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(54) Title: BAKING PROCESS AND A METHOD THEREOF

(57) Abstract: A method for improving dough characteristics without added sugar is disclosed. The process comprises of treating cereal flour with an enzyme to produce a composition containing fermentable sugars (glucose, fructose or maltose) and/or isomaltooligosaccharides. The composition can be used to form a dough mixture: shaping the dough mixture and baking the said shaped dough mixture to form baked products.

BAKING PROCESS AND A METHOD THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/340096, filed May 23, 2016, the disclosures of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present invention relates to a method of preparing a dough composition using one or more enzymes. It also relates to compositions (for example, dough compositions, baked products, bread, and the like) obtainable using the present method. The invention also relates to the use of a composition prepared using the method for improving the properties of dough and baked products prepared from the dough. The invention also relates to a method for preparing a composition containing isomaltooligosaccharides containing higher proportion of branched sugars.

SEQUENCE LISTING

[0003] The sequence listing submitted via EFS, in compliance with 37 C.F.R. §1.52(e), is incorporated herein by reference. The sequence listing text file submitted via EFS contains the file "20170511_NB41081WOPCT_SequenceListingST25.txt" created on May 10, 2015, which is 112 KB (115,446 bytes) in size.

BACKGROUND TO THE INVENTION

[0004] There is an industry-wide trend to reduce added sugars in not only baked goods, but all manufactured food products. There is also a market demand to replace high fructose corn syrup and other corn and beet sugars from product formulations. Consumers are also pressing manufacturers to reduce or eliminate added sugars to manufactured food products with emphasis on beverages, baked goods, and snack foods.

[0005] Dough "conditioners" are well known in the baking industry. The addition of conditioners to bread dough has resulted in improved machinability of the dough, improved texture, increased loaf volume, improved flavour, and increased softness (anti-staling) of the bread. Chemicals such as ascorbic acid, iodates, peroxides, and potassium bromate have

been suggested but have met with consumer resistance or are not permitted by regulatory agencies.

[0006] The use of natural enzymes as dough conditioners has been considered as an alternative to the chemical conditioners. A number of enzymes have been used recently as dough and/or bread improving agents, in particular, enzymes that act on components present in the dough. Examples of such enzymes are found within the groups of amylases, proteases, glucose oxidases, and hemicellulases; including pentosanases.

[0007] Traditionally, grains such as wheat, malt, sorghum (milo), millet, and particularly whole grains are used in nutrition as carriers of macro- and microelements: starch, proteins, fibre, vitamins, and minerals. The majority of cereal grains appeared to be too readily digested to play an effective role as a prebiotic or even as a nutraceutical.

[0008] Designing genetically modified, less digestible cereals, suitable as prebiotic to manipulate gut microflora, has been suggested (Gibson, G.R, and Robertford,M.B., *J.Nutr.* 1995, *125*, 1401-1412). The authors of this article define a prebiotic as a "non-digestible food ingredient that offers beneficial effect to the host by selectively stimulating the growth and or activity of one or limited number of bacteria in the colon that can improve the host health".

[0009] With growing consumer concern regarding clean label ingredients, there is a market demand for clean label and naturally sourced dough conditioners and sweeteners. One such class of conditioners is isomaltooligosaccharides (IMO) which can be used for anti-staling in pan breads and potentially other applications in various food areas including, but not limited to: confectionery and processed fruit products (for example, candy, fruit preparation, and syrups), bakery products (for example, breads, cakes, cookies, biscuits, and bars), and pet food.

[0010] Extensive work has been done on the production and application of oligosaccharides exhibiting this prebiotic activity, especially in Asia. These oligosaccharides typically include inulin, levan, fructooligosaccharides (FOS), galactooligosaccharides (GOS), mannooligosaccharides (MOS), and isomaltooligosaccharides (IMO). These oligosaccharides are relatively new functional food ingredients that have great potential to improve the quality of many foods.

[0011] Both the production and application of food-grade oligosaccharides are increasing rapidly. Major uses are in beverages, infant milk powder, confectionery, bakery products,

yoghurts, and dairy desserts. (Crittenden, R.G. and Playne, M.J., *Trends in Food Science & Technology* (1996) *7*, 353-361). A significant amount of isomaltooligosaccharides are now commercially produced in Japan, Korea, and China and applied in many food and beverage applications.

[0012] Isomaltooligosaccharides are generally regarded as safe (GRAS) by the US FDA. Addition of IMO in the baking process can result in replacing 25 to 50 % of the sucrose used in the process. Use of IMO results in dough expanding rapidly in fermenting state and volume becomes larger and stiffening of bread becomes slower due to high hygroscopicity of branched sugars (*Food Chemical News*, February 15, 1990).

[0013] Functional food oligosaccharides which are classified as “prebiotic” are receiving increased attention in food, beverage, and animal nutrition due to their health benefit by promoting the growth of beneficial bacteria, such as *Bifidobacteria* or *Lactobacilli*, in the colon (Hideo Tomomatsu, *Food Technology*, (1994) 61-65). There is continued interest in methods for producing grain compositions containing oligosaccharides exhibiting additional functional and nutritional properties from an enzymatically derived process from the source substrate, such as grain or cereals, without having to separate starch from other components.

[0014] US Patent 7,993,689 describes a process for making isomaltooligosaccharide-enriched flours at a temperature at or below the starch gelatinization temperature. The process comprises contacting an ungelatinized wheat having an endogenous maltogenic enzyme, *i.e.* beta amylase, with or without a starch solubilizing microbial enzyme to produce high maltose syrup. The maltose syrup was then contacted with a 1,4-α-glucan 6-α-glucosyltransferase to produce a substrate composition containing isomaltooligosaccharides. However, the process described does not involve the *in situ* production of IMO nor the application of a hydrolysate in bread production.

[0015] WO 00/27215 describes a process for preparing dough and or baked products with a glucose isomerase.

[0016] US 2003/0077369 describes a method to increase sweetness in baked goods using a combination of added IMO and high intensity sweeteners. However, IMO used in this method is not produced *in situ*.

[0017] US 4,377,602 describes a method in which whole grain products are subjected to enzymatic hydrolysis in order to extract all nutritionally important proteins. This patent describes an application for added fibre in food products, but does not address the replacement of sugar for sweetness and browning of breads. The wheat syrup produced by the process may subsequently be used in a baking composition. However, the proportion of wheat used in the initial enzymatic hydrolysis to total wheat used in the entire procedure exceeds that used in the first portion of wheat flour in the present invention.

[0018] US 4,859,474 describes a method for enzymatically producing fructose from moistened cereal grains using cellulase and glucose isomerase. This method requires heating under pressure and drying the final product prior to use in baked goods. This method describes the production of fermentable sugars by hydrolysis of starch components of flour and also the in situ production of IMO. In addition, it does not specifically disclose the subsequent addition of the fermentable sugar mixture to a further portion of cereal flour for use in a baking composition.

[0019] EP154135A describes a multi-step method for preparing bread dough by (a) heating and enzymatically treating a portion of wheat flour using an α-amylase, to produce a soluble, partially hydrolysed starch; (b) adding glucoamylase to the soluble, partially hydrolysed starch prepared in step (a); and (c) adding the composition prepared in step (b) to a mixture of wheat flour, water, and scrap dough. However, step (a) generally requires very high temperature cooking (greater than 90°C) for an extended period of time (typically over 10 hours): this results in both liquefaction and gelatinization of the starch and the high temperature above the starch gelatinization also results in the complete inactivation of wheat endogenous starch hydrolyzing enzyme activity. In addition, subjecting the composition to temperatures above 75°C results in the denaturation of wheat gluten proteins (Eliasson, A.C. and Hegg, P-O., *Cereal Chem.* (1980) *57*, 436-437) which may affect the gluten:starch complex structure in baked products.

SUMMARY OF THE INVENTION

[0020] In a first aspect, a method of preparing a dough composition is provided, said method comprising:

(a) preparing a composition by mixing a first portion of cereal flour, water and one or more enzymes capable of converting starch present in said first portion of cereal flour into a fermentable sugar, said one or more enzymes being introduced into said composition in a single step;

(b) allowing said enzyme to convert said starch into said fermentable sugar; and
(c) addition of a second portion of cereal flour;
wherein said first portion of cereal flour comprises 5 to 50% by weight of the total weight of said first and second portions of cereal flour; and said water used in step (a) comprises 50% to 100% by weight of the total weight of the water used in said method.

[0021] In a second aspect, a method of preparing a dough composition is provided, said method comprising:
(a) preparing a composition by mixing a first portion of cereal flour, water and an enzyme(s) capable of converting starch present in said first portion of cereal flour into an isomaltooligosaccharide;
(b) allowing said enzyme to convert said starch into said isomaltooligosaccharide; and
(c) adding a second portion of cereal flour; wherein said first portion of cereal flour comprises 5 to 50% by weight of the total weight of said first and second portions of cereal flour; and said water used in step (a) comprises 50% to 100% by weight of the total weight of the water used in said method.

[0022] In a third aspect, a method of preparing a baked product is provided, said method comprising: preparing a dough composition according to the present method; and
baking the dough composition to prepare the baked product.

[0023] In a fourth aspect, a composition is provided that is obtained or obtainable according to the present method.

[0024] In a fifth aspect, a baked product is provided that is obtained or obtainable according to the present method. Typically, such a baked product can be prepared without added sugars.

[0025] In a sixth aspect, a use is provided for the present methods and/or compositions for improving the properties of a dough and/or a baked product prepared from the dough. Examples of such improved properties include no added sugar; improved elasticity; improved texture; improved loaf volume; improved flavour; improved softness; and improved resistance to staling.

[0026] In a seventh aspect, a use related to *in situ* IMO as an anti-staling agent is also provided.

BRIEF DESCRIPTION OF THE BIOLOGICAL SEQUENCES

[0027] The following sequences comply with 37 C.F.R. §§ 1.821-1.825 ("Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures - the Sequence Rules") and are consistent with World Intellectual Property Organization (WIPO) Standard ST.25 (2009) and the sequence listing requirements of the European Patent Convention (EPC) and the Patent Cooperation Treaty (PCT) Rules 5.2 and 49.5(a-bis), and Section 208 and Annex C of the Administrative Instructions. The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. § 1.822.

SEQ ID NO: 1 is the amino acid sequence of an 1,4-α-glucan 6-α-glucosyltransferase from *Aspergillus niger* ("Transglucosidase L") from Amano International.

SEQ ID NO: 2 is the amino acid sequence of an alpha-amylase from *Geobacillus stearothermophilus.*

SEQ ID NO: 3 is the amino acid sequence of a glucoamylase from *Trichoderma reesei*.

SEQ ID NO: 4 is the amino acid sequence of a variant glucoamylase derived from SEQ ID NO: 3.

SEQ ID NO: 5 is the amino acid sequence of an 1,4-α-glucan 6-α-glucosyltransferase from *Aspergillus niger*.

SEQ ID NO: 6 is the amino acid sequence of an alpha-amylase derived from *Geobacillus stearothermophilus*.

SEQ ID NO: 7 is the amino acid sequence of a glucose isomerase from *Streptomyces rubiginosus*.

SEQ ID NO: 8 is the amino acid sequence of a 1,4-α-glucan 6-α-glucosyltransferase from GENBANK® BAA23616.1.

SEQ ID NO: 9 is the amino acid sequence of a 1,4-α-glucan 6-α-glucosyltransferase from GENBANK® BAD06006.1.

SEQ ID NO: 10 is the amino acid sequence of a 1,4-α-glucan 6-α-glucosyltransferase from GENBANK® BAA08125.1.

SEQ ID NO: 11 is the amino acid sequence of a 1,4-α-glucan 6-α-glucosyltransferase from GENBANK® XP_001271891.1.

SEQ ID NO: 12 is the amino acid sequence of a1,4-α-glucan 6-α-glucosyltransferase from GENBANK® XP_001266999.1.

SEQ ID NO: 13 is the amino acid sequence of a 1,4-α-glucan 6-α-glucosyltransferase from GENBANK® XP_751811.1.

SEQ ID NO: 14 is the amino acid sequence of a 1,4-α-glucan 6-α-glucosyltransferase from GENBANK® XP_659621.1.

SEQ ID NO: 15 is the amino acid sequence of a 1,4-α-glucan 6-α-glucosyltransferase from GENBANK® XP_001216899.1.
SEQ ID NO: 16 is the amino acid sequence of a1,4-α-glucan 6-α-glucosyltransferase from GENBANK® XP_001258585.1.

BRIEF DESCRIPTION OF THE FIGURES

[0028] Figure 1 is an ion chromatographic composition of IMO in the baking adjunct of the present invention as produced in Example 3.
[0029] Figure 2 is a chromatogram showing the effect of incubation time on the conversion of wheat flour starch to glucose and fructose under baking conditions.
[0030] Figures 3A to 3C shows the baked bread produced according to the methods of Examples 1-3 together with a control with added sucrose: Fig. 3A showing colour; Fig. 3B showing volume; and Fig. 3C showing open cells.
[0031] Figure 4 is a chromatogram showing the effect of exo-peptidase (SUMIZYME® Pf-G) during incubation of wheat flour with α-amylase (SPEZYME® RSL) and 1,4-α-glucan 6-α-glucosyltransferase (TR-TG) at pH 5.6, 55°C and 5 hours on the production of free amino acids.
[0032] Figure 5 shows a comparison of baked bread with and without added Example 1 with added sugar and Example 7 (without added sugar).

DETAILED DESCRIPTION

[0033] Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton, *et al.,* DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2ND ED., John Wiley and Sons, New York (1994), and Hale & Markham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, NY (1991) provide one of skill with a general dictionary of many of the terms used in this invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

[0034] The invention will now be described in detail by way of reference only using the following definitions and examples. All US patents and US patent application publications, including all sequences disclosed within such patents and publications, referred to herein are expressly incorporated by reference in their entirety. Numeric ranges are inclusive of the numbers defining the range.

The headings provided herein are not limitations of the various aspects or embodiments of the invention, which can be had by reference to the specification as a whole.

Definitions

[0035] As used herein, the term ‘saccharide’ in its broadest sense is intended to cover all saccharides (sugars), including naturally occurring and synthetic and semi-synthetic saccharides. The term encompasses monosaccharides (i.e. saccharides that cannot be hydrolyzed into simpler sugars), disaccharides (i.e. compounds having two monosaccharide units (moieties) joined together by a glycoside bond), oligosaccharides (i.e. compounds having 3 to 10 monosaccharide units joined together by glycoside bonds in a branched or unbranched chain or a ring (optionally having a saccharide side chain) and polysaccharides, i.e. compounds having over 10 monosaccharide units joined together by a glycoside bond in a branched or unbranched chain or a ring (optionally having a saccharide side chain).

[0036] The saccharide may be bonded to other molecules, such as biomolecules, for example peptides / proteins, lipids and nucleic acids. However, it is preferred for the purposes of the present invention that the saccharide is formed from monosaccharide units only.

[0037] In one embodiment, the saccharide is a monosaccharide, i.e. a saccharide that cannot be hydrolyzed into a simpler sugar. The monosaccharide may have the D- or L-configuration, and may be an aldose or ketose.

[0038] In one embodiment, the monosaccharide is a hexose, examples of which include aldohexoses such as glucose, galactose, allose, altrose, mannose, gulose, idose and talose and ketohexoses such as fructose, tagatose, psicose and sorbose. Preferably, the hexose is glucose or galactose.

[0039] In another embodiment, the monosaccharide is a pentose, examples of which include aldopentoses such as ribose, arabinose, xylose and lyxose and ketopentoses such as ribulose and xylulose. Preferably, the pentose is arabinose or xylose.

[0040] In an alternative embodiment, the saccharide is a higher saccharide, i.e. a saccharide comprising more than one monosaccharide moiety joined together by glycoside bonds and which are generally hydrolysable into their constituent monosaccharides. Examples of such higher saccharides include disaccharides (2 monosaccharide moieties),

oligosaccharides (3 to 10 monosaccharide moieties) and polysaccharides (more than 10 monosaccharide moieties). In this regard, the monosaccharide moieties which form the higher saccharide may be the same or different, and may each independently have the D- or L-configuration, and may each independently be aldose or ketose moieties.

[0041] The term "degree of polymerization (DP)" refers to the number (n) of monosaccharide units in a given saccharide. Examples of DP1 are the monosaccharides, such as glucose and fructose. Examples of DP2 are the disaccharides, such as maltose and sucrose. A $DP4^{+}$ (>DP4) denotes polymers with a degree of polymerization of greater than 4.

[0042] The monosaccharide units which form the higher saccharide may have the same or different numbers of carbon atoms. In one embodiment, the monosaccharide moieties of the higher saccharide are hexose moieties, examples of which include aldohexoses such as glucose, galactose, allose, altrose, mannose, gulose, idose and talose and ketohexoses such as fructose, tagatose, psicose and sorbose. Preferably, the hexose moieties of such a higher saccharide are glucose moieties.

