspressionsvektor, der omfatter nukleinsyrekonstruktionen ifølge krav 23.

25. Rekombinant værtscelle, der omfatter ekspressionsvektoren ifølge krav 24.

26. Fremgangsmåde til fremstilling af en lactase, som omfatter dyrkning af værtscellen ifølge krav 25 under betingelser, der fører til produktion af lactasen, og indvinding af lactasen.

27. Fremgangsmåde til fremstilling af en lactasepolypeptidvariant, hvilken fremgangsmåde omfatter:
(a) selektion af et polypeptid med lactaseaktivitet;
(b) substitution af mindst én aminosyrerest svarende til en hvilken som helst af 233, 257, 258, 263, 274, 284, 297, 415, 440, 483, 619, 621, 622, 633, 862 eller 1004, hvor positionen er defineret ved henvisning til SEQ ID NO: 2;
(c) eventuelt substitution af en eller flere yderligere aminosyrer som defineret i (b);
(d) frembringelse af varianten, der opnås fra trinene (a)-(c);
(e) bestemmelse af en egenskab for varianten;
(f) selektion af en variant med en ændret egenskab sammenlignet med lactasen, der omfatter sekvensen ifølge SEQ ID NO: 2, og selektion af en variant med mindst 80 % sekvensidentitet med SEQ ID NO: 2 til derved fremstilling af en lactasepolypeptidvariant.

28. Sammensætning, der omfatter variantpolypeptidet ifølge et hvilket som helst af kravene 1 til 21 eller variantpolypeptidet, der kan opnås ved hjælp af en fremgangsmåde ifølge krav 27, og mindst én bestanddel valgt blandt salt, konserveringsmiddel, polyol eller metal-ioner.

29. Anvendelse af et variant polypeptid ifølge et hvilket som helst af kravene 1 til 21 eller af en sammensætning ifølge krav 28 til fremstilling af et mejeriprodukt med lavt lactoseindhold eller er lactosefrit mejeriprodukt.

30. Fremgangsmåde til fremstilling af et mejeriprodukt, hvilken fremgangsmåde omfatter tilsætning af en effektiv mængde af et variant polypeptid ifølge et hvilket som helst af kravene 1 til 21 eller af en sammensætning ifølge krav 28 til et mejeriprodukt og muliggørelse af, at polypeptidet kan udøve dets enzymatiske aktivitet.