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(54) Title: MUTAROTASE IN CRYSTALLIZATION

(57) Abstract: This invention describes the use of mutarotase (also known as aldose-1-epimerase) in a process for carbohydrate crystallization. Preferably the carbohydrate is a reducing sugar, more preferably the carbohydrate is lactose. The mutarotase can be obtained from an organism, preferably a micro-organism, more preferably from a fungus, even more preferably from a fungus of the genus *Aspergillus*, most preferably from *Aspergillus niger*. Carbohydrate crystallization can be performed from a pure, partly purified or a crude solution of carbohydrate. In general, carbohydrates are first concentrated into a supersaturated solution, after which temperature decreases and optional seeding with carbohydrate crystals initiate crystallization. Mutarotase is preferably added before or during the crystallization process.



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MUTAROTASE IN CRYSTALLIZATION

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Field of the invention

The invention relates to the use of mutarotase in crystallization of carbohydrates.

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Background of the invention

Crystallization is one of the main separation and purification processes used in the production of a wide range of materials. It is used in many industries to remove a valuable substance from an impure mixture, glucose monohydrate from hydrolyzed starch, for example. Industrial crystallization processes in the chemical, pharmaceutical, and food industries mostly involve crystallization from solution. The crystallizing form can be anhydrous or hydrated depending on the operating conditions; however, usually only one crystal form is stable at a particular condition. D-Glucose, for example, can be crystallized from aqueous solution in three different forms: α -D-glucose monohydrate, anhydrous α -D-glucose, and anhydrous β -D-glucose. The first two forms are produced commercially, while the last one is available as a specialty chemical.

The crystallization of D-glucose also involves mutarotation. In solution both α -D-glucose and β -D-glucose anomers exist simultaneously and undergo reversible mutarotation along with crystallization. The equilibrium ratio of the concentration of β -D-glucose to α -D-glucose in aqueous solutions is approximately 1.5 independent of temperature and total glucose concentration. If a part of the α -anomer is crystallized out, part of the β -anomer slowly converts into the α -anomer and, if necessary, vice versa. If the rates of the mutarotation reactions are slower than the rates of crystallization, the crystallizing anomer will be depleted such that it is below the anomeric equilibrium in solution, leading to a decrease in the driving force for crystallization. This behavior has also been reported for fructose crystallized from aqueous ethanol solutions, where the mutarotation kinetics is particularly slow due to the less polar nature of the solvent. A similar behavior was described for galactose,

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including initial nucleation of the unstable crystalline form, followed by a solution-mediated (mutarotation controlled) phase transition to the stable α -crystalline form. Also for lactose the stable crystalline form is the α -anomer.

5 Lactose is the most abundant component of milk of most mammals. The approximate concentration in milk is between 2.0% and 10%. The lactose content of bovine milk ranges between 4.4 and 5.2 % averaging at 4.8% anhydrous lactose. This compares with 7% in human milk.

10 As one of the commonly used sugars, lactose is utilized in many areas. In the food industry, lactose is widely used as an ingredient in confectionary, beverages, infant foods, frozen foods or in prepared foods, etc. In the pharmaceutical industry lactose is used as additive for tableting. In the chemical industry, lactose can be used as a basis for the production of lactulose, lactitol, galactose oligosaccharides and lactobionic acid, etc. Lactose is also used for feed in the agricultural industry.

15 Lactose is a disaccharide comprising one glucose molecule linked to a galactose molecule, and can be described chemically as 4-O[^]-galactopyranosyl-D-glucopyranose. Lactose occurs in two anomeric forms in solution, α -lactose and β -lactose that differ by the position of the hydroxyl group at the C-1 position of the glucose moiety when lactose is in the pyranose form. Both anomers can convert in
20 each other via the open chain aldehyde of the sugar. This spontaneous process is called mutarotation. The rate of mutarotation is dependent on temperature: it is slow at low temperatures, but almost instantaneous at high temperatures. At room temperature it takes many hours before mutarotation equilibrium is reached; at 70 °C only a few minutes. Also the concentration has an effect on the mutarotation rate, being slower at
25 higher lactose concentration. An equilibrium solution of lactose at ambient temperature has a ratio of α -lactose to β -lactose of approximately 2:3.

30 The chemical composition of lactose has a large effect on the physical properties. The two anomeric forms of lactose differ considerably in solubility and in the temperature dependence of solubility; α -lactose is much less soluble than β -lactose at low temperatures. If α -lactose is brought in water, much less dissolves at the outset than later. This is because of mutarotation: α -lactose is converted into β -lactose, hence the α -concentration diminishes and more α can dissolve. If β -lactose is brought in

water, more dissolves at the outset than later: on mutarotation more α -lactose forms that start to crystallize.

Lactose solutions can be supersaturated easily and to a considerable extent. At concentrations over 2.1 times the saturation concentration, spontaneous crystallization occurs rapidly. At less than 1.6 times the saturation concentration, seeding with crystals usually is needed to induce crystallization, unless a very long time is waited. This solution is metastable.

Because of the poor solubility of α -lactose, cooling of supersaturated lactose solutions will lead to the specific crystallization of α -lactose. The β -lactose will initially stay in solution. Since crystallization of α -lactose will disturb the equilibrium between both anomers in solution, mutarotation of β -lactose to α -lactose will replete the crystallized α -lactose. With sufficient cooling, in theory almost all lactose can be crystallized this way.

Lactose production

Chemically, commercial lactose is available in the most stable form, namely, crystalline α -lactose monohydrate. Depending on the different requirements of the application, lactose products have different grades: crude, food (edible), pharmaceutical, etc. The quality aspects for lactose mainly include purity (content of lactose, impurities and moisture), color, crystal size distribution (mean size and deviations), microorganism cell count, odor, etc. Generally speaking, products with high quality should have a higher content of lactose, fewer impurities, less moisture, a higher whiteness, a desired size with narrow distribution and fewer microorganisms.

Crystallization is a means to obtain commercial products of lactose from whey permeate. However, to efficiently produce high quality α -lactose monohydrate, other operations combined with crystallization are necessary. The dry solids in whey are mainly lactose, proteins and minerals. Lactose is the largest portion of the whey solids and can be processed using evaporation, crystallization, mechanical separation, further purification and drying.

The first step in refining lactose is to have either whole whey or whey permeate from ultrafiltration, with or without reversed osmosis (RO) filtration and concentration in an evaporator. Prior to the evaporation step, it is possible to precipitate the calcium from

whey or permeate. The concentrated whey or whey permeate from the evaporator with a dry solids content of about 60%, is fed into crystallization tanks where a very controlled cooling regimen must be employed to develop lactose crystals at a maximum yield. Agitation is applied to the crystallization tanks during this process. The liquid becomes supersaturated for lactose after which lactose crystals are often added as seeding for the crystallization process. Crystallization proceeds slowly as the temperature of the suspension is gradually decreased from about 80 °C to about 25 °C.