[0043] In another embodiment, the monosaccharide moieties of the higher saccharide are aldopentose moieties such as ribose, arabinose, xylose and lyxose and ketopentoses such as ribulose and xylulose. Preferably, the pentose moieties of such a higher saccharide are arabinose or xylose moieties.

[0044] The monosaccharide moieties which form the higher saccharide are joined together by glycosidic bonds. When the monosaccharide moieties are hexose moieties, the glycosidic bonds may be 1,4'- glycosidic bonds (which may be 1,4'-α- or 1,4'-β-glycoside bonds), 1,6'-glycosidic bonds (which may be 1,6'-α- or 1,6'-β-glycoside bonds), 1,2'-glycosidic bonds (which may be 1,2'-α- or 1,2'-β-glycoside bonds), or 1,3'-glycosidic bonds (which may be 1,3'-α- or 1,3'-β-glycoside bonds), or any combination thereof.

[0045] In one embodiment, the higher saccharide comprises 2 monosaccharide units (i.e. a disaccharide). Examples of suitable disaccharides include lactose, maltose, cellobiose, sucrose, trehalose, isomaltulose, and trehalulose.

[0046] In another embodiment, the higher saccharide comprises 3 to 10 monosaccharide units (i.e. an oligosaccharide). The monosaccharide units may be in a chain, which may be branched or unbranched: such oligosaccharides are referred to in this specification as 'chain oligosaccharides'. Examples of such oligosaccharides include maltooligosaccharides (as

defined below) such as maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose, cellobiose, cellotriose, cellotetraose, cellopentaose, cellohexaose, and celloheptaose; as well as fructooligosaccharides (FOS) which consist of short chains of fructose molecules; mannanoligosaccharides, isomaltooligosaccharides, galactooligosaccharides, and xylooligosaccharides.

[0047] In another embodiment, the higher saccharide is a polysaccharide, comprising at least 10 monosaccharide units joined together by glycoside bonds. Typically, such polysaccharides comprise at least: 40, 100, 200, 500, 1000, 5000, 10000, 50000, 100000 or more monosaccharide units.

[0048] In some embodiments, the polysaccharide comprises from 10 to 500000 monosaccharide units. In other embodiments, the polysaccharide comprises from 100 to 1000 monosaccharide units. In other embodiments, the polysaccharide comprises from 1000 to 10000 monosaccharide units. In other embodiments, the polysaccharide comprises from 10000 to 100000 monosaccharide units. In some embodiments, the polysaccharide comprises from 40 to 3000, preferably 200 to 2500, monosaccharide units.

[0049] Examples of such polysaccharides include starch and derivatives thereof (as defined herein; such as cationic or anionic, oxidised or phosphated starch), amylose, amylopectin, glycogen, cellulose or a derivative thereof (such as carboxymethyl cellulose), alginic acid or a salt or derivative thereof, polydextrose, pectin, pullulan, carrageenan, locust bean gum and guar and derivatives thereof (such as cationic or anionic guar).

[0050] As used herein the term “fermentable sugar” refers to mono or di-saccharides capable of undergoing fermentation using a suitable fermentation microorganism, typically yeast. Examples of fermentable sugars include glucose, fructose and maltose.

[0051] As used herein the term "starch" refers to a polysaccharide consisting of a large number of glucose units joined by glycosidic bonds, having the formula $(C_6H_{10}O_5)_x$, wherein x can be any number. Typically, x ranges from 10 to 1,000,000; preferably x is from 50 to 500,000; and even more preferably x ranges from 100 to 100,000.

[0052] Starch consists of two types of molecules: amylose and amylopectin. Amylose typically consists of α-D-glucose units, bound to each other through α (1→4) glycosidic bonds). The chain typically comprises 50 to 50,000, preferably 100 to 10,000, more preferably 300 to 3,000 glucose units. Amylopectin typically consists of glucose units linked

in a linear way with α (1→4) glycosidic bonds and have branching with α (1→6) bonds occurring every 24 to 30 glucose units. The chain typically comprises 1,000 to 1,000,000, preferably 200 to 200,000 glucose units. Typically, the starch contains 15 to 30% amylose and 70 to 85% amylopectin by weight of the total weight of the starch. Preferably, the starch contains 20 to 25% amylose and 75 to 80% amylopectin by weight of the total weight of the starch.

[0053] Starch may be derived from any suitable plant-based material including, but not limited to grains, grasses, tubers and roots; and more specifically wheat, barley, corn, rye, rice, sorghum, legumes, cassava, millet, potato, sweet potato, and tapioca. In one embodiment, the starch is derived from wheat.

[0054] The term "granular starch" refers to uncooked (raw) starch, which has not been subject to gelatinization.

[0055] The term "starch gelatinization" means solubilization of a starch molecule to form a viscous suspension.

[0056] The term "gelatinization temperature" refers to the lowest temperature at which gelatinization of a starch substrate begins. The exact temperature depends upon the specific starch substrate and further may depend on the particular variety of plant species from which the starch is obtained and the growth conditions. Typically, the gelatinization temperature of starch ranges from 40 to 70°C, preferably from 50 to 60°C, and more preferably 55 to 60°C.

[0057] The term “DE” or “dextrose equivalent” is an industry standard for measuring the concentration of total reducing sugars, calculated as D-glucose on a dry weight basis. Un-hydrolyzed granular starch has a DE that is essentially 0 and D-glucose has a DE of 100.

[0058] The term “starch substrate” refers to granular starch or liquefied starch using refined starch, whole ground grains or fractionated grains.

[0059] The term “liquefied starch” refers to starch which has gone through solubilization process using starch liquefaction process.

[0060] The term "total sugar content" refers to the total sugar content present in a starch composition.

[0061] The term "ds" refers to dissolved solids in a solution.

[0062] The term "starch-liquefying enzyme" refers to an enzyme that affects the hydrolysis or breakdown of granular starch. Exemplary starch liquefying enzymes include α-amylases (E.C. 3.2.1.1).

[0063] The term "hydrolysis of starch" refers to the cleavage of glycosidic bonds between the glucose moieties of starch, with water molecule being added across the cleaved glycosidic bond.

[0064] The term "maltooligosaccharide" means a disaccharide or oligosaccharide consisting of short chains of glucose units (typically 2 to 10, preferably 2 to 5 glucose units) bound to each other through α (1→4) glycosidic bonds. Examples of maltooligosaccharides include maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose. The term "maltooligosaccharide" refers to the reaction products derived from the hydrolysis of starch substrate.

[0065] The term "isomaltooligosaccharide" or "IMO" means a disaccharide or oligosaccharide consisting of short chains of glucose units (typically 3 to 10, preferably 3 to 5 glucose units) bound to each other through alpha α (1→6) glycosidic bonds from the non-reducing ends. Isomaltooligosaccharides are glucose oligomers with α-D-(1,6)-linkages. Examples of isomaltooligosaccharides may include isomaltose, panose, isomaltotriose, isomaltotetraose, isomaltopentaose, nigerose, kojibiose, and higher branched oligosaccharides.

[0066] The term "contacting" is generally understood to refer to the placing of the respective enzymes in sufficiently close proximity to the respective substrate to enable the enzymes to convert the substrate to the end product. For example, those skilled in the art will recognize that mixing solutions of the enzyme with the respective substrates can effect contacting.

[0067] The term "improved property" is defined herein as any property of a dough and/or a product obtained from the dough, particularly a baked product. In one embodiment, the improved property is increased strength of the dough. In one embodiment, the improved property is increased elasticity of the dough. In one embodiment, the improved property is reduced stickiness of the dough. In one embodiment, the improved property is increased extensibility of the dough. In one embodiment, the improved property is improved machinability of the dough. In one embodiment, the improved property is increased volume

of the baked product. In one embodiment, the improved property is improved crumb structure of the baked product. In one embodiment, the improved property is improved anti-staling of the baked product. In one embodiment, the improved property is improved softness of the baked product. In one embodiment, the improved property is improved flavour of the baked product.

[0068] The term "*in situ* production" refers to production of the desired product (for example, a fermentable sugar or isomaltooligosaccharide) during the specified process, thereby eliminating the requirement for external addition of this product. In one embodiment, the term "*in situ* production" means that the required product is produced in step (a) and/or step (b) of the method of either aspect of the present invention, *i.e.* the product is prepared when the first portion of cereal flour, water and one or more enzymes are mixed and allowed to act so as to convert the starch into the desired product (such as a fermentable sugar or isomaltooligosaccharide), but before the addition of the second portion of cereal flour and the subsequent steps to produce the dough, and any subsequent step to produce a baked product prepared from the dough. In one embodiment, the term "*in situ* production" means that the required product is produced in a baking step subsequent to the steps of the method of the present invention. In one embodiment, the term "*in situ* production" means that the required product is produced partially in step (a) and/or step (b) of the method of either aspect of the present invention, and partially in a baking step subsequent to the steps of the method of the present invention.

[0069] As used herein, the term "baking adjunct" in its broadest sense refers to enzymatically produced syrups from grain or cereals containing fermentable sugars and/or isomaltooligosaccharides that are used in a subsequent baking process. In one embodiment, the term "baking adjunct" means that the required product is produced in step (a) and/or step (b) of the method of either aspect of the present invention, *i.e.* the intermediate composition prepared from the first portion of cereal flour, water and one or more enzymes, and the enzymes are allowed to act so as to convert the starch into the desired product (for example, a fermentable sugar or isomaltooligosaccharide). The baking adjunct is then added to the second portion of cereal flour used in the subsequent steps to produce the dough, and any subsequent step to produce a baked product prepared from the dough.

[0070] The term "effective amount" is defined herein as an amount of *in situ* production of fermentable sugars or branched oligosaccharides that is sufficient for proving a measurable effect on at least one property of interest of the dough and /or baked product.

Methods

[0071] The present inventors have identified that by *in situ* formation of fermentable sugars and/or IMO from wheat flour starch, it is possible to eliminate the need for addition of sugars (especially sucrose) to dough.

[0072] In one aspect, a method of preparing a dough composition is provided, said method comprising: (a) preparing a composition by mixing a first portion of cereal flour, water and one or more enzymes capable of converting starch present in said first portion of cereal flour into a fermentable sugar, said one or more enzymes being introduced into said composition in a single step; (b) allowing said enzyme to convert said starch into said fermentable sugar; and (c) addition of a second portion of cereal flour; wherein said first portion of cereal flour comprises 5 to 50% by weight of the total weight of said first and second portions of cereal flour; and said water used in step (a) comprises 50% to 100% by weight of the total weight of the water used in said method.

[0073] The method according confers the particular advantage that fermentable sugars (such as maltose, glucose or fructose) can be produced *in situ* by the action of the enzyme on the starch present in the wheat flour. This therefore avoids the requirement to add sucrose or other sugars to the composition.

[0074] In contrast to the process described in EP154135A, the present method requires the introduction of the one or more starch hydrolysing enzymes (particularly although not exclusively amylase, such as an alpha or beta amylase, and a glucoamylase) into the initial composition comprising water and the first portion of cereal flour in a single step. This avoids the requirement to heat the mixture to temperatures above wheat starch gelatinization temperature (i.e. around 60°C), thereby avoiding gelatinization of the starch, deactivating the wheat endogenous enzymes, and denaturing the wheat gluten. It also enables the processing time to be reduced from over 10 hours to less than 4 hours.

[0075] In another aspect, a method of preparing a dough composition is provided, said method comprising: (a) preparing a composition by mixing a first portion of cereal flour, water and an enzyme(s) capable of converting starch present in said first portion of cereal flour into an isomaltooligosaccharide; (b) allowing said enzyme to convert said starch into said isomaltooligosaccharide; and (c) adding a second portion of cereal flour; wherein said first portion of cereal flour comprises 5 to 50% by weight of the total weight of said first

and second portions of cereal flour; and said water used in step (a) comprises 50% to 100% by weight of the total weight of the water used in said method.

[0076] The isomaltooligosaccharides (IMO) can be produced *in situ* production of IMO by the hydrolytic action of an enzymes on the starch in wheat flour, typically to produce maltose or maltooligosaccharides; followed by action of an enzyme (typically a 1,4-α-glucan 6-α-glucosyltransferase) on the maltose or maltooligosaccharides to produce IMO. The IMO produced *in situ* can be used as a sweetening and/or softening agent in bread, thereby allowing sucrose to be removed from the formulation.

[0077] A dough composition and baked products prepared from the composition is provided, comprising an effective amount of functional oligosaccharides (in particular, containing a higher level of α-1,6 branched oligosaccharides, for example isomaltooligosaccharides, than was possible in the prior art) can be produced *in situ* under baking processing conditions.

[0078] In contrast to the method described in US 4,859,474, the present method allows the production of fructose as well as *in situ* IMO from a hydrolysate made from wheat flour, water, amylase, glucoamylase, and/or 1,4-α-glucan 6-α-glucosyltransferase.

[0079] The present methods involve a single step for hydrolyzing starch and, in one aspect, isomerisation of the hydrolysis products. The hydrolysis and/or isomerised products are used as baking adjuncts in the subsequent steps.

[0080] The starch used in the present methods may be derived from any suitable plant product. In a preferred embodiment, the starch is derived from a cereal crop, typical examples of which include wheat, barley, oats, rye and maize. In one embodiment the starch is derived from wheat. In a preferred embodiment, the starch is granular wheat starch.

[0081] Typically, the starch used as a starting material is insoluble in the aqueous suspension of cereal flour and water to which the enzyme is added. In a preferred embodiment, the starch is insoluble granular wheat starch.

[0082] In one embodiment, the present method is carried out *in situ* in a food composition. In one embodiment, the present method is carried out *in situ* in a baking composition.

[0083] In one embodiment, the present method uses enzymes which solubilize and hydrolyze the starch (such as wheat granular starch) are endogenous to wheat. Typically, the enzymes comprise α-amylase, β-amylase or mixtures thereof.

[0084] Step (a) of both aspects of the present method involves addition of water and the required enzyme to a first portion of cereal flour. The first portion of cereal flour comprises only a minor part of the total cereal flour used in the baking process as a whole. The proportion of the first cereal flour introduced at step (a) is expressed by weight of the total weight of cereal flour, i.e. the total of the first portion, introduced in step (a) and the second portion, introduced in step (c).

[0085] Typically, the first portion of cereal flour introduced at step (a) comprises 5 to 50% by weight of the total weight of said first and second portions of cereal flour. Preferably, the first portion of cereal flour introduced at step (a) comprises 10 to 40% by weight of the total weight of the first and second portions of cereal flour. More preferably, the first portion of cereal flour introduced at step (a) comprises 20 to 30% by weight of the total weight of the first and second portions of cereal flour.

[0086] Step (a) of both aspects of the present method involves addition of water and the required enzyme to a first portion of cereal flour. In this step the water used in step (a) comprises the major part of the total water used in the overall method, i.e. the method of each aspect of the invention comprising steps (a), (b) and (c). Typically the water used in step (a) comprises 50% to 100% by weight of the total weight of the water used in the overall method. Preferably, the water used in step (a) comprises 80% to 100% by weight of the total weight of the water used in the overall method. More preferably, the water used in step (a) comprises 90% to 100% by weight of the total weight of the water used in the overall method. Even more preferably, the water used in step (a) comprises 95% to 100% by weight of the total weight of the water used in the overall method. Most preferably, the water used in step (a) comprises 100% by weight of the total weight of the water used in the overall method.

[0087] The total amount of water used in the present method is a conventional amount used in the production of dough compositions. However, the addition in the first step of the method of only a minor portion of the flour, but the major part of the water, wherein the enzymes are added in a single step, has not previously been disclosed in the art.

[0088] Typically, the total amount of water used in the present method ranges from 20 to 100 grams of water per 100 grams of cereal flour. Preferably, the total amount of water used in the method of the invention ranges from 40 to 80 grams of water per 100 grams of cereal flour. More preferably, the total amount of water used in the method of the invention ranges from 50 to 70 grams of water per 100 grams of cereal flour.

[0089] When the flour is derived from wheat, typically, the total amount of water used ranges from 20 to 100 grams of water per 100 grams of wheat of the total used for making dough. Preferably, the total amount of water used ranges from 40 to 80 grams of water per 100 grams of wheat. More preferably, the total amount of water used ranges from 50 to 70 grams of water per 100 grams of wheat.

[0090] In one embodiment, the method comprises heating the composition. Typically the heating step is comprised in step (b) and this typically comprises heating the composition prepared in step (a). Heating typically increases the rate at which the enzyme(s) are of carrying out its hydrolytic activity and, where required, the conversion of maltooligosaccharides to IMO.

[0091] In one embodiment, the method is carried out below or at wheat starch gelatinization temperature. Typically, the method is carried out at a temperature below 60°C, such as below 58°C. In one embodiment, the method is carried out at a temperature of 40°C to 60°C, such as 50 to 58°C.

[0092] In one embodiment, the present method, typically step (b) thereof, comprises heating the composition for 1 to 24 hours. In one embodiment, the present method, typically step (b) thereof, comprises heating the composition for 2 to 6 hours.

[0093] The present method (particularly steps (a) and (b) thereof) enables the *in situ* production of fermentable sugars by incubation of the enzyme with a cereal flour, especially wheat flour, in water. When the fermentable sugar comprises glucose, the desired sugar is typically produced by the action of glucoamylase (gamma amylase) on the maltooligosaccharides produced by the action of amylase on the starting starch. When the fermentable sugar comprises fructose, the desired sugar is typically produced by the further action of glucose isomerase to convert glucose into fructose.

[0094] The *in situ* preparation of sugars or softening agent according to the method confers improvements on the properties of the dough composition produced thereby and/or baked products prepared from the dough.

[0095] In step c), the composition produced in steps a) and b), containing the fermentable sugars and/or IMO, is then mixed with the second portion of cereal flour and, if required, any remaining water to produce the dough composition. Typically, the second portion of cereal flour introduced at step (c) comprises 50 to 95% by weight of the total weight of said first and second portions of cereal flour. Preferably, the second portion of cereal flour introduced at step (a) comprises 60 to 90% by weight of the total weight of the first and second portions of cereal flour. More preferably, the second portion of cereal flour introduced at step (c) comprises 70 to 80% by weight of the total weight of the first and second portions of cereal flour.

[0096] In one embodiment, the method further comprises shaping the dough composition.

[0097] In one embodiment, the method further comprises baking the dough composition, particularly the shaped dough composition to form a baked product. Baking is carried out by conventional baking processes well known to those skilled in the art. Examples of baked products are set out below.

[0098] In one embodiment, step (c) of the method is carried out *in situ* in a food composition. In one embodiment, step (c) of the method is carried out *in situ* in a baking composition.

[0099] In one embodiment, the starch is hydrolyzed below or at wheat starch gelatinization temperature. Typically, the starch is hydrolyzed at a temperature below 60°C, such as below 58°C. In one embodiment, the starch is hydrolyzed at a temperature of 40°C to 60°C, such as 50 to 58°C.

[0100] In a preferred embodiment, the method comprises the steps of:

a) production of an aqueous slurry containing the first portion of cereal flour and 100% by weight of the total weight of the water used in the overall method;

a1) adding enzymes capable of converting starch into glucose, e.g. alpha- or beta-amylase and gamma-amylase (glucoamylase) in addition to any wheat endogenous starch hydrolyzing enzymes present in the mixture;

b) heating the mixture to below or at starch gelatinization temperature, preferably 55 to 60°C, for 1 to 24 hours, preferably less than 4 hours, such that hydrolysis of the starch to glucose occurs; and

c) mixing the product containing the starch hydrolysate with the second portion of cereal flour to produce a dough for baking.

[0101] In the above process, any subsequent baking step is typically carried out as per conventional baking process.

[0102] In an especially preferred embodiment of the first aspect of the present method, the cereal flour is wheat flour comprising wheat granular starch and the method comprises the steps of:

a) making an aqueous slurry containing a first portion of the wheat flour (preferably less than 30% of the total amount of the first and second portions of wheat flour);

a1) adding 100% by weight of the total weight of the water (preferably, 60 grams of water per 100 grams of wheat) used in the overall process;

a2) adding the enzymes capable of converting wheat granular starch into high glucose syrup (typically amylase and glucoamylase, in addition to wheat endogenous starch hydrolyzing enzymes);

b) heating to below or at wheat starch gelatinization temperature, such as 55-60°C, for 1 to 24 hours, preferably less than 4 hours such that hydrolysis of the starch to glucose occurs; and

c) mixing the wheat hydrolysate with the rest of the flour to produce a dough for baking.

[0103] In the above process, any subsequent baking step is typically carried out as per conventional baking process.

[0104] In a preferred embodiment of the second aspect of the present method, the method comprises the steps of:

a) formation of an aqueous slurry containing the first portion of cereal flour and 100% by weight of the total weight of the water used in the overall method;

a1) adding enzymes capable of converting starch into isomaltooligosaccharides (e.g. an amylase and a 1,4-α-glucan 6-α-glucosyltransferase) in addition to any wheat endogenous starch hydrolyzing enzymes present in the mixture;

b) heating the mixture to below or at starch gelatinization temperature, preferably 55 to 60°C, for 1 to 24 hours, preferably less than 4 hours, such that hydrolysis of the starch and isomerisation of any maltooligosaccharides to IMO occurs; and

c) mixing the product containing the IMO with the second portion of cereal flour to produce a dough for baking.

[0105] In the above process, any subsequent baking step is typically carried out as per conventional baking process.

[0106] In an especially preferred embodiment of the second aspect of the present method, the cereal flour is wheat flour comprising wheat starch and the method comprises the steps of:
a) making an aqueous slurry containing a first portion of the wheat flour (preferably less than 30% of the total amount of the first and second portions of wheat flour) and 100% by weight of the total weight of the water (preferably, 60 grams of water per 100 grams of wheat) used in the overall process;
a1) adding the enzymes capable of converting wheat granular starch into a syrup containing isomaltooligosaccharides, in addition to wheat endogenous starch hydrolyzing enzymes);
b) heating to below or at wheat starch gelatinization temperature, such as 55-60°C, for 1 to 24 hours, preferably less than 4 hours such that hydrolysis of the starch to maltooligosaccharides and the isomerisation of the maltooligosaccharides to IMO occurs; and
c) mixing the wheat hydrolysate containing the IMO with the rest of the flour to produce a dough for baking.

[0107] In the above process, any subsequent baking step is typically carried out as per conventional baking process.

Enzymes

[0108] The present methods involve the use of one or more enzymes to carry out the hydrolysis of the starch used as a starting material and, if necessary, the isomerisation of glucose to fructose or the isomerisation of the maltooligosaccharides to IMO. The enzymes used to carry out these functions are all well known to those skilled in the art.

[0109] In one embodiment, the enzyme or enzymes used in the process are endogenous to the cereal flour. In one embodiment, the enzyme or enzymes used in the process are added to the cereal flour. In one embodiment, the enzyme or enzymes used in the process comprise a mixture of an enzyme or enzymes endogenous to the cereal flour and an enzyme or enzymes added to the flour. In one embodiment, the enzyme or enzymes used

in the process comprise a mixture of up to 50% (by weight of the total weight of enzymes used) of an enzyme or enzymes endogenous to the cereal flour and more than 50% (by weight of the total weight of enzymes used) of an enzyme or enzymes added to the flour. In one embodiment, the enzyme or enzymes used in the process comprise a mixture of up to 25% (by weight of the total weight of enzymes used) of an enzyme or enzymes endogenous to the cereal flour and more than 75% (by weight of the total weight of enzymes used) of an enzyme or enzymes added to the flour. In one embodiment, the enzyme or enzymes used in the process comprise a mixture of more than 50% (by weight of the total weight of enzymes used) of an enzyme or enzymes endogenous to the cereal flour and up to 50% (by weight of the total weight of enzymes used) of an enzyme or enzymes added to the flour. In one embodiment, the enzyme or enzymes used in the process comprise a mixture of more than 75% (by weight of the total weight of enzymes used) of an enzyme or enzymes endogenous to the cereal flour and up to 25% (by weight of the total weight of enzymes used) of an enzyme or enzymes added to the flour.

[0110] In one embodiment of the method of the first aspect of the present invention, the one or more enzymes comprise an enzyme having granular starch hydrolysing activity (GSHE) (as defined below) or a mixture thereof. Typically, the GSHE is an amylase.

[0111] In the first aspect of the present method, the one or more enzymes is selected from the group consisting of an alpha amylase (E.C. 3.2.1.1), a beta amylase (E.C. 3.2.1.2) and a gamma amylase (glucoamylase; E.C. 3.2.1.3), or a mixture of any thereof.

[0112] In this embodiment, preferably said one or more enzymes comprises a mixture of an alpha amylase (E.C. 3.2.1.1) and a gamma amylase (glucoamylase; E.C. 3.2.1.3).

[0113] In a preferred embodiment, said one or more enzymes additionally comprises a glucose isomerase (E.C:5.3.1.5).

[0114] In the second aspect of the present method, preferably said one or more enzymes comprises a 1,4-α-glucan 6-α-glucosyltransferase (E.C. 2.4.1.24). Typically, said 1,4-α-glucan 6-α-glucosyltransferase enzyme catalyses the transfer of a glucose moiety from a malto-oligosaccharide to the 6-OH position of another saccharide.

[0115] In one embodiment, the one or more enzymes comprise a further enzyme having granular starch hydrolysing activity (GSHE) (as defined below) or a mixture thereof. Typically, the GSHE is an amylase.

[0116] In this aspect, preferably the further enzyme selected from the group consisting of an alpha amylase (E.C. 3.2.1.1), a beta amylase (E.C. 3.2.1.2) and a gamma amylase (glucoamylase; E.C. 3.2.1.3), or a mixture of any thereof.

[0117] In one embodiment, the enzyme used is of bacterial origin. In one embodiment, the enzyme used is of fungal origin. In one embodiment, the enzyme used is of animal origin. In one embodiment, the enzyme used is of plant origin.

[0118] In one embodiment, the enzyme used is a recombinant enzyme.

[0119] As understood by those in the art, the quantity of enzyme used in the methods of the present invention will depend on the nature and activity of the enzyme, the nature and amount of the cereal flour, and the desired reaction. Typically, the enzyme is used in an amount of about 1 g to 10 kg of the enzyme per metric ton of the first portion of the cereal flour used in step (a) of the process of the invention (calculated on a dry solids basis, i.e. excluding the weight of the water used in this step). In some embodiments the enzyme is used in an amount about 10 g to 5.0 kg per metric ton of the first portion of the cereal flour. In some embodiments the enzyme is used in an amount about of about 0.5 kg to 2.0 kg per metric ton of the first portion of the cereal flour. In other embodiments, the enzyme is used in an amount of about 0.1 kg to 1.0 kg per metric ton of the first portion of the cereal flour.

[0120] The enzyme used in the present invention may have side activities in addition to their primary activity as defined below. In some embodiments the primary activity of the enzyme comprises more than 50% of the total activity of the enzyme. In some embodiments the primary activity of the enzyme comprises more than 75% of the total activity of the enzyme. In some embodiments the primary activity of the enzyme comprises more than 90% of the total activity of the enzyme. In some embodiments the primary activity of the enzyme comprises more than 95% of the total activity of the enzyme. In some embodiments the primary activity of the enzyme comprises more than 97% of the total activity of the enzyme. In some embodiments the primary activity of the enzyme comprises more than 98% of the total activity of the enzyme. In some embodiments the primary activity of the enzyme comprises more than 99% of the total activity of the enzyme. In some embodiments the primary activity of the enzyme comprises 100% of the total activity of the enzyme.

[0121] The various enzymes used are defined generally and specifically below.

Enzymes Having Granular Starch Hydrolyzing Activity (GSHEs)

[0122] In one embodiment, the enzyme used is an enzyme having granular starch hydrolyzing activity. As used herein, the term “enzyme having granular starch hydrolyzing activity” or “GSHE” or “GSH” means an enzyme capable of catalysing the hydrolysis of granular starch.

[0123] In one embodiment, the enzyme having GSH activity is an amylase (as defined below).

[0124] In one embodiment, the enzyme having GSH activity is an enzyme having glucoamylase activity (as defined below). In one embodiment, the enzyme having GSH activity is an enzyme having alpha-amylase activity (as defined below). In one embodiment, the enzyme having GSH activity is an enzyme having both glucoamylase activity and alpha-amylase activity. In one embodiment, the enzyme having GSH activity is a mixture of enzymes, at least one enzyme having both glucoamylase activity and at lease one, different enzyme having alpha-amylase activity.
In one embodiment, the enzyme having GSH activity is of fungal origin. In one embodiment, the enzyme having GSH activity is of bacterial origin. In one embodiment, the enzyme having GSH activity is of plant origin. These enzymes have been recovered from fungal, bacterial and plant cells such as *Bacillus sp.*, *Penicillium sp.*, *Humicola sp.*, *Trichoderma sp. Aspergillus sp. Mucor sp.* and *Rhizopus sp.*

[0125] In some embodiments, a particular group of enzymes having GSH activity include enzymes having glucoamylase activity and/or alpha-amylase activity (Tosi *et al*, *Can. J. Microbiol.* (1993) 39: 846 –855). A *Rhizopus oryzae* GSHE has been described in Ashikari *et al.*, *Agric. Biol. Chem.,* (1986) 50: 957-964 and US patent 4,863,864. A *Humicola grisea* GSHE has been described in Allison *et al.*, *Curr. Genet.* (1992) 21: 225-229; International patent application publication no. WO 05/052148, and European patent application publication no. EP 171218A. An *Aspergillus awamori var. kawachi* GSHE has been described by Hayashida *et al.*, *Agric. Biol. Chem.* (1989) 53: 923-929. An *Aspergillus shirousami* GSHE has been described by Shibuya *et al.*, *Agric. Biol. Chem.* (1990) 54: 1905-1914.

[0126] In some embodiments, a GSHE may have glucoamylase activity and is derived from a strain of Humicola *grisea*, particularly a strain of *Humicola grisea var. thermoidea* (see US patent 4,618,579). In some preferred embodiments, the *Humicola* enzyme having GSH

activity will have at least 85%, 90%, 92%, 94%, 95%, 96%, 97%, 98% OR 99% sequence identity to the amino acid sequence of SEQ ID NO: 3 of WO 2005/052148.

[0127] In other embodiments, a GSHE may have glucoamylase activity and is derived from a strain of *Aspergillus awamori*, particularly a strain of *A. awamori var. kawachi*. In some preferred embodiments, the *A. awamori var. kawachi* enzyme having GSH activity will have at least 85%, 90%, 92%, 94%, 95%, 96%, 97%, 98% and 99% sequence identity to the amino acid sequence of SEQ ID NO: 6 of WO 2005/052148.

[0128] In other embodiments, a GSHE may have glucoamylase activity and is derived from a strain of *Rhizopus*, such as *R. niveus* or *R. oryzae*. The enzyme derived from the Koji strain *R. niveus* is sold under the trade name “CU CONC” or the enzyme from *Rhizopus* sold under the trade name GLUZYME® (Novozymes A/S).

[0129] In other embodiments, the GSHE having glucoamylase activity is SPIRIZYME™ Plus (Novozymes A/S), which also includes acid fungal amylase activity.

[0130] In other embodiments, a GSHE may have alpha-amylase activity and is derived from a strain of *Aspergillus* such as a strain of *A. awamori*, *A. niger*, *A. oryzae*, or *A. kawachi* and particularly a strain of *A. kawachi*. In some preferred embodiments, the *A. kawachi* enzyme having GSHE activity will have at least 85%, 90%, 92%, 94%, 95%, 96%, 97%, 98% and 99% sequence identity to the amino acid sequence of SEQ ID NO: 3 of WO 2005/118800 and WO 2005/003311.

[0131] In some embodiments, the enzyme having amylase activity or GSH activity is a hybrid enzyme, for example one containing a catalytic domain of an alpha amylase such as a catalytic domain of an *Aspergillus niger* alpha amylase, an *Aspergillus oryzae* alpha amylase or an *Aspergillus kawachi* alpha amylase and a starch binding domain of a different fungal alpha amylase or glucoamylase, such as an *Aspergillus kawachi* or a *Humicola grisea* starch binding domain. In other embodiments, the hybrid enzyme having GSH activity may include a catalytic domain of a glucoamylase, such as a catalytic domain of an *Aspergillus sp.*, a *Talaromyces sp.*, an *Althea sp.*, a *Trichoderma sp.* or a *Rhizopus sp.* and a starch binding domain of a different glucoamylase or an alpha amylase. Some hybrid enzymes having GSH activity are disclosed in WO 2005/003311, WO 2005/045018; Shibuya *et al.*, *Biosci. Biotech. Biochem.* (1992) 56*:* 1674-1675, and Cornett *et al.*, *Protein Engineering* (2003) 16: 521.