The slurry from the crystallization tanks is then passed through a decanter or centrifuge, where the main portion of the lactose crystals is separated. The crystals are then washed in order to remove residuals of the mother liquor, after which it is dried, often on a fluid bed dryer. The average crystal size (200-250 μm) is of importance for the fluidization velocity that can be used. Crystal size influences the performance of the fluid bed, as smaller crystals would require lower fluidization velocity or else too high carry-over of product to the bag filter would be the result. A pre-crystallization step of 48 hours is often used to "grow" the size of lactose crystals.

After drying the powder is often cooled with dehumidified air. Lactose produced this way is termed crude lactose. More refined pharmaceutical lactose is produced by re-dissolving the washed crystals from above mentioned decanter and then re-crystallizing a second time by cooling followed by another decanting/washing prior to the final drying. The lactose powder exiting the dryer is very often classified with different crystal sizes. Milling of the powder after the fluid bed and final sifting is often required.

Crystallization is a complex physico-chemical phenomenon of phase transition. Many factors including concentration, temperature, viscosity of the solution, agitation intensity, etc., have an impact on crystallization. To efficiently obtain high quality lactose, crystallization must be optimally controlled. However, the methods used in the crystallization process currently known in the art are far from optimal. In the currently existing processes, the filling of the crystallizer takes about 6 hours. Cooling and crystallization last for 14-18 hours. Therefore, the currently existing process takes 20-24 hours for crystallization. Crystallization is therefore the most important operation step in the process and affects the efficiency of the process and the product quality. The crystallization step leads to a lengthy occupancy of large crystallization tanks.

Consequently, this leads to large capital investments for the lactose producer, and a slow process operation. Additionally, long occupancy of crystallization tanks also increases the chance of bacterial contamination and spoilage of the content.

5 Additionally, the yield of the crystallization process is poor. A large part of the lactose is not crystallized and stays soluble in the whey (permeate), even after the crystallization process. AU2005239622 revealed that maximum lactose yield increases from 61.6% at 60 °C to 79.8 % at 40 °C.

10 AU2005239622 teaches specifically that the level of supersaturation, the mutarotation rate, the viscosity and the nucleation rate, are important parameters that influence the rate of lactose crystallization. At high temperatures the rate of mutarotation will be high, but the supersaturation, viscosity and nucleation rate will be low. At lower temperatures the supersaturation, viscosity and nucleation rate will be high, but the mutarotation rate will be low. AU2005239622 teaches that the optimum
15 temperature for fast lactose crystallization will be approximately 50 degrees Celsius, which is an acceptable average to obtain sufficient lactose yield in time. Although lowering the crystallization temperature can clearly increase lactose yield, the process becomes inhibitory slow. Therefore, crystallization rate decreases at lower temperature, requiring a longer occupancy of the crystallization tanks, with additional risks and costs.

20 A method that would improve carbohydrate crystallization in general and lactose crystallization in specific would be beneficial for industry.

Summary of the invention

25 This invention describes the use of mutarotase (also known as aldose-1-epimerase) in a process for carbohydrate crystallization. Preferably the carbohydrate is a reducing sugar, more preferably the carbohydrate is lactose. The mutarotase can be obtained from an organism, preferably a micro-organism, more preferably from a fungus, even more preferably from a fungus of the genus *Aspergillus*, most preferably from *Aspergillus niger*. Carbohydrate crystallization can be performed from a pure,
30 partly purified or a crude solution of carbohydrate. In general, carbohydrates are first concentrated into a supersaturated solution, after which temperature decreases and optional seeding with carbohydrate crystals initiate crystallization. Mutarotase is preferably added before or during the crystallization process.

The use of a mutarotase during carbohydrate crystallization has advantages over the prior art in that it can speed up crystallization thereby decreasing residence time in the crystallization tank. A shorter crystallization process will also minimize risks for spoilage of the crystallization solution. Additional benefits are an increase in purity, quality and/or uniformity of the carbohydrate crystals. Additional, the use of mutarotase during crystallization may lead to a yield increase of the carbohydrate.

Description of SEQ ID numbers

SEQ ID NO: 1 protein sequence of an *Aspergillus niger* mutarotase; a possible processing site in the amino acid sequence of SEQ ID NO: 1 is after amino acid 22 or 23; i.e. the mature protein is defined by the sequence as represented by amino acids 23-403 or 24-403

SEQ ID NO: 2 genomic sequence

SEQ ID NO: 3 coding sequence

SEQ ID NO: 4 forward primer

SEQ ID NO: 5 reverse primer

Detailed description of the invention

In a first embodiment, the invention provides a method for improving carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate.

In a more preferred first embodiment, the invention provides a method for improving carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate and wherein said protein having mutarotase activity is active during crystallization.

Whether or not a process for carbohydrate crystallization is improved is determined by comparing the claimed process to a control process which does not include the addition of a protein having mutarotase activity and in which all other process parameters are identical (for example time and temperature). Features that can be improved are: time needed for crystallization (preferably, said time is decreased), purity of final product (preferably, said purity is increased), , uniformity (i.e. the predetermined crystal size and optionally a narrow size distribution/deviation) of final

product (preferably, the uniformity is increased) or yield of final product (preferably, said yield is increased). The invention therefore also provides:

- a method for reducing carbohydrate crystallization time comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate,
- a method for increasing the purity of a product of carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate,
- a method for increasing the uniformity of a product of carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate, or
- a method for increasing yield of carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate.

More preferably, the invention provides:

- a method for reducing carbohydrate crystallization time comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate and wherein said protein having mutarotase activity is active during crystallization,
- a method for increasing the purity of a product of carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate and wherein said protein having mutarotase activity is active during crystallization,
- a method for increasing the uniformity of a product of carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate and wherein said protein having mutarotase activity is active during crystallization, or
- a method for increasing yield of carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate and wherein said protein having mutarotase activity is active during crystallization.

The phrase "and wherein said protein having mutarotase activity is active during crystallization" is used to refer to the fact that at least 50% of the total amount of added

enzyme is active during crystallization. Even more preferably, at least 60, 70 or 80% of the total amount of added enzyme is active during crystallization. Most preferred at least 85, 90 or 95% of the total amount of added enzyme is active during crystallization.

In a preferred embodiment, at least two features of carbohydrate crystallization are improved, for example yield and process time, i.e. increased yield and decreased processing time.

A protein having mutarotase activity / mutarotase

Mutarotase (aldose 1-epimerase) accelerates the establishment of equilibrium between the alpha- and beta-anomers of aldoses, for example between alpha- and beta-glucose or alpha- and beta-galactose. The current main application of the enzyme is in analytical biochemistry for the acceleration of enzymatic detection reactions for aldoses by means of enzymes specific for the alpha- or beta-form, where the establishment of an equilibrium between the two anomers can become the rate-determining step, for example in sugar determination methods with glucose-dehydrogenase, glucose-oxidase, galactose-dehydrogenase or galactose-oxidase. Such determinations are conventional and are described in U.S. Pat. No. 3,964,974 and Anal. Biochem. 43, 312 (1971).

Mutarotase is very widespread in nature. It occurs in various microorganisms (bacteria, yeasts, thread fungi), in plants and in animal tissues.

Bacterial galactose mutarotases play a key role in the Leloir pathway by catalyzing the interconversion of β -D-galactose and α -D-galactose. In several bacterial species, especially in *Escherichia coli* and in *Lactococcus lactis*, the enzyme and its gene galM, a cistron of the gal operon, have been studied in detail. In contrast to bacteria, in various yeast species such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis* and *Pachysolen tannophilus* the mutarotase activity is assumed to be located at the C-terminal domain of the Galp protein, while the N-terminal domain of Galp has the UDP-glucose 4-epimerase activity needed for the interconversion of UDP-galactose and UDP-glucose in the galactose metabolism.

Significant enzyme contents which allow isolation of mutarotase on an industrial scale have so far only been found in the kidneys of mammals (cattle, pigs) and the known commercial products are prepared from kidneys. It is known from Bailey (Meth. Enzymol. 1975, page 478) that bovine kidneys contain more than 60 times the mutarotase activity per g of fresh weight than, for example, *Escherichia coli*.

Until recently little was known about possible genes encoding mutarotase in filamentous fungi. A gal10 gene has been identified in the ascomycete fungus *Hypocrea jecorina*. The gene codes for an enzyme that only shows strict conservation of the amino acid residues and motifs characteristic of the UDP-glucose 4-epimerases at the N-terminal domain of the yeast homologues. It was, therefore, speculated that the *H. jecorina* Gall op lacks the mutarotase activity and that the UDP-glucose 4-epimerase and the mutarotase are two independent enzymes in this organism. Additionally, it was reported that a stress inducible protein, encoded by the cDNA from the zygomycete filamentous fungus *Rhizopus nigricans* (GenBank Accession No. Y10414), exhibits moderate amino acid sequence similarity in a 113 amino acid overlap with the galactose mutarotase from *Acinetobacter calcoaceticus* and the C-terminal domains of Gall Ops from *K. lactis* and *S. cerevisiae*. The alignment analysis also revealed the presence of two histidine residues, strictly conserved among different mutarotases. The mutarotase protein could be isolated from mycelial extracts of *R. nigricans* and the gene coding for the mutarotase was expressed in insect cells and mutarotase activity of a recombinant expressed enzyme was determined.

Additionally, Bentley and Bhate (1960) (J. Biol. Chem. 235, 1219-1233) have described the partial isolation and characterization of a mutarotase enzyme from the mycelial biomass of the fungus *Penicillium notatum*. *P. notatum* mutarotase was shown to be active on both glucose and galactose. In Biochim. Biophys. Acta 662, 285 (1981), Kinoshita et al. describe a process for the microbiological preparation of mutarotase from a strain of *Aspergillus niger*. According to this, a mutarotase activity of 4.4 mU/ml of culture broth was obtained from the best strain, the Michaelis constant was 50 mM, and the pH optimum was in the range 5-7. The *Aspergillus* mutarotase isolated by Kinoshita et al. has an estimated size of 260,000 Da, which appears to be a homodimer existing of 2 subunits of 130,000 Da. Like with *Penicillium notatum*, the *Aspergillus* enzyme was isolated from the mycelium of the fungus, this time using a homogenizer

and glass-bead agitation. These results show that all the currently described fungal mutarotases are naturally located intracellularly.

The *Aspergillus niger* mutarotase described by Kinoshita et al. was also tested in the mutarotation of different sugars. While the enzyme was shown to be highly active on both glucose and galactose, on other sugars (among which was lactose) stimulation of mutarotation compared to the spontaneous reaction was only small. Similar results have been found with the *Penicillium notatum* mutarotase and mutarotase from hog kidney.

The mechanistic aspects of mutarotation have been reviewed by Samuel and Tanner (Nat. Prod. Rep. 2002, 19, 261-277). Theoretically, mutarotation could be brought about by breaking any of the four bonds at C-1 of an aldose. The C(1)-O(ring) bond could be broken by a ring-opening reaction involving general acid/general base catalysis. Alternatively, the C(1)-H bond could be broken in a mechanism involving transient oxidation. However, this would require the presence of a cofactor. A third possibility is a mechanism similar to that of glycosidases, and involves cleavage of the C(1)-O(anomeric) bond with the addition of water. In the mutarotase isolated from *Penicillium notatum* no significant exchange of the C-1 oxygen or hydrogen has been observed and no primary deuterium kinetic isotope effect has been observed at C-1. This argues against the last two mechanisms. The formation of β -D-galactofuranose as a minor product when α -D-galactopyranose is allowed to equilibrate in water in the presence and absence of galactose mutarotase supports the mechanism involving ring opening. Thus, it seems that both the non-enzymatic and enzymatic reactions occur via an initial ring opening followed by ring closure with addition of the opposite face of the aldehyde. An enzymatic base of pKa 5.5 has been implicated as the catalytic residue. *E. coli* galactose mutarotase has been cloned, over-expressed and purified to homogeneity. It has been shown to be a part of the gal operon and is involved in the Leloir pathway. The enzyme lacks metal ions and oxidoreduction cofactors. Using the recombinant mutarotase, it has been shown that the enzyme catalyzes both the ring opening as well as closure. Mutagenesis studies on two conserved histidine residues suggest that one of them might be involved in catalysis.

The phrase "a protein having mutarotase activity" refers to a protein which is capable of converted a sugar in alpha conformation to a sugar in beta conformation and vice versa. Whether or not a protein has mutarotase activity is for example determined by assays that are known in the art (Miwa (1972) Analytical Biochemistry 45, 441-447; Miwa and Okuda (1974) J. Biochem. 75, 1177-1179; [www.bbienzymes.com/userfiles/file/ap46mutarotase01 .pdf](http://www.bbienzymes.com/userfiles/file/ap46mutarotase01.pdf)). An example of such a mutarotase assay is described in Example 2 .The protein does not necessarily have to be annotated as a mutaroase or as an aldose 1-epimerase. It might well be that a protein domain having mutarotase activity is fused to another protein sequence or protein domain and that the fusion product is not annotated as being a mutarotase.

In a preferred embodiment, the invention provides a method for improving carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate and wherein said protein having mutarotase activity is active during crystallization, wherein said protein having mutarotase activity is a protein having at least 50% identity with the protein of SEQ ID NO: 1.

In yet another preferred embodiment, the invention provides a method for improving carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate and wherein said protein having mutarotase activity is active during crystallization, wherein said protein has at least mutarotase activity for said carbohydrate, i.e. the carbohydrate that needs to be crystallized. For example, the invention provides a method for improving lactose crystallization comprising adding a protein having mutarotase activity before or during crystallization of said lactose and wherein said protein having mutarotase activity is active during crystallization, wherein said protein has at least mutarotase activity for lactose.

In another embodiment, the invention provides a method for improving carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate and wherein said protein having mutarotase activity is active during crystallization and wherein said protein having mutarotase activity is immobilized on a carrier. Preferably said carrier and immobilized protein having mutarotase activity can easily be separated from the final product (i.e. the crystallized carbohydrate).