[0132] As understood by those in the art, the quantity of the enzyme having GSH activity used in the methods of the present invention will depend on nature and the activity of the enzyme having GSH activity, the nature and amount of the cereal flour, and the desired reaction. When the enzyme having GSH activity is a glucoamylase, in general, the enzyme is used in an amount of about 0.01 Glucoamylase Activity Units (GAU) to 10.0 GAU per gram of the first portion of the cereal flour used in step (a) of the processes of the invention (calculated on a dry solids basis, *i.e.* excluding the water used in this step). In some embodiments, the glucoamylase is used in an amount about 0.5 to 5.0 GAU per gram dry solids of the first portion of the cereal flour. In some embodiments, the glucoamylase is used in an amount of about 1.0 to 2.0 GAU per gram dry solids of the first portion of the cereal flour. The units of glucoamylase enzyme activity can be calculated according to the assay methods set out below.

Amylases

[0133] In some of the embodiments, the enzyme used is an amylase. The term "amylase" refers generally to an enzyme that catalyzes the hydrolysis of starches. All amylases are glycoside hydrolases and act on α-1,4-glycosidic bonds.

[0134] In some embodiments, the amylase is an amylase selected from the group consisting of an alpha amylase (E.C. 3.2.1.1), a beta amylase (E.C. 3.2.1.2) and a gamma amylase or glucoamylase (E.C. 3.2.1.3). In some embodiments, the amylase is an alpha amylase (E.C. 3.2.1.1). In some embodiments, the amylase is a beta amylase (E.C. 3.2.1.2). In some embodiments, the amylase is a gamma amylase or glucoamylase (E.C. 3.2.1.3).

[0135] In one embodiment, the amylase is an alpha-amylase. Alpha amylases are classified in E.C. 3.2.1.1. The term "alpha-amylase" refers to enzymes that catalyze the hydrolysis of α-1,4-glucosidic linkages in polysaccharides, particularly starch. Alternative names for this enzyme include 1,4-α-D-glucan glucanohydrolase or glycogenase. Alpha-amylases can act at random locations along the starch chain, in order to break down the glucose chain, typically yielding: maltotriose and maltose from amylose; or maltose, glucose and "limit dextrin" from amylopectin.

[0136] In some embodiments, the alpha amylase is of microbial origin. In some embodiments, the alpha amylase is of bacterial origin.

[0137] In some embodiments, the alpha amylase is a thermostable bacterial alpha amylase. Suitable alpha amylases may be naturally occurring as well as recombinant and mutant alpha amylases.

[0138] In particularly preferred embodiments, the alpha amylase is derived from a bacterium of the genus *Bacillus*. Preferred *Bacillus* species include *B. subtilis*, *B. stearothermophilus*, *B. lentus*, *B. licheniformis*, *B. coagulans*, and *B. amyloliquefaciens* (disclosed in United States patent Nos. US 5,763,385; US 5,824,532; US 5,958,739; US 6,008,026; and US 6,361,809, respectively).

[0139] Particularly preferred alpha amylases are derived from *Bacillus* species selected from the group consisting of *B. stearothermophilus*, *B. amyloliquefaciens*, and *B. licheniformis*.

[0140] In one embodiment, the enzyme is derived from the strains having the accession number ATCC 39709; ATCC 11945; ATCC 6598; ATCC 6634; ATCC 8480; ATCC 9945A, and NCIB 8059.

[0141] Commercially available alpha amylases contemplated for use in the methods of the invention include; SPEZYME™ AA; SPEZYME™ FRED; GZYME™ G997 (Genencor International Inc.) and TERMAMYL™ 120-L, LC, SC and SUPRA (Novozymes).

[0142] As understood by those in the art, the quantity of alpha amylase used in the methods of the present invention will depend on nature and activity of the alpha amylase enzyme, the nature and amount of the cereal flour, and the desired reaction. In general, an amount of about 0.01 to 5.0 kg of the alpha amylase is used per metric ton of the first portion of the cereal flour used in step (a) of the process of the invention (calculated on a dry solids basis, i.e. excluding the weight of the water used in this step). In some embodiments, the alpha amylase is used in an amount about 0.5 to 2.0 kg per metric ton of the first portion of the cereal flour. In some embodiments, the alpha amylase is used in an amount of about 0.1 to 1.0 kg per metric ton of the first portion of the cereal flour.

[0143] Typically, the alpha amylase enzyme is used in an amount of about 0.01 units of alpha amylase activity (AAU) to 10 AAU per g of the first portion of the cereal flour used in step (a) of the process of the invention (calculated on a dry solids basis, i.e. excluding the weight of the water used in this step). In some embodiments, the enzyme is used in an amount about 0.1 to 2 AAU per g dry solids of the first portion of the cereal flour. In some embodiments, the enzyme is used in an amount of about 0.4 to 0.5 AAU per g dry solids of

the first portion of the cereal flour. The units of alpha amylase enzyme activity can be calculated according to the assay methods set out below.

[0144] In particular embodiments, other quantities are utilized. For example, when the enzyme is GZYME™ 997 or SPEZYME™ FRED (DuPont Industrial Bioscience) generally an amount of between about 0.01 to 1.0 kg of GZYME™ 997 or SPEZYME™ FRED is added per metric ton of starch. In other embodiments, the enzyme is added in an amount between about 0.05 to 1.0 kg; between about 0.1 to 0.6 kg; between about 0.2 to 0.6 kg and between about 0.4 to 0.6 kg of GZYME™ 997 and SPEZYME™ FRED per metric ton of starch.

[0145] In some embodiments, the alpha amylase is of fungal origin. Alpha amylases (1,4-α-D-glucan glucanohydrolase, E.C. 3.2.1.1) of fungal origin are known in the art.

[0146] In preferred embodiments, the alpha amylase is derived from a fungus of the genus *Aspergillus* or *Rhizopus*. In particularly preferred embodiments, the alpha amylase is derived from a fungus of the species *Aspergillus niger, Aspergillus fumigatus, Aspergillus kawachi, Aspergillus awamori, Aspergillus clavatus, Aspergillus oryzae* or *Rhizopus oryzae.* Fungal alpha amylase from *Aspergillus oryzae* has been extensively used in the manufacture of high maltose syrup using enzyme liquefied starch substrate. Examples of such alpha-amylases include CLARASE® from DuPont Industrial Bioscience and Fungamyl® from Novozymes.

Maltogenic enzyme

[0147] In another embodiment, the enzyme is a maltogenic enzyme. As used herein, the term “maltogenic enzymes” refers to an enzyme or mixture of enzymes capable of producing maltose, maltotriose and/or maltotetraose by hydrolysis of starch substrate, preferably in an amount of greater than 20% by weight of maltose, maltotriose and maltotetraose (the amount by weight being measured as the total weight of maltose, maltotriose and maltotetraose produced relative to the total weight of all sugars produced), more preferably during their optimum conditions of pH and temperature.

In one embodiment, the maltogenic enzyme is an amylase (as defined above).
[0148] In one embodiment, the maltogenic enzyme is an alpha amylase (E.C. 3.2.1.1). In one embodiment, the maltogenic enzyme is a beta amylase (E.C. 3.2.1.2). In one embodiment, the maltogenic enzyme is a mixture of an alpha amylase and a beta amylase.

[0149] In one embodiment, the maltogenic enzyme may be a high DP2 forming enzyme, such as a fungal alpha amylase or a plant and microbial beta amylase (e.g. derived from barley, soya bean or wheat). In one embodiment, the maltogenic enzyme may be a high DP 3 forming enzyme, examples of which are of bacterial origin (e.g. derived from *Mycobacterium, Chloroflexus aurantiacus*). In one embodiment, the maltogenic enzyme may be a high DP4 forming enzyme, examples of which are of bacterial origin and include those derived from *Pseudomonas* species (OPTIMALT™ 4G; DuPont Industrial Biosciences).

[0150] As understood by those in the art, the quantity of maltogenic enzyme used in the present methods will depend on nature and activity of the maltogenic enzyme, the nature and amount of the cereal flour, and the desired reaction. In general, an amount of enzyme added to the first portion of the wheat flour resulting in greater than 20% by weight of maltose, maltotriose, and maltotetraose; the amount by weight being measured as the total weight of maltose, maltotriose, and maltotetraose produced relative to the total weight of all sugars produced.

[0151] When the maltogenic enzyme is an alpha amylase, typically, the maltogenic enzyme is added to the first portion of the wheat flour in an amount of about resulting in greater than 20% by weight of maltose, maltotriose and maltotetraose. The units of alpha-amylase enzyme activity can be calculated according to the assay methods set out below.

Glucoamylase

[0152] In one embodiment, the amylase is a glucoamylase (E.C. 3.2.1.3). Glucoamylases, also known as gamma amylases, γ-amylases, 1,4-α-glucosidases or 1,4-α-D-glucan glucohydrolases, are enzymes that remove successive glucose units from the non-reducing ends of starch. The enzyme can hydrolyse both linear and branched glycosidic linkages of starch, both the amylose and amylopectin portions.

[0153] In some embodiments, the glucoamylase is of plant origin. In some embodiments, the glucoamylase is of bacterial origin. In some embodiments, the glucoamylase is of fungal origin.

[0154] Preferred glucoamylases used in the present methods are derived from fungal strains. In preferred embodiments, the glucoamylase is derived from a fungus of the genus *Aspergillus*, *Rhizopus*, *Humicola* or *Mucor*. In a preferred embodiment, the glucoamylase is

derived from a fungus of the species *Aspergillus niger*, *Aspergillus awamori*, *Talaromyces,* species and its variants, Rhizopus *niveus, Rhizopus oryzae, Mucor miehei, Humicola grisea, Aspergillus shirousami,* and *Humicola (Thermomyces) lanuginosa.* Examples of such enzymes are disclosed in, Boel *et al.* (1984) *EMBO J.* 3:1097-1102; WO 92/00381; WO 00/04136; Chen *et al*., (1996) *Prot. Eng.* 9:499-505; Taylor *et al*., (1978) *Carbohydrate Res.* 61:301 – 308 and Jensen *et al*., (1988) *Can. J. Microbiol.* 34, 218 – 223).

[0155] Enzymes having glucoamylase activity are commercially available. Examples include that produced from *Aspergillus niger* (OPTIDEX™ L-400 and G-ZYME™ G990 4X from DuPont Industrial Biosciences) or that produced from *Rhizopus* species (CU.CONC™ from Shin Nihon Chemicals, Japan and GLUCZYME™ from Amano Pharmaceuticals, Japan).

[0156] In an alternative embodiment, the glucoamylase enzyme is a recombinant glucoamylase enzyme. Examples include a glucoamylase enzyme obtainable (or obtained) from a *Trichoderma* host, such as those described in WO 2005/052148. In other embodiments, the *Trichoderma* host expresses a heterologous polynucleotide which encodes a *Humicola grisea* glucoamylase enzyme, particularly a *Humicola grisea* var. *thermoidea* glucoamylase enzyme. In other embodiments, the *Trichoderma* expresses a recombinant granular starch hydrolysing enzyme (GSHE) wherein the heterologous polynucleotide encodes a GSHE having at least 50% sequence identity with the sequence of the *Trichoderma* glucoamylase having SEQ ID NO: 3 of WO 2005/052148. This enzyme and its variants are commercially available as DIAZYME® –TGA from DuPont Industrial Biosciences.

[0157] As understood by those in the art, the quantity of glucoamylase used in the methods will depend on nature and activity of the glucoamylase enzyme, the nature and amount of the cereal flour, and the desired reaction. In general, an amount of about 0.025 to 25.0 kg of the glucoamylase is used per metric ton of the first portion of the cereal flour used in step (a) of the process of the invention (calculated on a dry solids basis, i.e. excluding the weight of the water used in this step). In some embodiments, the glucoamylase is used in an amount about 0.5 to 15.0 kg per metric ton of the first portion of the cereal flour. In some embodiments, the glucoamylase is used in an amount of about 5.0 to 12.5 kg per metric ton of the first portion of the cereal flour.

[0158] In general, the glucoamylase enzyme is used in an amount of about 0.01 Glucoamylase Activity Units (GAU) to 10.0 GAU per g of the first portion of the cereal flour

used in step (a) of the process of the invention (calculated on a dry solids basis, i.e. excluding the weight of the water used in this step). In some embodiments, the glucoamylase is used in an amount of about 0.5 to 5.0 GAU per gram dry solids of the first portion of the cereal flour. In some embodiments, the glucoamylase is used in an amount of about 1.0 to 2.0 GAU per gram dry solids of the first portion of the cereal flour. The units of glucoamylase enzyme activity can be calculated according to the assay methods set out below.

1,4-α-glucan 6-α-glucosyltransferase (transglucosidase)

[0159] In some embodiments, the enzyme comprises a 1,4-α-glucan 6-α-glucosyltransferase (transglucosidase, EC 2.4.1.24). A 1,4-α-glucan 6-α-glucosyltransferase (systematic name: 1,4-α-D-glucan: 1,4-α-D-glucan(D-glucose) 6-α-D-glucosyltransferase) catalyzes both hydrolytic and transfer reactions on incubation with α-D-glucooligosaccharides. Transfer of a glucose residue occurs to the primary hydroxyl group (HO-6) of a glucose unit, producing isomaltose from D-glucose and panose from maltose.

[0160] As a result of 1,4-α-glucan 6-α-glucosyltransferase reactions, the maltooligosaccharides resulting from the partial hydrolysis of starch are converted to isomaltooligosaccharides which contain high proportions of glucosyl residues linked by an α-D-1,6 linkages from the non-reducing end.

[0161] In one embodiment, the 1,4-α-glucan 6-α-glucosyltransferase is a 1,4-α-glucan 6-α-glucosyltransferase having an action on maltose which produces equimolar concentration of panose and glucose.

Any suitable 1,4-α-glucan 6-α-glucosyltransferase enzyme finds use in the present methods. Specific examples of such products include those described in Pazur *et al.*, *Carbohydr. Res.* (1986), 149:137-147 and in Nakamura *et al.*, *J. Biotechnol.* (1997) 53: 75-84).

[0162] In some embodiments, the 1,4-α-glucan 6-α-glucosyltransferase is of plant origin. In some embodiments, the 1,4-α-glucan 6-α-glucosyltransferase is of bacterial origin. In some embodiments, the 1,4-α-glucan 6-α-glucosyltransferase is of fungal origin.

[0163] Preferred 1,4-α-glucan 6-α-glucosyltransferases are derived from fungal strains. In preferred embodiments, the 1,4-α-glucan 6-α-glucosyltransferase is derived from a fungus of the genus *Aspergillus*, *Trichoderma* or *Neosartorya*. In preferred embodiments, the 1,4-α-

glucan 6-α-glucosyltransferase is derived from a fungus of the species *Aspergillus niger, Aspergillus awamori, Aspergillus oryzae, Aspergillus clavatus, Aspergillus fumigatus, Aspergillus nidulans, Neosartorya fischeri* or *Trichoderma reesei.*

[0164] In some embodiments, the 1,4-α-glucan 6-α-glucosyltransferase enzyme that finds use in the present method is commercially available. Examples of commercially available 1,4-α-glucan 6-α-glucosyltransferase enzymes include, but are not limited to enzymes obtained from Megazyme, Wicklow, Ireland, those from DuPont Industrial Bioscience or those from Amano International, Japan.

[0165] In some embodiments, the 1,4-α-glucan 6-α-glucosyltransferase enzyme is an *Aspergillus niger* 1,4-α-glucan 6-α-glucosyltransferase produced in *Trichoderma reesei* cells. Specific example of such enzymes include TRANSGLUCOSIDASE L-2000 (from DuPont Industrial Bioscience) and Transglucosidase L (SEQ ID NO: 1; from Amano International, Japan).

[0166] In some embodiments, the 1,4-α-glucan 6-α-glucosyltransferase is a wild type fungal 1,4-α-glucan 6-α-glucosyltransferase (including, but not limited to a fungal 1,4-α-glucan 6-α-glucosyltransferase having an amino acid sequence deposited in NCBI's GENBANK® database as accession number : BAA23616.1; *Aspergillus niger*; SEQ ID NO: 8), or a variant thereof that has an amino acid sequence that is at least about 70 % identical, at least 80 % identical, at least 85 % identical, at least 90 % identical, at least 95 % identical, or at least 98% identical to a wild type fungal 1,4-α-glucan 6-α-glucosyltransferase.

[0167] In some embodiments, the 1,4-α-glucan 6-α-glucosyltransferase is a wild type fungal 1,4-α-glucan 6-α-glucosyltransferase (including but not limited to a fungal 1,4-α-glucan 6-α-glucosyltransferase having an amino acid sequence deposited in NCBI's GENBANK® database as accession number : BAD06006.1 (*Aspergillus awamori*), or a variant thereof that has an amino acid sequence that is at least about 70 % identical, at least 80 % identical, at least 85 % identical, at least 90 % identical, at least 95 % identical, or at least 98% identical to a wild type fungal 1,4-α-glucan 6-α-glucosyltransferase.