All currently known mutarotases are intracellular proteins. Consequently, productivity of such enzymes is poor and industrial production of such enzymes for the use in the food, pharma or chemical industry is not (economically) feasible. Mutarotase isolated from porcine kidney is available but is very expensive and only suitable for laboratory scale testing. For instance, the use of mutarotase in the development of glucose sensors has been described. Furthermore, all described mutarotases are poorly active on lactose and generally prefer mono-hexoses or pentoses as substrate. E.g. the mutarotase from *Escherichia coli* has a K_m of 475 mM for lactose, while the K_m for glucose and galactose is 15 and 6.5 mM respectively (Hucho and Wallenfels (1971) Eur. J. Biochem. 23, 489-496). Consequently, the efficient mutarotation of lactose is not possible with currently available enzymes.

Here we describe the overexpression of a mutarotase that is active on lactose as substrate. The efficient production of this enzyme makes its use in crystallization of carbohydrates for the food, pharma or chemical industry (economically) feasible.

The nucleotide sequence of the gene expressing the mutarotase protein used in the experimental part has been published as NCBI Reference Sequence NT_166519.1. The protein sequence deduced from this gene sequence is publically available under accession number CAK44320.1 at GenBank and is furthermore presented as SEQ ID NO: 1 in the sequence listing accompanying this patent application. This protein sequence has 25-30% sequence identity when it is compared with galactose mutarotases from different mammalia, such as mouse, rat, human, bovine or porcine, using the pBLAST algorithm on the NCBI server (<http://www.ncbi.nlm.nih.gov/>). Identity of the *Aspergillus niger* mutarotase amino acid sequence with putative mutarotase from bacteria run up to 32% when using the same algorithm. Similar identities can be found with putative mutarotases from plants. Identity of the *Aspergillus* mutarotase with the yeast *Gall op* is only 25%.

These relative poor identities with known mutarotases indicate that the *Aspergillus niger* mutarotase is drastically different from all currently described mutarotases. For the one skilled in the art it will be obvious that the *Aspergillus niger* mutarotase can be substituted for any other mutarotase active in the application, for the purpose of this invention. Preferably such other mutarotase is a secreted mutarotase and even more

preferably such mutarotase is a secreted fungal mutarotase. In another embodiment such mutarotase would be active on carbohydrates, preferably active on sugars, and more preferably on lactose.

5 Advantageously the protein having mutarotase activity of the invention is an extracellular enzyme. Moreover the protein having mutarotase activity is preferably of a fungal origin. An exoenzyme, or extracellular enzyme, is an enzyme that is secreted by a cell and that works outside that cell. It is usually used for breaking up large molecules that would not be able to enter the cell otherwise. It is an enzyme that organisms
10 secrete into the environment which acts outside the microbe. The opposite of an exoenzyme is called an endoenzyme or intracellular enzyme.

 The present invention provides a method for applying the protein having mutarotase activity which has been identified in the fungus *Aspergillus niger* in carbohydrate crystallization.

15 Advantageously the present invention meets the demand for a protein having mutarotase activity that can be produced in high amounts. Preferably, such a protein having mutarotase activity is secreted from a host cell. Active secretion is of paramount importance for an economical production process because it enables the recovery of the enzyme in an almost pure form without going through cumbersome purification
20 processes. Overexpression of such an actively secreted protein having mutarotase activity by a food grade fungal host such as *Aspergillus*, yields a food grade enzyme and a cost effective production process, and is therefore preferable. The presently secreted protein having mutarotase activity is for the first time found in filamentous fungi. Processes are disclosed for the production of a protein having mutarotase activity
25 in large amounts by the food-grade production host *Aspergillus niger*.

 From an economic point of view there exists a clear need for an improved means of producing a protein having mutarotase activity in high quantities and in a relatively pure form, compared to the poor productivity of the mammalian and bacterial
30 mutarotases. A preferred way of doing this is via the overproduction of such a mutarotase using recombinant DNA techniques. A particularly preferred way of doing this is via the overproduction of a fungal derived protein having mutarotase activity and a most preferred way of doing this is via the overproduction of an *Aspergillus* derived

protein having mutarotase activity. To enable the latter production route unique sequence information of an *Aspergillus* derived mutarotase is essential. More preferable the whole nucleotide sequence of the encoding gene has to be available.

An improved means of producing the newly identified secreted protein having mutarotase activity in high quantities and a relatively pure form is via the overproduction of the *Aspergillus* encoded enzyme using recombinant DNA techniques. A preferred way of doing this is via the overproduction of such a secreted protein having mutarotase activity in a food grade host microorganism. Well known food grade microorganisms include *Aspergilli*, *Trichoderma*, *Fusarium*, *Streptomyces*, *Bacilli* and yeasts such as *Saccharomyces* and *Kluyveromyces*. An even more preferred way of doing this is via overproduction of the secreted *Aspergillus* derived protein having mutarotase activity in a food grade fungus such as *Aspergillus*. Most preferred is the over production of the secreted protein having mutarotase activity in a food grade fungus in which the codon-usage of the mutarotase-encoding gene has been optimized for the food grade expression host used. In general, to enable the latter optimization routes, unique sequence information of a secreted protein having mutarotase activity is desirable. More preferable the whole nucleotide sequence of the protein having mutarotase activity encoding gene has to be available. Once the gene encoding a secreted protein having mutarotase activity is transformed in a preferred host, selected strains can be used for fermentation and isolation of the secreted protein having mutarotase activity protein from the fermentation broth.

A polypeptide, used in a method of the invention, which has mutarotase activity (i.e. protein having mutarotase activity) may be in an isolated form. As defined herein, an isolated polypeptide is an endogenously produced or a recombinant polypeptide which is essentially free from other non-mutarotase polypeptides, and is typically at least about 20% pure, preferably at least about 40% pure, more preferably at least about 60% pure, even more preferably at least about 80% pure, still more preferably about 90% pure, and most preferably about 95% pure, as determined by SDS-PAGE. The protein having mutarotase activity may be isolated by centrifugation, filtration and chromatographic methods, or any other technique known in the art for obtaining pure proteins from crude solutions. It will be understood that the protein having mutarotase activity may be mixed with carriers or diluents which do not interfere with the intended

purpose of the protein having mutarotase activity, and thus the protein having mutarotase activity in this form will still be regarded as isolated. It will generally comprise the protein having mutarotase activity in a preparation in which more than 20%, for example more than 30%, 40%, 50%, 80%, 90%, 95% or 99%, by weight of the proteins in the preparation is a protein having mutarotase activity.

Preferably, the protein having mutarotase activity is obtainable from a microorganism which possesses a gene encoding an enzyme with mutarotase activity. More preferably the protein having mutarotase activity is secreted from a microorganism. Even more preferably the microorganism is fungal, and optimally is a filamentous fungus. Preferred donor organisms are thus of the genus *Aspergillus*, such as those of the species *Aspergillus niger*.

In a preferred embodiment, the invention provides a method for improving carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate and wherein said protein having mutarotase activity is active during crystallization, wherein said protein having mutarotase activity has an amino acid sequence which has a degree of amino acid sequence identity to amino acids 1 to 403 of SEQ ID NO: 1 of at least 50% or 60%, preferably at least 70%, more preferably at least 80%, even more preferably at least 90%, still more preferably at least 95%, and most preferably at least 97, 98 or 99%, and which has at least mutarotase activity for the carbohydrate that needs to be crystallized.

For the purposes of the present invention, the degree of identity between two or more amino acid sequences is determined by BLAST P protein database search program (Altschul et al., 1997, Nucleic Acids Research 25: 3389-3402) with matrix Blosum 62 and an expected threshold of 10.

In a more preferred embodiment, the invention provides a method for improving carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate and wherein said protein having mutarotase activity is active during crystallization, wherein said protein having mutarotase activity comprises the amino acid sequence set forth in SEQ ID NO: 1 or a fragment thereof. In general, the amino acid sequence shown in SEQ ID NO: 1 is preferred.

Also part of the invention is the use of a part of the amino acid sequence shown in SEQ ID NO: 1 in the claimed method. Secreted enzymes, like the amino acid sequence of SEQ ID NO: 1, are often synthesized including a pre- or signal-sequence and / or a pro-sequence. These sequences are often removed from the protein either during or
5 after the secretion process. The mature secreted protein therefore often does not contain these pre- and pro-sequences anymore. A processed version of SEQ ID NO: 1 lacking possible pre- or pro-sequences can also be used in a method of the invention. A possible processing site in the amino acid sequence of SEQ ID NO: 1 is after amino acid 22 or 23. The mature enzyme will in this case start at amino acid number 23 or 24.
10 Other modifications from the amino acid sequence of SEQ ID NO: 1 due to further processing are allowed as long that they do not disturb the activity of the enzyme.

In yet another preferred embodiment, the invention provides a method for improving carbohydrate crystallization comprising adding a protein having mutarotase
15 activity before or during crystallization of said carbohydrate and wherein said protein having mutarotase activity is active during crystallization, wherein said protein having mutarotase activity comprises a naturally occurring variant or species homologue of the polypeptide of SEQ ID NO: 1.

A variant is a polypeptide that occurs naturally in, for example, fungal, bacterial,
20 yeast or plant cells, the variant having mutarotase activity and a sequence substantially similar to the protein of SEQ ID NO: 1. The term "variants" refers to polypeptides which have the same essential character or basic biological functionality as the mutarotase of SEQ ID NO: 1, and includes allelic variants. Preferably, a variant polypeptide has at least the same level of mutarotase activity as the polypeptide of SEQ ID NO: 1.
25 Variants include allelic variants either from the same strain as the polypeptide of SEQ ID NO: 1 or from a different strain of the same genus or species.

Similarly, a species homologue of the protein having mutarotase activity is an equivalent protein of similar sequence which is a mutarotase and occurs naturally in another species.

30 Variants and species homologues can be found using homology search of the amino acid sequence of SEQ ID NO: 1 against protein or nucleotide databases, using methods known in the art. Also possible is to use a probe (designed on the corresponding nucleotide sequence of the protein of SEQ ID NO: 1) to probe DNA

libraries made from yeast, bacterial, fungal or plant cells in order to obtain clones expressing variants or species homologues of the polypeptide of SEQ ID NO: 1. The methods that can be used to isolate variants and species homologues of a known gene are extensively described in literature, and known to those skilled in the art. These genes can be manipulated by conventional techniques to generate a polypeptide of the invention which thereafter may be produced by recombinant or synthetic techniques known *per se*.

The sequence of the polypeptide of SEQ ID NO: 1 and of variants and species homologues can also be modified to provide polypeptides for use in a method of the invention. Amino acid substitutions may be made, for example from 1, 2 or 3 to 10, 20 or 30 substitutions. The same number of deletions and insertions may also be made. These changes may be made outside regions critical to the function of the protein having mutarotase activity, as such a modified protein having mutarotase activity will retain its mutarotase activity.

A method according to the invention can also use a protein having mutarotase activity which protein is a fragment of the above mentioned full length polypeptide and of variants thereof, including fragments of the sequence set out in SEQ ID NO: 1. Such fragments will typically retain activity as a mutarotase (at least for the carbohydrate that needs to be crystallized). Fragments may be at least 50, 100 or 200 amino acids long or may be this number of amino acids short of the full length sequence shown in SEQ ID NO: 1.

A protein having mutarotase activity can, if necessary, be produced by synthetic means although usually they will be made recombinantly as described below. Synthetic and recombinant polypeptides may be modified, for example, by the addition of histidine residues or a T7 tag to assist their identification or purification, or by the addition of a signal sequence to promote their secretion from a cell.

Thus, the variants sequences may comprise those derived from strains of *Aspergillus* other than the strain from which the polypeptide of SEQ ID NO: 1 was isolated. Variants can be identified from other *Aspergillus* strains by looking for mutarotase activity and cloning and sequencing as described herein. Variants may include the deletion, modification or addition of single amino acids or groups of amino acids within the protein sequence, as long as the peptide maintains the basic biological functionality of the mutarotase of SEQ ID NO: 1.

Amino acid substitutions may be made, for example from 1, 2 or from 3 to 10, 20 or 30 substitutions. The modified a protein having mutarotase activity will generally retain activity as a mutarotase. Conservative substitutions may be made; such substitutions are well known in the art.

5 Shorter or longer polypeptide sequences can also be used in a method according to the invention. For example, a peptide of at least 50 amino acids or up 100, 150, 200, 300, 400, 500, 600, 700 or 800 amino acids in length is considered to fall within the scope of the invention as long as it demonstrates the basic biological functionality of the mutarotase of SEQ ID NO: 1. In particular, but not exclusively, this
10 aspect of the invention encompasses the situation in which the protein is a fragment of the complete protein sequence.

The present invention also relates to a polynucleotide which encodes a protein having mutarotase activity, said polynucleotide comprises a polynucleotide sequence which encodes amino acid SEQ ID NO: 1.

15 For the present invention it is especially relevant that the protein having mutarotase activity is actively secreted into the growth medium. Secreted proteins are normally originally synthesized as pre-proteins and the pre-sequence (signal sequence) is subsequently removed during the secretion process. The secretion process is basically similar in prokaryotes and eukaryotes: the actively secreted pre-protein is
20 threaded through a membrane, the signal sequence is removed by a specific signal peptidase, and the mature protein is (re)-folded. Also for the signal sequence a general structure can be recognized. Signal sequences for secretion are located at the amino-terminus of the pre-protein, and are generally 15-35 amino-acids in length. The amino-terminus preferably contains positively charged amino-acids, and preferably no acidic
25 amino-acids. It is thought that this positively charged region interacts with the negatively charged head groups of the phospholipids of the membrane. This region is followed by a hydrophobic, membrane-spanning core region. This region is generally 10-20 amino-acids in length and consists mainly of hydrophobic amino-acids. Charged amino-acids are normally not present in this region. The membrane spanning region is followed by
30 the recognition site for signal peptidase. The recognition site consists of amino-acids with the preference for small-X-small. Small amino-acids can be alanine, glycine, serine or cysteine. X can be any amino acids. Using such rules an algorithm has been written that is able to recognize such signal sequences from eukaryotes and prokaryotes

(Bendtsen, Nielsen, von Heijne and Brunak. (2004) J. Mol. Biol., 340:783-795). The SignalP program to calculate and recognize signal sequences in proteins is generally available (<http://www.cbs.dtu.dk/services/SignalP/>).