[0168] In some embodiments, the 1,4-α-glucan 6-α-glucosyltransferase is a wild type fungal 1,4-α-glucan 6-α-glucosyltransferase (including but not limited to a fungal 1,4-α-glucan 6-α-glucosyltransferase having an amino acid sequence deposited in NCBI's GENBANK® database as accession number : BAA08125.1 (*Aspergillus oryzae*), or a variant thereof that has an amino acid sequence that is at least about 70 % identical, at least 80 % identical, at

least 85 % identical, at least 90 % identical, at least 95 % identical, or at least 98% identical to a wild type fungal 1,4-α-glucan 6-α-glucosyltransferase.

[0169] In some embodiments, the 1,4-α-glucan 6-α-glucosyltransferase is a wild type fungal 1,4-α-glucan 6-α-glucosyltransferase (including but not limited to a fungal 1,4-α-glucan 6-α-glucosyltransferase having an amino acid sequence deposited in NCBI's GENBANK® database as accession number : XP_001271891.1 (*Aspergillus clavatus*), or a variant thereof that has an amino acid sequence that is at least about 70 % identical, at least 80 % identical, at least 85 % identical, at least 90 % identical, at least 95 % identical, or at least 98% identical to a wild type fungal 1,4-α-glucan 6-α-glucosyltransferase.

[0170] In some embodiments, the 1,4-α-glucan 6-α-glucosyltransferase is a wild type fungal 1,4-α-glucan 6-α-glucosyltransferase (including but not limited to a fungal 1,4-α-glucan 6-α-glucosyltransferase having an amino acid sequence deposited in NCBI's GENBANK® database as accession number : XP_001266999.1 (*Neosartorya fischeri*), or a variant thereof that has an amino acid sequence that is at least about 70 % identical, at least 80 % identical, at least 85 % identical, at least 90 % identical, at least 95 % identical, or at least 98% identical to a wild type fungal 1,4-α-glucan 6-α-glucosyltransferase.

[0171] In some embodiments, the 1,4-α-glucan 6-α-glucosyltransferase is a wild type fungal 1,4-α-glucan 6-α-glucosyltransferase (including but not limited to a fungal 1,4-α-glucan 6-α-glucosyltransferase having an amino acid sequence deposited in NCBI's GENBANK® database as accession number : XP_751811.1 (*Aspergillus fumigatus*), or a variant thereof that has an amino acid sequence that is at least about 70 % identical, at least 80 % identical, at least 85 % identical, at least 90 % identical, at least 95 % identical, or at least 98% identical to a wild type fungal 1,4-α-glucan 6-α-glucosyltransferase.

[0172] In some embodiments, the 1,4-α-glucan 6-α-glucosyltransferase is a wild type fungal 1,4-α-glucan 6-α-glucosyltransferase (including but not limited to a fungal 1,4-α-glucan 6-α-glucosyltransferase having an amino acid sequence deposited in NCBI's GENBANK® database as accession number : XP_659621.1 (*Aspergillus nidulans*), or a variant thereof that has an amino acid sequence that is at least about 70 % identical, at least 80 % identical, at least 85 % identical, at least 90 % identical, at least 95 % identical, or at least 98% identical to a wild type fungal 1,4-α-glucan 6-α-glucosyltransferase.

[0173] In some embodiments, the 1,4-α-glucan 6-α-glucosyltransferase is a wild type fungal 1,4-α-glucan 6-α-glucosyltransferase (including but not limited to a fungal 1,4-α-glucan 6-α-

glucosyltransferase having an amino acid sequence deposited in NCBI's GENBANK® database as accession number : XP_001216899.1 (*Aspergillus terreus*) or a variant thereof that has an amino acid sequence that is at least about 70 % identical, at least 80 % identical, at least 85 % identical, at least 90 % identical, at least 95 % identical, or at least 98% identical to a wild type fungal 1,4-α-glucan 6-α-glucosyltransferase.

[0174] In some embodiments, the 1,4-α-glucan 6-α-glucosyltransferase is a wild type fungal 1,4-α-glucan 6-α-glucosyltransferase (including but not limited to a fungal 1,4-α-glucan 6-α-glucosyltransferase having an amino acid sequence deposited in NCBI's GENBANK® database as accession number : XP_001258585.1 (*Neosartorya fischeri*), or a variant thereof that has an amino acid sequence that is at least about 70 % identical, at least 80 % identical, at least 85 % identical, at least 90 % identical, at least 95 % identical, or at least 98% identical to a wild type fungal 1,4-α-glucan 6-α-glucosyltransferase.

[0175] As understood by those in the art, the quantity of 1,4-α-glucan 6-α-glucosyltransferase used in the methods of the present invention will depend on the nature and activity of the 1,4-α-glucan 6-α-glucosyltransferase enzyme, the nature and amount of the cereal flour, and the desired reaction. In general, an amount of about 0.20 to 5.0 kg of the 1,4-α-glucan 6-α-glucosyltransferase is used per metric ton of the first portion of the cereal flour used in step (a) of the process of the invention (calculated on a dry solids basis, i.e. excluding the weight of the water used in this step).In some embodiments the 1,4-α-glucan 6-α-glucosyltransferase is used in an amount about 0.5 to 2.0 kg per metric ton of the first portion of the cereal flour. In some embodiments, the 1,4-α-glucan 6-α-glucosyltransferase is used in an amount of about 1.0 to 1.5kg per metric ton of the first portion of the cereal flour.

[0176] In general, the 1,4-α-glucan 6-α-glucosyltransferase enzyme is used in an amount of about 0.5 to 10 units of 1,4-α-glucan 6-α-glucosyltransferase activity (TGU) per gram of the first portion of the cereal flour used in step (a) of the present invention (calculated on a dry solids basis, i.e. excluding the water used in this step). In some embodiments the 1,4-α-glucan 6-α-glucosyltransferase is used in an amount about 1.0 to 5.0 TGU per gram dry solids of the first portion of the cereal flour. In some embodiments, the 1,4-α-glucan 6-α-glucosyltransferase is used in an amount of about 2.0 to 3.0 TGU per dry solids of the first portion of the cereal flour. The units of 1,4-α-glucan 6-α-glucosyltransferase enzyme activity can be calculated according to the assay methods set out below.

Glucose Isomerases (Xylose isomerases)

[0177] In some embodiments, the enzyme comprises a glucose isomerase (E.C:5.3.1.5). Glucose isomerases catalyze the isomerization of glucose to fructose and are found in a large number of different microorganisms. Generally glucose isomerases are also described as xylose isomerases.

[0178] In some embodiments, the glucose isomerase (xylose isomerase) is of plant origin. In some embodiments, the glucose isomerase (xylose isomerase) is of bacterial origin. In some embodiments, the glucose isomerase (xylose isomerase) is of fungal origin.

[0179] Preferred xylose isomerases are derived from bacterial strains. In one embodiment, the xylose isomerase is derived from a bacterium of the genus *Streptomyces, Actinoplanes,* Bacillus, or *Flavobacterium*. In another embodiment, the xylose isomerase is derived from a fungus, such as a species belonging to the class *Basidiomycetes*. Preferably, the xylose isomerase is derived from a bacterium of the genus *Streptomyces.*

[0180] Examples of xylose isomerase producing microorganisms from the bacterial genus Streptomyces have been described in the patent literature: US 3,616,221 (S. *flavovirens, S. albus, S. achromogenus, S. wedmorensis,* and *S. echinatus*); US 3,622,463 (*S. olivochromogenes* and *S. venezuelae*); US 4,351,903 (*S. griseoflavus*); US 4,137,126 (*S. glaucescens*); US 3,625,828 (S. *olivaceus);* HU 12,415 (*S. galbus, S. niveus, S. gracilis, S. platensis,* and *S. matensis*); DE 2417642 (S. *violaceoniger);* US 4,399,222 *(S. acidodurans*); and JP 6-928473 (*S. phaeochromogenes, S. californicus, S. fradiae, S. vanaceus, S. rosechromogenes, S. virginiae,* and *S. olivaceus).*

[0181] In one embodiment, the glucose isomerase is an immobilized glucose isomerase. The high cost of glucose isomerase and the problems associated with using liquid glucose isomerase for commercial application limited the use of liquid glucose isomerase on a large scale production of high fructose corn syrup. This resulted in the development of immobilized glucose isomerase. Examples of glucose isomerases include GENSWEET®-IGI from DuPont Industrial Bioscience and Sweetzyme®-T from Novozymes. Liquid glucose isomerase product, GENSWEET®-SGI from DuPont Industrial Bioscience is now commercially available for limited applications and is used in the present invention to produce a baking adjunct containing fructose.

[0182] As understood by those in the art, the quantity of glucose isomerase used in the methods of the present invention will depend on the nature and activity of the glucose isomerase enzyme, the nature and amount of the cereal flour, and the desired reaction. In general, an amount of about 2.0 to 20.0 kg of the glucose isomerase is used per metric ton of the first portion of the cereal flour used in step (a) of the process of the invention (calculated on a dry solids basis, i.e. excluding the weight of the water used in this step). In some embodiments, the glucose isomerase is used in an amount about 5.0 to 15.0 kg per metric ton of the first portion of the cereal flour. In some embodiments, the glucose isomerase is used in an amount of about 8.0 to 12.5 kg per metric ton of the first portion of the cereal flour.

[0183] In general, the glucose isomerase enzyme is used in an amount of about 5 to 500 units of glucose isomerase activity (GIU) per g of the first portion of the cereal flour used in step (a) of the present invention (calculated on a dry solids basis, i.e. excluding the water used in this step). In some embodiments the glucose isomerase is used in an amount about 25 to 100 GIU per g dry solids of the first portion of the cereal flour. In some embodiments, the glucose isomerase is used in an amount of 50 to 75 GIU per g dry solids of the first portion of the wheat flour. The units of glucose isomerase enzyme activity can be calculated according to the assay methods set out below.

[0184] When the glucose isomerase enzyme is the liquid glucose isomerase product GENSWEET®-SGI, this enzyme is typically used in an amount of about 0.5 to 10.0 kg per metric ton of the first portion of the cereal flour used in step (a) of the process of the present invention (calculated on a dry solids basis, i.e. excluding the water used in this step). In some embodiments, 3 and 6 kg of GENSWEET®-SGI is used per ton dry solids of the first portion of the cereal flour. In some embodiments, about 2 to 8 kg of GENSWEET®-SGI is used per ton dry solids of the first portion of the cereal flour. In other embodiments, about 5.0 to 8.0 kg of GENSWEET®-SGI is used per ton dry solids of the first portion of the cereal flour.

Assay Methods

Carbohydrate composition by High Pressure Liquid Chromatographic (HPLC) method

[0185] The composition of the reaction products of oligosaccharides was measured by high pressure liquid chromatographic method (Beckman System Gold 32 Karat Fullerton, California, USA) equipped with a HPLC column (Rezex 8 u8% H, Monosaccharides),

maintained at 50 °C fitted with a refractive index (RI) detector (ERC-7515A, RI Detector from The Anspec Company, Inc.).

[0186] Dilute sulfuric acid (0.01 N) was used as the mobile phase at a flow rate of 0.6 mL per minute. 20 µL of 4.0% solution was injected on to the column.

[0187] The column separates based on the molecular weight of the saccharides. For example, a designation of DP1 is a monosaccharide, such as glucose or fructose; a designation of DP2 is a disaccharide, such as maltose; a designation of DP3 is a trisaccharide, such as maltotriose; and the designation "DP4^{+}" is an oligosaccharide having a degree of polymerization (DP) of 4 or greater.

Ion Chromatographic separation of malto-and isomalto-oligosaccharides:

[0188] The standard sugars (malto- and isomaltooligosaccharides) were dried at 80°C until constant mass, and 1 g/L stock solutions were made for each compound. The solutions were stored at -20°C.

[0189] Sample preparation: IMO syrup was diluted to a suitable concentration and filtered through a 0.22 mm membrane before analysis.

[0190] The analysis was performed on a Dionex ICS-5000 ion chromatography (Sunnyvale, CA, USA) composed of a GP50 gradient pump, an ED 40 electrochemical detector including a pulsed amperometric detection cell consisting of a gold electrode and a pH-Ag/AgCl reference electrode.

[0191] Separations were performed using a CarboPak™ PA200 column (3*250mm) with a guard column (3*50mm). The flow rate was at 0.5 mL/min, and a column temperature was maintained at 30°C. Injection volume was 25 µL.

[0192] The optimal gradient for CarboPak™ PA10 column was found as: Gradient: 0-10 min, 50 mM NaOH; 10-15 min, 50-100 mM NaOH, curve 6; 15-25 min, 100 mM NaOH, 0-40 mM sodium acetate (NaAc), curve 2; 25-35 min, 40-100 mM NaAc, 100 mM NaOH; 35.1-39min, 250 mM NaAc, 50 mM NaOH; 39.1-48min, 50 mM NaOH.

[0193] The following pulse potentials and durations were used: E1 = 0.1V, t1 = 400 ms; E2 = −2V, t2 = 20 ms; E3 = 0.6V, t3 = 10 ms; E4 = −0.1V, t4 = 70 ms. Data acquisition and integration were performed using Chromeleon 6.8 workstation.

Glucoamylase Activity Units (GAU)

[0194] The PNPG assay is based on the activity of glucoamylase enzyme to catalyze the hydrolysis of *p*-nitrophenyl-α-D-glucopyranoside (PNPG) to glucose and *p*-nitrophenol. At an alkaline pH the nitrophenol forms a yellow colour that is measured spectrophotometrically at 400 nm used in the calculation for GAU. One Glucoamylase Unit is the amount of enzyme that will liberate one gram of reducing sugars calculated as glucose from a soluble starch substrate per hour under the specified conditions of the assay.

Alpha Amylase activity Units (AAU)

[0195] AAU was determined by the rate of starch hydrolysis, as reflected in the rate of decrease of iodine-staining capacity measured spectrophotometrically. One AAU of bacterial alpha-amylase activity is the amount of enzyme required to hydrolyze 10 mg of starch per min under standardized conditions (pH 6.0 and 60°C). Alpha-amylase activity can also be determined as soluble starch unit (SSU) and is based on the degree of hydrolysis of soluble potato starch substrate (4% DS) by an aliquot of the enzyme sample at pH 4.5 and 50°C. The reducing sugar content is measured using the DNS method as described in Miller, G. L. *Anal. Chem.* (1959) 31:426 - 428.

1,4-α-glucan 6-α-glucosyltransferase Activity Units (TGU):

[0196] One 1,4-α-glucan 6-α-glucosyltransferase activity unit (transglucosidase activity unit, TGU) is defined as the amount of 1,4-α-glucan 6-α-glucosyltransferase which converts 1 micromole of maltose substrate in 1 minute under standard assay conditions of pH 4.8 and 37°C. It also can be defined as the amount of 1,4-α-glucan 6-α-glucosyltransferase which produces 1 micromole of panose per minute under standard assay conditions of pH 4.8 and 37°C.

Glucose Isomerase Activity Units (GIU):

[0197] The activity of soluble glucose isomerase is determined from the rate of conversion of glucose to fructose under controlled conditions for thirty minutes (2.6 M glucose in 200 mM maleate, 20 mM $MgSO_4.7H_2O$, 1mM $CoCl_2.6H_2O$, pH 6.85). Glucose isomerase activity is then calculated by measuring the amount of fructose converted from glucose via HPLC. One glucose isomerase activity Unit (GIU) is defined as that activity which will produce one micromole of fructose per minute under the conditions of this assay.

Product

[0198] In the first aspect of the present invention, the intermediate product is a composition in which at least part, in some embodiments all, of the starch present in said first portion of cereal flour has been converted into a fermentable sugar. This is described in the examples below as a “baking adjunct”.

[0199] Typically, the fermentable sugar produced *in situ* is selected from glucose, fructose and maltose.

[0200] In the second aspect of the present invention, the intermediate product is a composition in which at least part or all of the starch present in said first portion of cereal flour has been converted into an isomaltooligosaccharide. This is also described in the examples below as a “baking adjunct”.

[0201] Typically, the isomaltooligosaccharide produced *in situ* has a degree of polymerisation of less than 5. Preferably, said isomaltooligosaccharide has a degree of polymerisation of less than 4. More preferably, said isomaltooligosaccharide has a degree of polymerisation of less than 3. Most preferably, said isomaltooligosaccharide is selected from the group consisting of isomaltose, panose, and mixtures thereof.

Food Product

[0202] The present invention further relates to the use of the composition obtained or obtainable according to the methods of the present invention. The composition is useful as a food ingredient, particularly though not exclusively for incorporation into flour compositions for preparing dough and baked products, such as bread, prepared from dough.