Relevant for the present invention is that signal sequences can be recognized
5 from the deduced protein sequence of a sequenced gene. If a gene encodes a protein where a signal sequence is predicted using the SignalP program, the chance that this protein is secreted is high.

In a further embodiment, the present invention provides a method for improving carbohydrate crystallization comprising adding a protein having mutarotase activity
10 before or during crystallization of said carbohydrate and wherein said protein having mutarotase activity is active during crystallization, wherein said protein having mutarotase activity is encoded by polynucleotides which hybridize or are capable of hybridizing under low stringency conditions, more preferably medium stringency conditions, and most preferably high stringency conditions, with (i) the nucleic acid
15 sequence of SEQ ID NO:2 or SEQ ID NO:3 or (ii) a nucleic acid fragment comprising at least a portion of SEQ ID NO:2 or SEQ ID NO:3, or (iii) having bases differing from the bases of SEQ ID NO:2 or SEQ ID NO:3; or (iv) with a nucleic acid strand complementary to SEQ ID NO:2 or SEQ ID NO:3.

The term "capable of hybridizing" means that the target polynucleotide can
20 hybridize to the nucleic acid used as a probe (for example, the nucleotide sequence set forth in SEQ ID NO: 2 or SEQ ID NO:3, or a fragment thereof, or the complement of SEQ ID NO: 2 or SEQ ID NO:3, or a fragment thereof) at a level significantly above background. The nucleotide sequence may be RNA or DNA, including genomic DNA, synthetic DNA or cDNA. Preferably, the nucleotide sequence is DNA and most
25 preferably, a genomic DNA sequence. Typically, a polynucleotide comprises a contiguous sequence of nucleotides which is capable of hybridizing under selective conditions to the coding sequence or the complement of the coding sequence of SEQ ID NO: 2 or SEQ ID NO:3. Such nucleotides can be synthesized according to methods well known in the art.

30 Such nucleotides can hybridize to the coding sequence or the complement of the coding sequence of SEQ ID NO: 3 at a level significantly above background. Background hybridization may occur, for example, because of other cDNAs present in a cDNA library. The signal level generated by the interaction between polynucleotide as

described herein and the coding sequence or complement of the coding sequence of SEQ ID NO: 3 is typically at least 10 fold, preferably at least 20 fold, more preferably at least 50 fold, and even more preferably at least 100 fold, as intense as interactions between other polynucleotides and the coding sequence of SEQ ID NO: 3. The intensity of interaction may be measured, for example, by radiolabelling the probe, for example with ^{32}P . Selective hybridization may typically be achieved using conditions of low stringency (0.3M sodium chloride and 0.03M sodium citrate at about 40°C), medium stringency (for example, 0.3M sodium chloride and 0.03M sodium citrate at about 50°C) or high stringency (for example, 0.3M sodium chloride and 0.03M sodium citrate at about 60°C).

A polynucleotide as described herein also includes synthetic genes that can encode for the polypeptide of SEQ ID NO: 1 or variants thereof. It is sometimes preferable to adapt the codon usage of a gene to the preferred bias in a production host. Techniques to design and construct synthetic genes are generally available (i.e. <http://www.dnatwopointo.com/>).

Modifications

Polynucleotides as described herein may comprise DNA or RNA. They may be single or double stranded. They may also be polynucleotides which include within them synthetic or modified nucleotides including peptide nucleic acids. A number of different types of modifications to polynucleotides are known in the art. These include a methylphosphonate and phosphorothioate backbones, and addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art.

It is to be understood that skilled persons may, using routine techniques, make nucleotide substitutions that do not affect the polypeptide sequence encoded by the polynucleotides as described herein to reflect the codon usage of any particular host organism in which the polypeptides as described herein are to be expressed.

The coding sequence of SEQ ID NO: 3 or the genomic sequence of SEQ ID NO: 2 may be modified by nucleotide substitutions, for example from 1, 2 or 3 to 10, 25, 50, 100, or more substitutions. The polynucleotide of SEQ ID NO: 3 or SEQ ID NO: 2 may alternatively or additionally be modified by one or more insertions and/or deletions

and/or by an extension at either or both ends. The modified polynucleotide generally encodes a polypeptide which has mutarotase activity. Degenerate substitutions may be made and/or substitutions may be made which would result in a conservative amino acid substitution when the modified sequence is translated, for example as discussed
5 with reference to polypeptides later.

Homologues

A nucleotide sequence which is capable of selectively hybridizing to the complement of the DNA coding sequence of SEQ ID NO:3 or the genomic sequence of
10 SEQ ID NO: 2 is included for use in a method of the invention and will generally have at least 50% or 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98% or at least 99% sequence identity to the coding sequence of SEQ ID NO:3 or the genomic sequence of SEQ ID NO: 2 over a region of at least 60, preferably at least 100, more preferably at least 200 contiguous nucleotides or most preferably over the
15 full length of SEQ ID NO:3 or SEQ ID NO: 2. Likewise, a nucleotide which encodes an active mutarotase and which is capable of selectively hybridizing to a fragment of a complement of the DNA coding sequence of SEQ ID NO:3 or the genomic sequence of SEQ ID NO: 2, is also embraced for use in a method of the invention.

Any combination of the above mentioned degrees of identity and minimum sizes
20 may be used to define polynucleotides as described herein, with the more stringent combinations (i.e. higher identity over longer lengths) being preferred. Thus, for example, a polynucleotide which is at least 80% or 90% identical over 60, preferably over 100 nucleotides, forms one aspect of the invention, as does a polynucleotide which is at least 90% identical over 200 nucleotides.

25 The BLAST N algorithm can be used to calculate sequence identity or to line up sequences (such as identifying equivalent or corresponding sequences, for example on their default settings).

Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This algorithm
30 involves first identifying high scoring sequence pair (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold. These initial

neighborhood word hits act as seeds for initiating searches to find HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extensions for the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

The BLAST algorithm performs a statistical analysis of the similarity between two sequences. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a sequence is considered similar to another sequence if the smallest sum probability in comparison of the first sequence to the second sequence is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

Primers and Probes

Polynucleotides as described herein include and may be used as primers, for example as polymerase chain reaction (PCR) primers, as primers for alternative amplification reactions, or as probes for example labeled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, for example at least 20, 25, 30 or 40 nucleotides in length. They will typically be up to 40, 50, 60, 70, 100, 150, 200 or 300 nucleotides in length, or even up to a few nucleotides (such as 5 or 10 nucleotides) short of the coding sequence of SEQ ID NO: 3 or the genomic sequence of SEQ ID NO: 2.