[0203] The composition may be used as – or in the preparation of - a food product. Here, the term “food product” is used in a broad sense – and covers food for humans as well as food for animals (i.e. a feed). In some aspects, the food is for human consumption. The food may be in the form of a solution or as a solid – depending on the use and/or the mode of application and/or the mode of administration.
In some embodiments, the composition according to the invention is added directly in the production of the food product. In other alternative embodiments, the composition according to the invention may be used without further separation or harvesting directly in production of food products.

[0204] The composition may also be used as a food ingredient. As used herein the term “food ingredient” includes a formulation which is or can be added to functional foods or foodstuffs, for example, as a nutritional supplement and/or fibre supplement. The term food ingredient as used here also refers to formulations which can be used at low levels in a wide variety of products that require gelling, texturising, stabilising, suspending, film-forming and structuring, retention of juiciness and improved mouthfeel, without adding viscosity. The food ingredient may be in the form of a solution or as a solid – depending on the use and/or the mode of application and/or the mode of administration.

[0205] In one embodiment, the composition may be – or may be added to - food supplements. In one embodiment, the composition may be – or may be added to - functional foods. As used herein, the term “functional food” means food which is capable of providing not only a nutritional effect and/or a taste satisfaction, but is also capable of delivering a further beneficial effect to consumer. Accordingly, functional foods are ordinary foods that have components or ingredients (such as those described herein) incorporated into them that impart to the food a specific functional – e.g. medical or physiological benefit - other than a purely nutritional effect. Although there is no legal definition of a functional food, most of the parties with an interest in this area agree that they are foods marketed as having specific health effects.

[0206] Some functional foods are nutraceuticals. Here, the term “nutraceutical” means a food which is capable of providing not only a nutritional effect and/or a taste satisfaction, but is also capable of delivering a therapeutic (or other beneficial) effect to the consumer. Nutraceuticals cross the traditional dividing lines between foods and medicine.

[0207] Therefore, the invention further provides a food product containing the present composition(s).

[0208] In one embodiment, the composition comprises a dough composition. The term "dough" is defined herein as a mixture of flour and other ingredients firm enough to knead or roll. This may be fresh, frozen, pre-baked, or prebaked.

[0209] In one embodiment, the dough composition is baked to form a baked product. The term "baked products" is defined herein as any product prepared from dough, either of a soft or a crisp character. Examples of the baked products, whether of a white, light or dark type, which may be advantageously produced by the present invention are bread (in particular white, wholemeal or rye bread), typically in the form of loaves or rolls, French baguette-type bread, pasta, pita bread, tortillas, tacos, cakes, pancakes, biscuits, cookies, pie crusts, steamed bread, and crisp bread, and the like. Suitable baking conditions are well known to the person skilled in the art.

[0210] Accordingly, a baked product is also provided (obtained) obtainable by baking the dough composition of the invention. In one embodiment, said baked product comprises bread.

EXAMPLES

[0211] The present invention is described in further detail in the following examples which are not in any way intended to limit the scope of the invention as claimed. The attached Figures are meant to be considered as integral parts of the specification and description of the invention. All references cited are herein specifically incorporated by reference for all that is described therein. The following examples are offered to illustrate, but not to limit the claimed invention.

In the disclosure and experimental section which follows, the following abbreviations apply:
[0212] % w/w (weight percent)
dH_2O (deionized water);
dIH_2O (deionized water, Milli-Q filtration);
U (units of enzyme activity – the activity depending on that of the specified enzyme);
MW (molecular weight);
DO (dissolved oxygen);
ds or DS (dry solids content);

MT (metric ton).

Example 1

[0213] A baking adjunct containing very high soluble sugars was prepared by incubating 200 grams of wheat flour slurred with 1,200 g water followed by dosing with an alpha-amylase (SEQ ID NO: 2; SPEZYME® RSL from DuPont) at 0.5 AAU/g ds at pH 5.6 and 60°C for 6 hours. Samples were withdrawn at 6 hours for HPLC.

[0214] The data in Table 1 (A & B) show that 543.6 mg of total soluble sugars were produced per 1 gram of wheat flour, indicating that most of starch in wheat flour was solubilized by alpha-amylase and wheat endogenous starch hydrolyzing enzymes under the specified conditions of incubation.

Table 1A - Total soluble sugars (mg/g wheat flour)

Enzyme	**Incubation Time, hours**	**Total soluble sugars**	**Total soluble glucose**	**Total soluble disaccharide**	**Total soluble trisaccharide**	**Total soluble higher sugars**
No added Enzyme	6	428.89	2.97	261.39	13.17	151.35
SPEZYME® RSL	6	543.6	13.8	362.1	34.5	133.2

Table 1B - Relative sugar composition

Enzyme	**Incubation Time, hours**	**% glucose**	**% disaccharide**	**% trisaccharide**	**% higher sugars**
None	6	0.69	60.95	3.07	35.29
SPEZYME® RSL	6	2.54	66.61	6.35	24.51

[0215] As it can be seen in Tables 1A and 1B, a significant amount of the wheat granular starch was solubilized (>75 %) without the addition of any external enzymes (alpha amylase) indicating the presence of very high level of endogenous wheat granular starch hydrolyzing enzymes. However, the soluble sugar composition of the incubated sample showed high level of maltose, > 60 % indicating the presence of beta amylase activity. This will offer an excellent substrate containing high maltose without the addition of external beta amylase for producing *in situ* IMO using 1,4-α-glucan 6-α-glucosyltransferase.

Example 2

[0216] Baking adjunct containing very high glucose was prepared by incubating 200 grams of wheat flour slurred with 1,200 g water followed by dosed with alpha amylase (SEQ ID NO: 2; SPEZYME® RSL) at 0.5 AAU/g ds and glucoamylase (SEQ ID NO: 4; "TrGA CS4" variant from wild type glucoamylase SEQ ID NO: 3 from *Trichoderma reesei*; "GC321") at 2 GAU/ g ds at pH 5.6 and 60°C for 6 hours. Samples were withdrawn at 6 hours for HPLC.

[0217] Table 2A shows that 487.8 mg of glucose were produced per 1 gram of wheat flour, indicating that most of starch in wheat flour was solubilized and converted to glucose by alpha-amylase and glucoamylase. Total soluble sugars concentration (mg/g wheat flour, Table 2A) and the percentage of the solution sugars (Table 2B) were shown below.

Table 2A - Total soluble sugars (mg/g wheat flour)

Hour	**Total soluble sugars**	**Total soluble glucose**	**Total soluble disaccharide**	**Total soluble trisaccharide**	**Total soluble higher sugars**
6	521.2	487.8	9.4	2.8	21.3

Table 2B - Relative sugar composition

Hour	**% glucose**	**% disaccharide**	**% trisaccharide**	**% higher sugars**
6	93.58	1.80	0.53	4.09

Example 3

[0218] A baking adjunct containing very high isomaltooligosaccharide (IMO) was prepared by incubating 200 grams of wheat flour slurred with 1,200 g water followed by dosing with alpha amylase (SEQ ID NO:2; SPEZYME® RSL) at 2 or 0.5 AAU/g ds and 1,4-α-glucan 6-α-glucosyltransferase (SEQ ID NO:5; TRANSGLUCOSIDASE L-2000 ("TG L-2000") from DuPont Industrial Bioscience) at 2.5 TGU/g ds at pH 5.6 and 60°C for 6 hours. Samples were withdrawn at 6 hours for HPLC.

[0219] Tables 3A and 3B show that significantly elevated trisaccharide (88.8 mg) was produced while 553.5 mg of total soluble sugars produced per 1 gram of wheat flour, indicating that most of starch in wheat flour was solubilized and converted to IMO by alpha-amylase and 1,4-α-glucan 6-α-glucosyltransferase.

Table 3A - Total soluble sugars (mg/g wheat flour)

AAU/g ds	Hour	Total soluble sugars	Total soluble glucose	Total soluble disaccharide	Total soluble trisaccharide	Total soluble higher sugars
2	6	588	130.6	156.9	131.7	168.7
0.5	6	553.5	180.1	136.4	88.8	148.2

Table 3B - Relative sugar composition

AAU/g ds	Hour	% glucose	% disaccharide	% trisaccharide	% higher sugars
2	6	27.10	24.12	21.10	27.68
0.5	6	32.54	24.64	16.05	26.77

[0220] The content of isomaltooligosaccharides in the sample treated with 2 AAU/g ds and 2.5 TGU/g ds were then determined using the Ion Chromatographic separation method described above. The total composition of the sugar content of the syrup is given in Table 4.

Table 4

Sugar	% w/w
Glucose	27.04
Fructose	1.25
Isomaltose	10.73
Maltulose	1.27
Kojibiose	1.21
Isomaltotriose	2.75
Nigerose	0.97
Maltose	19.21
Isomaltotetraose	0.41
Panose	27.04
Maltotriose	3.03
>DP3 Oligosaccharides (including isomaltotetraose)	7.84

[0221] The results in Table 4 showed that the syrup contained approximately 50 % isomaltooligosaccharides consisting of 10.73% isomaltose, 27.04% panose and 2.75 % isomaltotriose in addition to other branched reduced digestible sugars. The composition of this IMO syrup is very close to an IMO-55 syrup commercially available in Japan and China.

Example 4

[0222] Baking Studies: The baking adjuncts from Examples 1, 2 and 3 were used to make dough by adding remaining 800 grams of wheat flour to each and mixed with other baking ingredients listed in Table 5. Additional sugar was added to Example 1 prior to making

dough for baking. White pan breads were prepared using a no-time dough system; as follows:

[0223] Each hydrolysate was used in place of water in the dough. No sucrose was added to each dough. These are shown in Table 5 below as Samples 2, 3, and 4 respectively. Sample 1 is a control made using 8% sucrose.

[0224] The pH of dough was taken before and after proof. Breads were proofed to ¼ in (0.63 cm) to ½" (1.27 cm) above the edge of the pan. Breads were unsliced and individually packaged in plastic bags and stored overnight under ambient conditions. Breads were sliced and tasted.

Table 5 - Baking ingredients

Ingredient	**1 - Control**	**2 - SPEZYME® RSL (Example 1)**	**3 - SPEZYME® RSL +TRGA (Example 2)**	**4 - SPEZYME® RSL +TG L-2000 (Example 3)**
Flour, Polar Bear	100.00%	90.00%	90.00%	90.00%
Water	60.00%	-	-	-
Slurry Mixture (10% Flour/ 60% Water)	-	70.00%	70.00%	70.00%
Sugar, Fine Granulated	8.00%	-	-	-
Sodium Bicarbonate	0.25%	0.25%	0.25%	0.25%
Salt	2.00%	2.00%	2.00%	2.00%
Soybean Oil Sysco	2.00%	2.00%	2.00%	2.00%
Yeast, Compressed	3.50%	3.50%	3.50%	3.50%
Panodan 205	0.50%	0.50%	0.50%	0.50%
HP 75	1.00%	1.00%	1.00%	1.00%
POWERBAKE® 4205	0.1875%	0.1875%	0.1875%	0.1875%
	ppm	ppm	ppm	ppm
Ascorbic Acid	75	75	75	75

In the list of ingredients in Table 5 above:

[0225] PANODAN® 205 (Dupont) is a diacetyl tartaric acid ester of mono- and diglycerides (DATEM) made from edible, refined vegetable fat;
HP 75 is a distilled monoglyceride made from edible, fully hydrogenated palm based oil; and POWERBAKE® 4205 is an enzyme blend having xylanase, oxidase and lipase activities and is produced by fermentation with selected microbial strains.
All are commercially available from DuPont Nutrition Biosciences ApS.

[0226] All ingredients listed in Table 5 were added to a spiral mixer and were mixed to development for a final dough temperature of 26.5°C (80°F). Dough was scaled to 0.680 kg (24 oz) pieces. Dough was rounded, sheeted and moulded, and then placed into a proof box set at 40.5 to 43.3°C (105-110°F) and 85-90% relative humidity. Loaves were proofed to 0.635 to 1.27 cm (¼ - ½ inches) above the pan lip (approximately 1 hour). Loaves were baked at 204.4°C (400°F) for 23 minutes to an internal temperature of 93.3-97.8°C (200-208°F).

The results are summarized in Table 6.

Table 6 - Baked loaves and internal structure

Test	**Crust Colour**	**Crumb**	**Flavour / Texture**
Control	Medium/dark brown	closed/tight cells	standard
SPEZYME® RSL (Example 2)	Medium brown	Slightly more open; slight grey colour	slightly salty; gummy; breaks down quickly
SPEZYME® RSL + TR-GA CS4 (Example 3)	Medium/dark brown	Slightly more open	Very similar to control in flavour and texture
SPEZYME® RSL + TG L-2000 (Example 4)	Medium brown	Slightly more open	slightly salty; gummy and chewy; slight enzyme afternote

[0227] There were no significant differences in dough handling or mix time. Dough made with SPEZYME® only (Example 2) took about 1.5 times longer to proof than other test variables. Crust colour of SPEZYME® only and SPEZYME® RSL + TG L-2000 (Example 4) were very similar. Both were lighter than the control. Crust colour of bread made with SPEZYME® RSL + TRGA CS4 (Example 3) was very similar to the control. Crumb of all three test variables were slightly more open than the control, but acceptable. Volumes of all three variables were slightly lower than control. The flavour of bread made with SPEZYME® only and TG L-2000 had a saltier note. Bread made with TG L-2000 had a slight enzyme aftertaste. Bread made with TRGA CS4 was very similar to control. Texture of bread made with SPEZYME® only was slightly gummy with quick breakdown during chew. Texture of bread made with TRGA CS4 was very similar to control. Bread made with TG L-2000 had a slightly gummy and chewy texture with slightly longer breakdown during chew.

Example 5

[0228] To test the effect of different concentration of glucose isomerase on *in situ* production of fructose in the dough system, 20 grams of wheat flour was slurried with 60 grams of water with pH adjusted at 7.0 and incubated at 60°C for 4 hours following addition of 5 GAU/g ds of TrGA CS4 and 0.5, 3 and 6 kg of glucose isomerase (SEQ ID NO: 7; GENSWEET®-SGI) /ton ds of the first portion of the wheat flour, respectively. Samples were withdrawn to check sugar composition by HPLC.

The results are shown in Tables 7A and 7B.

Table 7A - Total soluble sugars (mg/g wheat flour)

SGI (kg/ ton ds)	Total soluble sugars	Total soluble fructose	Total soluble glucose	Total soluble disaccharide	Total soluble trisaccharide	Total soluble higher sugars
0.5	726.3	3.9	665	14.5	7.7	67.3
3	654.4	16.2	588.5	12.4	3.5	33.9
6	598.3	53.1	501.7	10	2.8	30.7

Table 7B - Relative sugar composition

SGI (kg / ton ds)	% fructose	% glucose	% disaccharide	% trisaccharide	% higher sugars
0.5	0.54	91.56	2.00	0.61	5.30
3	2.47	89.92	1.89	0.54	5.17
6	8.88	83.87	1.66	0.46	5.13

Example 6

[0229] To investigate the conversion of wheat starch to fructose using glucose isomerase, the wheat slurry was made as described in Example 1 (200 gram of wheat flour + 1,200 grams of water). The pH of the slurry was adjusted to pH 7.0 using sodium bicarbonate, then alpha-amylase (SEQ ID NO: 6; SPEZYME® Xtra (0.5 AAU/g wheat flour)), *Trichoderma* glucoamylase, (SEQ ID NO: 3; GC321®)(2.00 GAU / g wheat flour) and glucose isomerase (SEQ ID NO: 7; GENSWEET®-SGI glucose isomerase (DuPont Industrial Bioscience)) (2.5 mL) were added, then the slurry was incubated at 60°C with constant mixing. The samples were withdrawn at different intervals of time, centrifuged to remove the insoluble and the clear supernatant was analyzed for sugar composition by HPLC. The results are shown in Figure 2 and Tables 8A and 8B.

Table 8A - Total soluble sugars (mg/g wheat flour)

Hour	Total soluble sugars	Total soluble fructose	Total soluble glucose	Total soluble disaccharide	Total soluble trisaccharide	Total soluble higher sugars
5	651.8	55.7	263.6	195.9	10.2	126.3

Table 8B - Relative sugar composition

Hour	% fructose	% glucose	% disaccharide	% trisaccharide	% higher sugars
5	8.54	40.44	30.06	1.57	19.38

[0230] The results showed that the insoluble wheat starch was solubilized and then hydrolyzed to glucose by alpha amylase and glucoamylase. The glucose was then isomerized to fructose by glucose isomerase under the incubation conditions. The hydrolysate was then subjected to baking as described in Example 4.

[0231] The baked bread using baking adjunct prepared with GENSWEET®-SGI (Example 6) produced bread with slight to moderate sweetness, slightly more elastic, medium brown in colour, slightly more open and a decrease in loaf volume (6.06 cm^3/g vs 6.38 cm^3/g) compared to conventional bread with added sugar.

Example 7

[0232] The effect of proteases, exhibiting both end- and exo-activity containing leucine aminopeptidase activity was studied during *in situ* production of fermentable sugars under baking conditions. The fermentable sugars production conditions are similar to as described in Example 2 with additional 200 ppm of SUMIZYME® PF-G (a protease preparation exhibiting both end- and exo-activity containing leucine aminopeptidase activity; Shin Nihon Chemicals, Japan) added and incubated for 5 hours at 60°C, and pH 5.6. The results are shown in Tables 9A and 9B.

Table 9A -Total soluble sugars (mg/g wheat flour)

Hour	Total soluble sugars	Total soluble glucose	Total soluble disaccharide	Total soluble trisaccharide		Total soluble higher sugars
5	645.2	605	11.9	3.6		24.7

Table 9B - Relative sugar composition

Hour	% glucose	% disaccharide	% trisaccharide	% higher sugars
5	93.77	1.84	0.56	3.83

[0233] As it can be seen from HPLC chromatograms (Figure 4) that the addition of SUMIZYME® PF-G (Shin Nihon Chemicals, Japan) containing exopeptidase activity during *in situ* production of fermentable sugars (Example 7) resulted in the hydrolysis of wheat proteins and produced soluble fraction containing a very high level of free amino acids, i.e., arginine, valine, leucine, and tryptophan.

[0234] The baking trials were then conducted in white pan bread to compare the bread with sugar added and bread without sugar added with respect to mixing time, dough, handling, proof time, crust colour, crumb structure, and flavour. The fermentable syrup from Example 7 was then mixed with remaining 800 grams of wheat flour in addition to all ingredients listed in Table 5 and were then taken in a spiral mixer. It was then mixed to development for a final dough temperature of 26.5°C (80°F). Dough was scaled to 0.680 kg (24 oz) pieces. Dough was rounded, sheeted and moulded, and then placed into a proof box set at 40.5 to 43.3°C (105-110°F) and 85-90% relative humidity. Loaves were proofed to 0.635 to 1.27 cm (¼ - ½ inches) above the pan lip (approximately 1 hour). Loaves were baked at 204.4°C (400°F) for 23 minutes to an internal temperature of 93.3-97.8°C (200-208°F). The results are summarized in Tables 10A and 10B.

Table 10A - Baked loaves and mix through proof

Test	Slurry Flavour	Mixing	Dough handling/properties	Proof time to 1.27 cm (½”)
Example 1 (with sugar)	n/a	To development	Soft, visco-elastic	63 min
Example 7 (without sugar)	Slight to moderate sweetness	No significant difference	Slightly more extensible	71 min

Table 10B - Baked loaves and internal structure

Test	**Crust colour**	**Crumb**	**Flavour**	**Specific Volume, cm^3/g**
Example 1 (with sugar)	Medium/dark brown	Slightly open and uneven	standard	6.38
Example 7 (without sugar)	Medium/dark brown	Slightly more closed and even	Similar to control	6.4

[0235] The bread produced using process described in Example 7 with exopeptidase showed the results comparable to conventional bread, with added sugar with respect to mixing time, proof time, volume, crumb structure, crust colour, dough handling, and flavour.

Example 8

[0236] In this example, a baking adjunct containing very high glucose was prepared by mixing 200 grams of wheat flour with 1,200 g water and incubated with only glucoamylase (*Tr*-GA GC321 at 5 GAU/g ds) at pH 5.6 and 60°C for 6 hours. Samples were withdrawn at 6 hours for HPLC.

[0237] In another experiment, a baking adjunct containing IMO was prepared by mixing 200 grams of wheat flour with 1,200 grams water and incubated with alpha amylase (0.3 AAU SPEZYME® Xtra)/g ds and 5 TGU units of TRANSGLUCOSIDASE L-2000 /g ds of wheat flour at pH 5.2 and 60°C for 6 hours. Samples were withdrawn at 6 hours for HPLC.

[0238] The results are shown in Tables 11A and 11B. Data shown in Tables 11A and 11B below are based on the test series using the following test variables:
Sample 1) 8% (w/w) sucrose;
Sample 2) 0.5% (w/w) DIMODAN® distilled monoglyceride;
Sample 3) glucoamylase, GC321 (*Tr*-GA; SEQ ID NO: 3) at 5 GUA/g ds; and
Sample 4) alpha amylase (SPEZYME® XTRA; SEQ ID NO: 6) at 0.3 AAU/g ds + 1,4-α-glucan 6-α-glucosyltransferase (TRANSGLUCOSIDASE L-2000; SEQ ID NO: 5) at 5 TGU/g ds.

Table 11A - Total soluble sugars (mg/g wheat flour)

Sample No	**Total soluble sugars**	**Total soluble glucose**	**Total soluble disaccharide**	**Total soluble trisaccharide**	**Total soluble higher sugars**
3	292.8	272.9	6.2	1.7	12
4	311.8	130	69.4	28.2	84.2

Table 11B - Relative sugar composition

Sample No	**% glucose**	**% disaccharide**	**% trisaccharide**	**% higher sugars**
3	93.21	2.13	0.57	4.09
4	41.68	22.27	9.05	27.00

[0239] Breads were prepared using a no time dough method. Hydrolysates were used in place of 10% flour and all of water in Samples 3 and 4. Breads were unsliced and individually packaged in plastic bags and stored under ambient conditions. Breads were sliced and tested on days 1 and 2. There were no significant differences in mixing, dough handling, proof time or baking.

[0240] The loaf volume of the bread from different treatment, cm^3/gram was measured for each of the above four Samples. The data are given in Table 12A.

Table 12A - Effect of *in situ* glucose and IMO production under baking conditions without added sugar on the loaf volume of the bread after storage for 1 day at room temperature

Sample No	**Sample Description**	**Loaf Volume (cm^3/g)**
1	Control (8% w/w sucrose)	6.32
2	0.5% w/w DIMODAN® HS 150	6.11
3	glucoamylase, GC 321 (*Tr*-GA) at 5 GUA/g ds	6.25
4	Alpha-amylase SPEZYME® XTRA (0.3 AAU/g ds + TRANSGLUCOSIDASE L-2000 at 5 TGU/g ds.	6.45

[0241] In the above Table, the DIMODAN® HS 150 used in Sample 2 is a softener commercially available from DuPont Nutrition Biosciences ApS comprising distilled monoglycerides made from edible, refined hydrogenated vegetable oil.

[0242] The results in Table 12A showed a slight differences in loaf volume of the bread produced using different conditions. However, the loaf volume of the bread produced from *in situ* glucose was comparable or slightly better than control or containing conventional softener, i.e. DIMODAN® HS 150.

[0243] Results of TPA softness suggest that the test variable using just glucoamylase alone for producing no-sugar added (Sample no. 3) resulted in an acceptable softness when compared to a standard formula containing 8% sucrose and a formula containing distilled monoglycerides (a standard softening agent; Sample no. 2).

[0244] These results are shown in Table 12B, wherein the descriptions of the samples are the same as those having the corresponding sample number in Table 12A.

[0245] Two of the four loaves were sliced on each testing date. The 8 centermost slices of each loaf were tested sets of two with the centermost slice facing up. Testing was done using the TAXT Plus using a 35 mm metal probe. Softness is measured as the force required to compress each set of slices. Adhesiveness is measured as the negative work between two compressions. Cohesiveness is measured as the area of work during the second compression divided by the area of work during the first compression. Resilience is measured as how well the bread slices regain their original height after the first compression.

Table 12B - Effect of *in situ* glucose and IMO production under baking conditions without added sugar on the functional properties of bread

Sample No	**Day**	**Softness (g/s)**	**Adhesiveness (g/s)**	**Cohesiveness (area work f / area work i)**	**Resilience (height f / height i)**
1	1	292.579	-5.091	0.66	0.254
2	1	292.139	-5.413	0.464	0.237
3	1	270.983	-4.028	0.701	0.295
4	1	298.582	-4.723	0.664	0.268
1	2	387.238	-4.256	0.605	0.221
2	2	392.013	-4.302	0.605	0.216
3	2	345.78	-3.489	0.643	0.252
4	2	380.498	-2.643	0.609	0.239

[0246] Based on the results from the Softness TPA test, all test variables staled at about the same rate (similar slopes). Sample 3 (*Tr*-GA GC 321) was softer than all other variables on both days of testing. The results of the adhesiveness test also showed lower adhesiveness at similar rates with exception of the Sample 4, in which IMO had been produced *in situ*, which became less adhesive at a faster rate.

[0247] Based on the results from the Cohesiveness TPA test, all variables became less cohesive at similar rates. Based on the results from the Resilience TPA test, Sample 3 (*Tr*-GA GC 321) had the most resilience showing more spring back in the bread.

[0248] Based on the results from Specific Volume testing, there are slight differences in volume. Sample 3 (using glucoamylase GC321) has a similar volume to the control, the comparative Sample 2 (DIMODAN® HS 150) has a slightly lower volume, and Sample 4, where IMO was produced *in situ* using alpha amylase (SPEZYME® Xtra) + 1,4-α-glucan 6-α-glucosyltransferase (TRANSGLUCOSIDASE L-2000) has a slightly higher volume.

CLAIMS

1. A method of preparing a dough composition, said method comprising:
   (a) preparing a composition by mixing a first portion of cereal flour, water and one or more enzymes capable of converting starch present in said first portion of cereal flour into a fermentable sugar, said one or more enzymes being introduced into said composition in a single step;
   (b) allowing said enzyme to convert said starch into said fermentable sugar;
   and
   (c) addition of a second portion of cereal flour;
   wherein said first portion of cereal flour comprises 5 to 50% by weight of the total weight of said first and second portions of cereal flour; and said water used in step (a) comprises 50% to 100% by weight of the total weight of the water used in said method.

2. The method of claim 1, wherein said starch is granular-insoluble starch.

3. The method of claim 1, wherein said fermentable sugar is selected from the group consisting of glucose, fructose and maltose, or a mixture thereof.

4. The method of claim 1 or claim 2, wherein said one or more enzymes comprises an enzyme having granular starch hydrolysing activity (GSHE) or a mixture thereof.

5. The method of claim 4, wherein said one or more enzymes is selected from the group consisting of an alpha amylase (E.C. 3.2.1.1), a beta amylase (E.C. 3.2.1.2), and a gamma amylase (glucoamylase; E.C. 3.2.1.3), or a mixture of any thereof.

6. The method of claim 4, wherein said one or more enzymes comprises a mixture of an alpha amylase (E.C. 3.2.1.1) and a gamma amylase (glucoamylase; E.C. 3.2.1.3).

7. The method of any one of claims 4 to 6, wherein said one or more enzymes additionally comprises a glucose isomerase (E.C. 5.3.1.5).

8. A method of preparing a dough composition, said method comprising:
   (a) preparing a composition by mixing a first portion of cereal flour, water and an enzyme(s) capable of converting starch present in said first portion of cereal flour into an isomaltooligosaccharide;
   (b) allowing said enzyme to convert said starch into said isomaltooligosaccharide;

and:

(c) addition of a second portion of cereal flour; wherein said first portion of cereal flour comprises 5 to 50% by weight of the total weight of said first and second portions of cereal flour; and said water used in step (a) comprises 50% to 100% by weight of the total weight of the water used in said method.

9. The method of claim 8, wherein step (a) comprises introduction of said one or more enzymes into said composition in a single step.

10. The method of claim 8 or claim 9, wherein said starch is granular insoluble starch.

11. The method of any one of claims 8 to 10, wherein said one or more enzymes comprises a 1,4-α-glucan 6-α-glucosyltransferase (E.C. 2.4.1.24).

12. The method of claim 11, wherein said 1,4-α-glucan 6-α-glucosyltransferase enzyme catalyses the transfer of a glucose moiety from a malto-oligosaccharide to the 6-OH position of another saccharide.

13. The method of any one of claims 8 to 12, wherein said one or more enzymes additionally comprises a further enzyme having granular starch hydrolysing activity (GSHE) or a mixture thereof.

14. The method of claim 13, wherein said one or more enzymes additionally comprises a further enzyme selected from the group consisting of an alpha amylase (E.C. 3.2.1.1), a beta amylase (E.C. 3.2.1.2), and a gamma amylase (glucoamylase; E.C. 3.2.1.3), or a mixture of any thereof.

15. The method of any one of claims 8 to 14, wherein said isomaltooligosaccharide has a degree of polymerisation of less than 5.

16. The method of claim 15, wherein said isomaltooligosaccharide has a degree of polymerisation of less than 4.

17. The method of claim 16, wherein said isomaltooligosaccharide has a degree of polymerisation of less than 3.

18. The method of any one of claims 8 to 17, wherein said isomaltooligosaccharide is selected from the group consisting of isomaltose, panose and mixtures thereof.

19. The method of any one of claims 1 to 18, wherein said first portion of cereal flour comprises 10 to 40% by weight of the total weight of said first and second portions of cereal flour.

20. The method of claim 19, wherein said first portion of cereal flour comprises 20 to 30% by weight of the total weight of said first and second portions of cereal flour.

21. The method of any one of claims 1 to 20, wherein said water used in step (a) comprises 80% to 100% by weight of the total weight of the water used in said method.

22. The method of claim 21, wherein said water used in step (a) comprises 90% to 100% by weight of the total weight of the water used in said method.

23. The method of claim 22, wherein said water used in step (a) comprises 100% by weight of the total weight of the water used in said method.

24. The method of any one of claims 1 to 23, wherein the one or more enzymes used in step (a) are endogenous to the cereal flour.

25. The method according to claim 24, wherein the enzyme endogenous to the cereal flour is an alpha amylase, a beta amylase, or a mixture thereof.

26. The method of any one of claims 1 to 25, wherein the one or more enzymes used in step (a) are added to the cereal flour.

27. The method of any one of claims 1 to 26, wherein step (b) comprises heating the composition prepared in step (a) to a temperature below the gelatinisation temperature of starch.

28. The method of claim 27, wherein the temperature in step (b) is from 40 to 60 °C.

29. The method of claim 27 or claim 28, wherein step (b) comprises heating the composition for 1 to 24 hours.

30. The method of claim 29, wherein step (b) comprises heating the composition for 2 to 6 hours.

31. The method of any preceding claim, wherein step (c) comprises adding further ingredients to prepare the dough composition.

32. A method of preparing a baked product, said method comprising:
preparing a dough composition according to the method of any preceding claim; and baking the dough composition to prepare the baked product.

33. A composition obtainable according to the method of any one of claims 1-31.

34. A baked product obtainable according to the method of claim 32.

35. Use of a composition according to claim 33 for improving the properties of a dough and/or a baked product prepared from dough.