In general, primers will be produced by synthetic means, involving a step-wise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this and protocols are readily available in the art. Longer polynucleotides will generally be produced using recombinant means, for example

using PCR cloning techniques. This will involve making a pair of primers (typically of about 15-30 nucleotides) to amplify the desired region of the mutarotase to be cloned, bringing the primers into contact with mRNA, cDNA or genomic DNA obtained from a yeast, bacterial, plant, prokaryotic or fungal cell, preferably of an *Aspergillus* strain, performing a polymerase chain reaction under conditions suitable for the amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector, such as described in Example 1.

Alternatively, synthetic genes can be constructed that encompass the coding region of the secreted mutarotase or variants thereof. Polynucleotides that are altered in many positions, but still encode the same protein can be conveniently be designed and constructed using these techniques. This has as advantage that the codon usage can be adapted to the preferred expression host, so productivity of the protein in this host can be improved. Also the polynucleotide sequence of a gene can be changed to improve mRNA stability or reduced turnover. This can lead to improved expression of the desired protein or variants thereof. Additionally, the polynucleotide sequence can be changed in a synthetic gene such that mutations are made in the protein sequence that have a positive effect on secretion efficiency, stability, proteolytic vulnerability, temperature optimum, specific activity or other relevant properties for industrial production or application of the protein. Companies that provide services to construct synthetic genes and optimize codon usage are generally available.

Such techniques may be used to obtain all or part of the polynucleotides encoding a protein having mutarotase activity. Introns, promoter and trailer regions can be used and may also be obtained in an analogous manner (e.g. by recombinant means, PCR or cloning techniques), starting with genomic DNA from a fungal, yeast, bacterial plant or prokaryotic cell.

The polynucleotides or primers may carry a revealing label. Suitable labels include radioisotopes such as ^{32}P or ^{35}S , fluorescent labels, enzyme labels, or other protein labels such as biotin. Such labels may be added to polynucleotides or primers as described herein and may be detected using techniques known to persons skilled in the art.

Polynucleotides or primers (or fragments thereof) labeled or unlabelled may be used in nucleic acid-based tests for detecting or sequencing a mutarotase or a variant thereof in a fungal sample. Such detection tests will generally comprise bringing a fungal sample suspected of containing the DNA of interest into contact with a probe comprising a polynucleotide or primer as described herein under hybridizing conditions, and detecting any duplex formed between the probe and nucleic acid in the sample. Detection may be achieved using techniques such as PCR or by immobilizing the probe on a solid support, removing any nucleic acid in the sample which is not hybridized to the probe, and then detecting any nucleic acid which is hybridized to the probe. Alternatively, the sample nucleic acid may be immobilized on a solid support, the probe hybridized and the amount of probe bound to such a support after the removal of any unbound probe detected.

The probes as described herein may conveniently be packaged in the form of a test kit in a suitable container. In such kits the probe may be bound to a solid support where the assay format for which the kit is designed requires such binding. The kit may also contain suitable reagents for treating the sample to be probed, hybridizing the probe to nucleic acid in the sample, control reagents, instructions, and the like. The probes and polynucleotides of the invention may also be used in microassay.

Preferably, the polynucleotide as described herein is obtainable from the same organism as the polypeptide, such as a fungus, in particular a fungus of the genus *Aspergillus*.

Production of polynucleotides

Polynucleotides which do not have 100% identity with SEQ ID NO: 3 or SEQ ID NO: 2 can be obtained in a number of ways. Thus, variants of the mutarotase sequence described herein may be obtained for example, by probing genomic DNA libraries made from a range of organisms, such as those discussed as sources of the polypeptides as described herein. In addition, other fungal, plant or prokaryotic homologues of mutarotase may be obtained and such homologues and fragments thereof in general will be capable of hybridising to SEQ ID NO:3 or SEQ ID NO: 2. Such sequences may be obtained by probing cDNA libraries or genomic DNA libraries from other species, and probing such libraries with probes comprising all or part of SEQ ID NO: 2 or 3 under conditions of low, medium to high stringency (as described earlier).

Nucleic acid probes comprising all or part of SEQ ID NO: 2 or 3 may be used to probe cDNA or genomic libraries from other species, such as those described as sources for a polypeptide having mutarotase activity.

Species homologues may also be obtained using degenerate PCR, which uses
5 primers designed to target sequences within the variants and homologues which encode conserved amino acid sequences. The primers can contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

Alternatively, such polynucleotides may be obtained by site directed
10 mutagenesis of the mutarotase sequences or variants thereof. This may be useful where, for example, silent codon changes to sequences are required to optimize codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be made in order to introduce restriction enzyme recognition sites, or to alter the property or function of the polypeptides
15 encoded by the polynucleotides.

The invention also describes double stranded polynucleotides comprising a polynucleotide as described above and its complement.

The present invention also describes polynucleotides encoding the polypeptides of the invention described above. Since such polynucleotides will be useful as
20 sequences for recombinant production of a protein having mutarotase activity, it is not necessary for them to be capable of hybridising to the sequence of SEQ ID NO: 2 or SEQ ID NO:3, although this will generally be desirable. Otherwise, such polynucleotides may be labeled, used, and made as described above if desired.

Recombinant Polynucleotides

The invention also describes vectors comprising a polynucleotide as described above, including cloning and expression vectors, and in another aspect methods of growing, transforming or transfecting such vectors into a suitable host cell, for example under conditions in which expression of a polypeptide as, or encoded by a sequence
30 as, described above. Provided also are host cells comprising a polynucleotide or vector as herein described wherein the polynucleotide is heterologous to the genome of the host cell. The term "heterologous", usually with respect to the host cell, means that the polynucleotide does not naturally occur in the genome of the host cell or that the

polypeptide is not naturally produced by that cell. Preferably, the host cell is a yeast cell, for example a yeast cell of the genus *Kluyveromyces*, *Pichia*, *Hansenula* or *Saccharomyces* or a filamentous fungal cell, for example of the genus *Aspergillus*, *Penicillium*, *Trichoderma* or *Fusarium*.

5

Vectors

The vector into which the expression cassette as described herein is inserted may be any vector that may conveniently be subjected to recombinant DNA procedures, and the choice of the vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extra-chromosomal entity, the replication of which is independent of chromosomal replication, such as a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicates together with the chromosome(s) into which it has been integrated.

Preferably, when a polynucleotide as described herein is in a vector it is operably linked to a regulatory sequence which is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence such as a promoter, enhancer or other expression regulation signal "operably linked" to a coding sequence is positioned in such a way that expression of the coding sequence is achieved under production conditions.