36. Use according to claim 35, wherein said improved property comprises improved elasticity.

37. Use according to claim 35, wherein said improved property comprises improved texture.

38. Use according to claim 35, wherein said improved property comprises improved loaf volume.

39. Use according to claim 35, wherein said improved property comprises improved flavour.

40. Use according to claim 35, wherein said improved property comprises improved softness.

41. Use according to claim 35, wherein said improved property comprises improved resistance to staling.

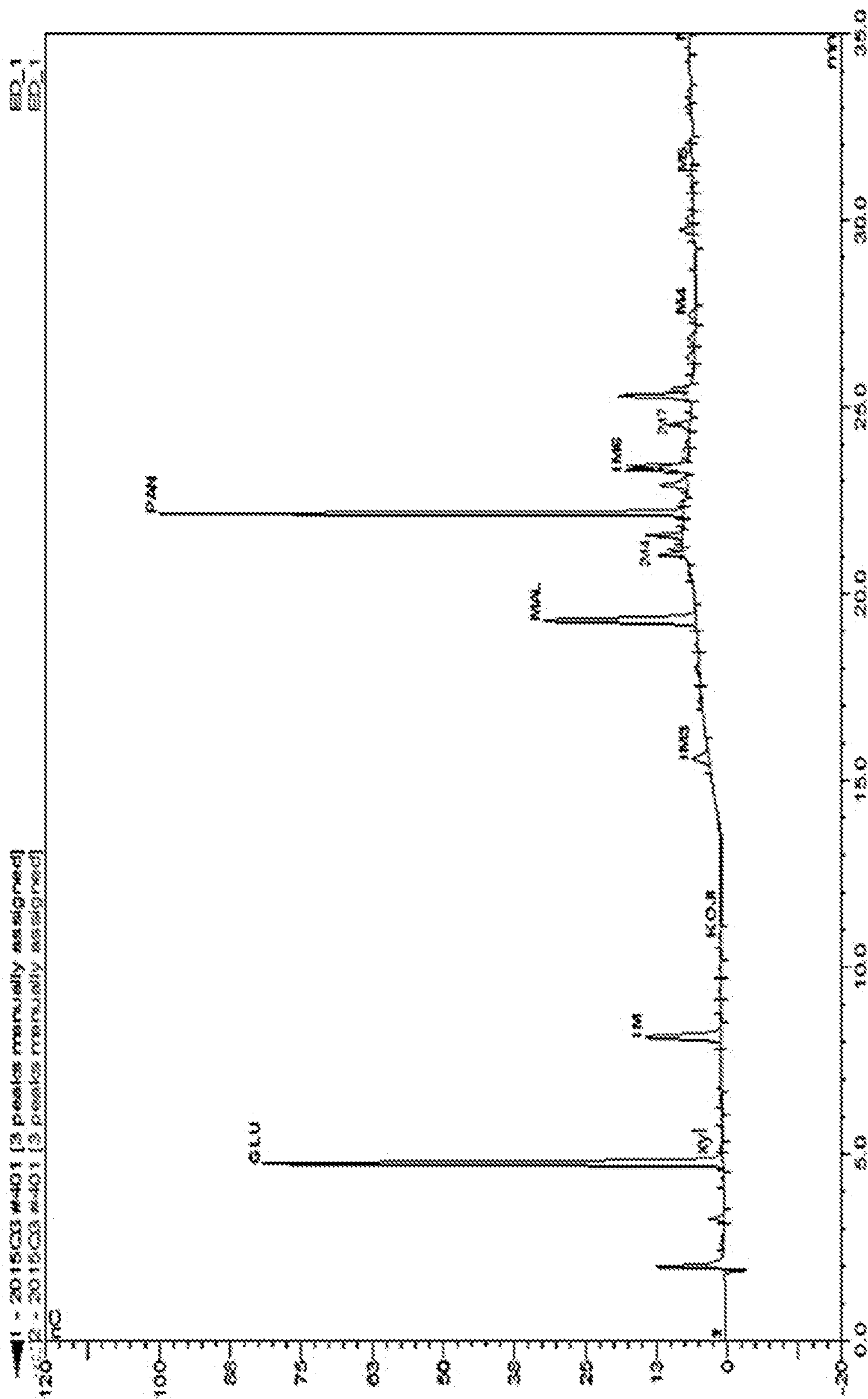


FIG. 1

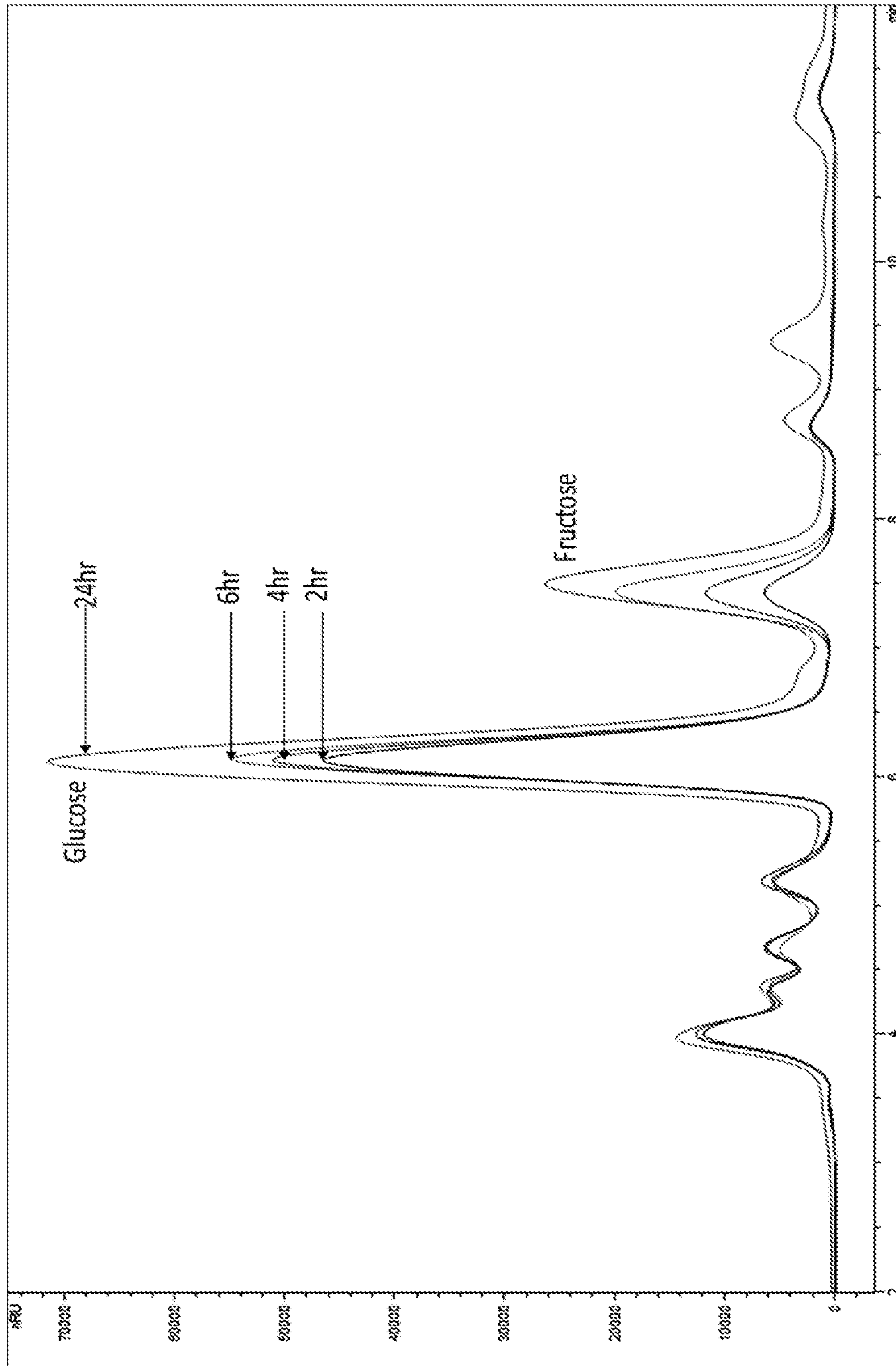


FIG. 2

FIG. 3A

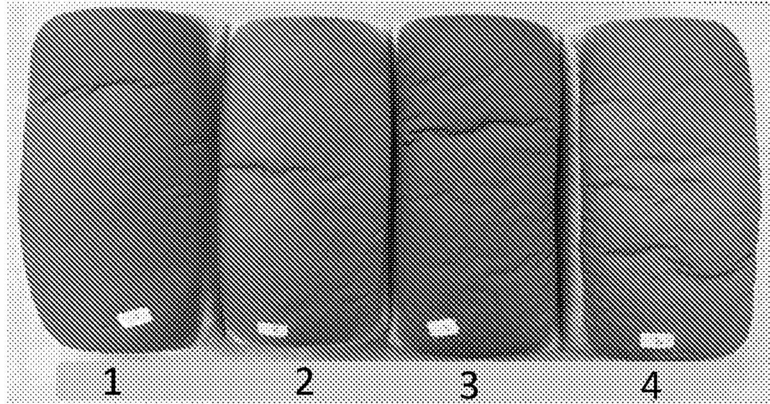


FIG. 3B

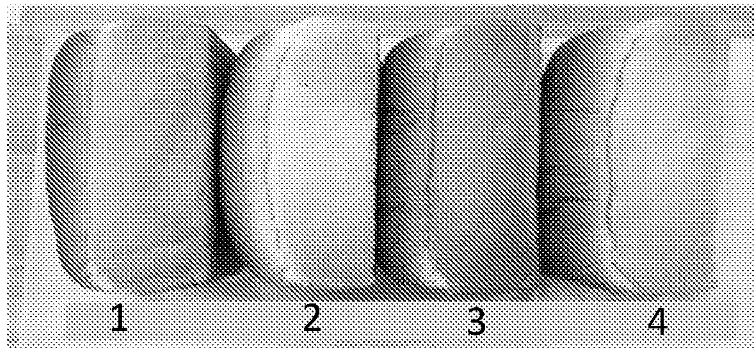
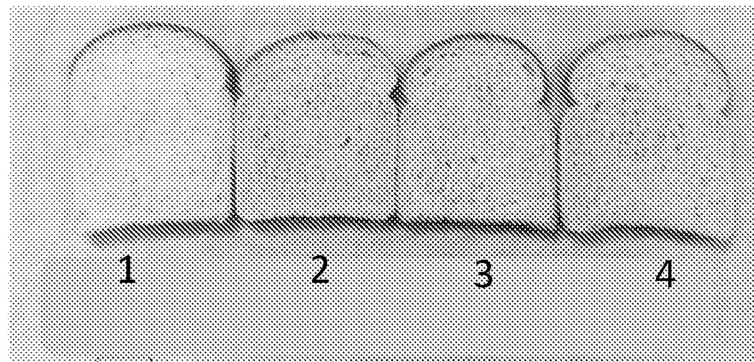


FIG. 3C



Control with Sucrose Example # 2 Example # 3 Example # 4

Control Spezyme RSL TRGA CS4 TG L-2000

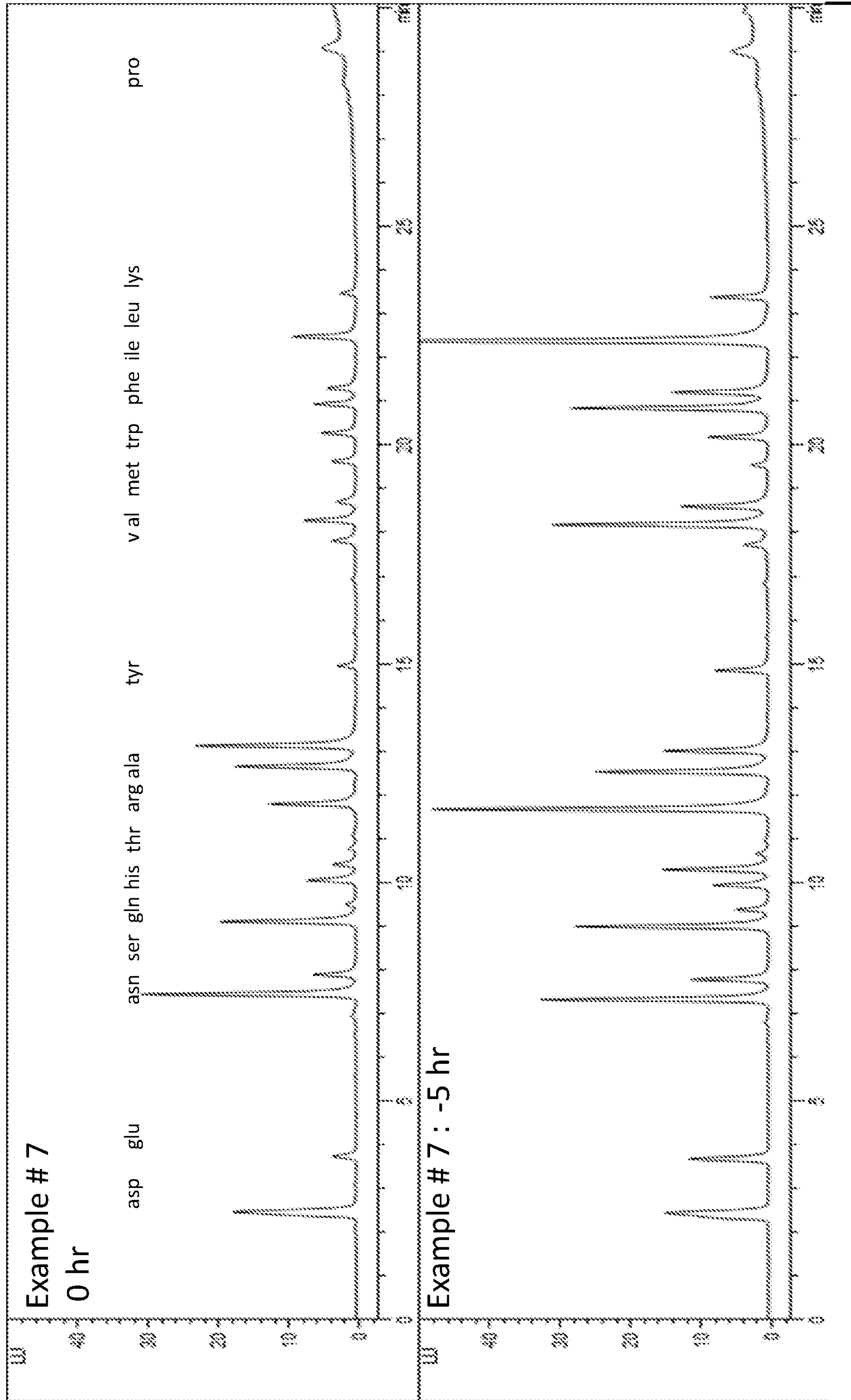


FIG. 4

Example # 1 Example # 7

(With added sugar-Table # 5)

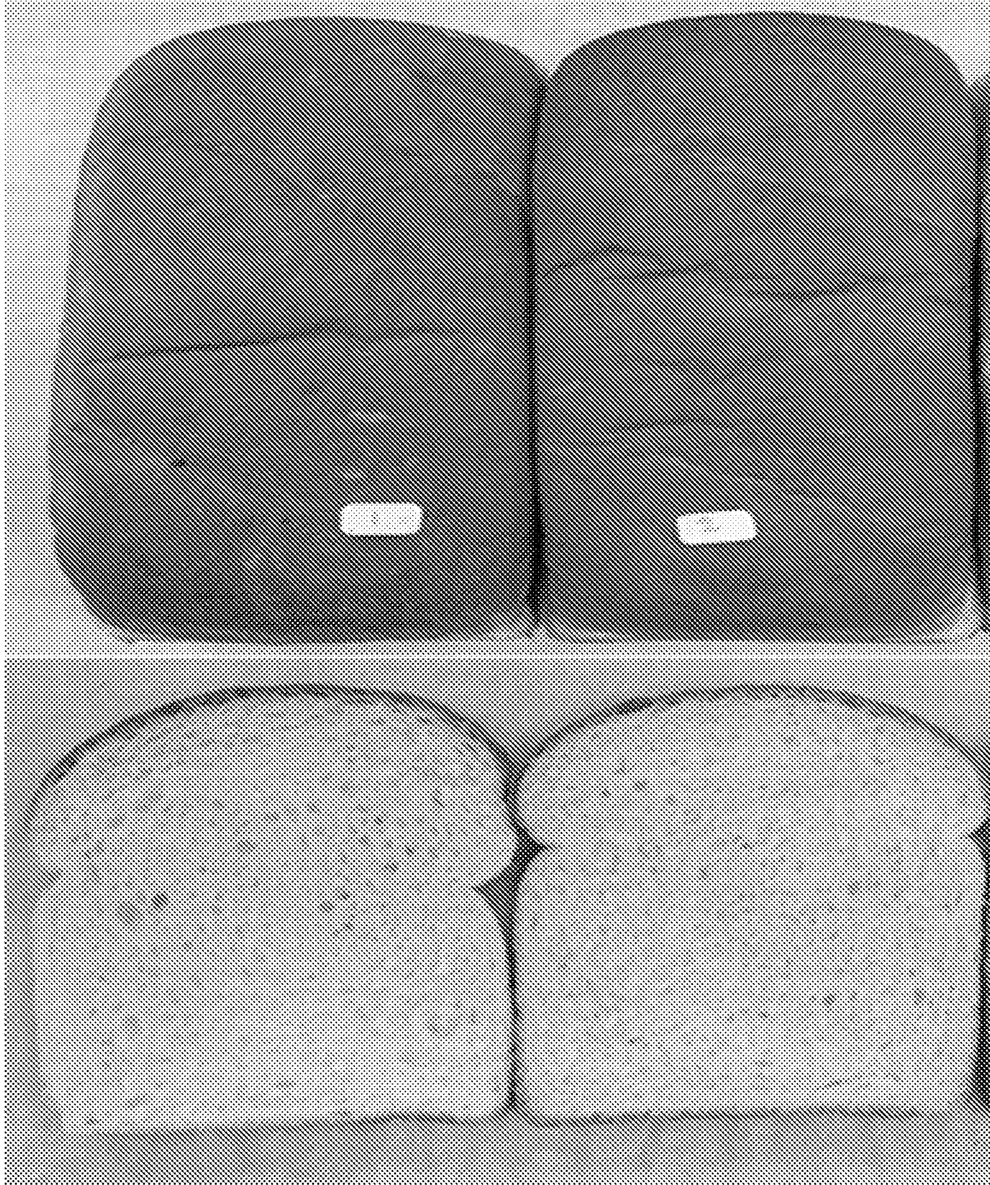


FIG. 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/033942

A. CLASSIFICATION OF SUBJECT MATTER
INV. A21D8/04 A21D13/062
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A21D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
4 August 2017	23/08/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Couzy, François

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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