The vectors may, for example in the case of plasmid, cosmid, virus or phage vectors, be provided with an origin of replication, optionally a promoter for the expression of the polynucleotide and optionally an enhancer and/or a regulator of the promoter. A terminator sequence may be present, as may be a polyadenylation sequence. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used *in vitro*, for example for the production of RNA or can be used to transfect or transform a host cell.

The DNA sequence encoding a protein having mutarotase activity is preferably introduced into a suitable host as part of an expression construct in which the DNA sequence is operably linked to expression signals which are capable of directing

expression of the DNA sequence in the host cells. For transformation of the suitable host with the expression construct transformation procedures are available which are well known to the skilled person. The expression construct can be used for transformation of the host as part of a vector carrying a selectable marker, or the
5 expression construct is co-transformed as a separate molecule together with the vector carrying a selectable marker. The vectors may contain one or more selectable marker genes.

Preferred selectable markers include but are not limited to those that complement a defect in the host cell or confer resistance to a drug. They include for
10 example versatile marker genes that can be used for transformation of most filamentous fungi and yeasts such as acetamidase genes or cDNAs (the *amdS*, *niaD*, *facA* genes or cDNAs from *A.nidulans*, *A.oryzae*, or *A.niger*), or genes providing resistance to antibiotics like G418, hygromycin, bleomycin, kanamycin, phleomycin or benomyl resistance (*benA*). Alternatively, specific selection markers can be used such
15 as auxotrophic markers which require corresponding mutant host strains: e.g. *URA3* (from *S.cerevisiae* or analogous genes from other yeasts), *pyrG* or *pyrA* (from *A.nidulans* or *A.niger*), *argB* (from *A.nidulans* or *A.niger*) or *trpC*. In a preferred embodiment the selection marker is deleted from the transformed host cell after introduction of the expression construct so as to obtain transformed host cells capable
20 of producing the polypeptide which are free of selection marker genes.

Other markers include ATP synthetase subunit 9 (*oliC*), orotidine-5'-phosphate-decarboxylase (*pvrA*), the bacterial G418 resistance gene (useful in yeast, but not in filamentous fungi), the ampicillin resistance gene (*E. coli*), the neomycin resistance gene (*Bacillus*) and the *E. coli uidA* gene, coding for glucuronidase (GUS). Vectors may
25 be used *in vitro*, for example for the production of RNA or to transfect or transform a host cell.

For most filamentous fungi and yeast, the expression construct is preferably integrated into the genome of the host cell in order to obtain stable transformants. However, for certain yeasts suitable episomal vector systems are also available into
30 which the expression construct can be incorporated for stable and high level expression. Examples thereof include vectors derived from the 2 μ m, CEN and pKD1 plasmids of *Saccharomyces* and *Kluyveromyces*, respectively, or vectors containing an AMA sequence (e.g. AMA1 from *Aspergillus*). When expression constructs are

integrated into host cell genomes, the constructs are either integrated at random loci in the genome, or at predetermined target loci using homologous recombination, in which case the target loci preferably comprise a highly expressed gene. A highly expressed gene is a gene whose mRNA can make up at least 0.01% (w/w) of the total cellular mRNA, for example under induced conditions, or alternatively, a gene whose gene product can make up at least 0.2% (w/w) of the total cellular protein, or, in case of a secreted gene product, can be secreted to a level of at least 0.05 g/l.

An expression construct for a given host cell will usually contain the following elements operably linked to each other in consecutive order from the 5'-end to 3'-end relative to the coding strand of the sequence encoding the polypeptide of the first aspect: (1) a promoter sequence capable of directing transcription of the DNA sequence encoding the polypeptide in the given host cell, (2) preferably, a 5'-untranslated region (leader), (3) optionally, a signal sequence capable of directing secretion of the polypeptide from the given host cell into the culture medium, (4) the DNA sequence encoding a mature and preferably active form of the polypeptide, and preferably also (5) a transcription termination region (terminator) capable of terminating transcription downstream of the DNA sequence encoding the polypeptide.

Downstream of the DNA sequence encoding a polypeptide for use in a method of the invention, the expression construct preferably contains a 3' untranslated region containing one or more transcription termination sites, also referred to as a terminator. The origin of the terminator is less critical. The terminator can for example be native to the DNA sequence encoding the polypeptide. However, preferably a bacterial terminator is used in bacterial host cells, a yeast terminator is used in yeast host cells and a filamentous fungal terminator is used in filamentous fungal host cells. More preferably, the terminator is endogenous to the host cell in which the DNA sequence encoding the polypeptide is expressed.

Enhanced expression of the polynucleotide encoding a protein having mutarotase activity may also be achieved by the selection of heterologous regulatory regions, e.g. promoter, signal sequence and terminator regions, which serve to increase expression and, if desired, secretion levels of the protein of interest from the chosen expression host and/or to provide for the inducible control of the expression of the polypeptide of the invention.

Aside from the promoter native to the gene encoding a protein having mutarotase activity, other promoters may be used to direct expression of said protein. The promoter may be selected for its efficiency in directing the expression of the polypeptide of the invention in the desired expression host.

5 Promoters/enhancers and other expression regulation signals may be selected to be compatible with the host cell for which the expression vector is designed. For example, prokaryotic promoters may be used, in particular those suitable for use in *E.coli* strains. When expression of a protein having mutarotase activity is carried out in mammalian cells, mammalian promoters may be used. Tissues-specific promoters, for
10 example hepatocyte cell-specific promoters, may also be used. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, herpes simplex virus promoters or adenovirus promoters.

15 Suitable yeast promoters include the *S. cerevisiae* GAL4 and ADH promoters and the *S. pombe* nmt1 and adh promoter. Mammalian promoters include the metallothionein promoter which can be induced in response to heavy metals such as cadmium. Viral promoters such as the SV40 large T antigen promoter or adenovirus promoters may also be used. All these promoters are readily available in the art.

20 Mammalian promoters, such as β -actin promoters, may be used. Tissue-specific promoters, in particular endothelial or neuronal cell specific promoters (for example the DDAH I and DDAH II promoters), are especially preferred. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human
25 cytomegalovirus (CMV) IE promoter, adenovirus, HSV promoters (such as the HSV IE promoters), or HPV promoters, particularly the HPV upstream regulatory region (URR). Viral promoters are readily available in the art.

A variety of promoters can be used that are capable of directing transcription in the host cells as described herein. Preferably the promoter sequence is derived from a
30 highly expressed gene as previously defined. Examples of preferred highly expressed genes from which promoters are preferably derived and/or which are comprised in preferred predetermined target loci for integration of expression constructs, include but are not limited to genes encoding glycolytic enzymes such as triose-phosphate

isomerases (TPI), glyceraldehyde-phosphate dehydrogenases (GAPDH), phosphoglycerate kinases (PGK), pyruvate kinases (PYK), alcohol dehydrogenases (ADH), as well as genes encoding amylases, glucoamylases, proteases, xylanases, cellobiohydrolases, β -galactosidases, alcohol (methanol) oxidases, elongation factors and ribosomal proteins. Specific examples of suitable highly expressed genes include e.g. the LAC4 gene from *Kluyveromyces* sp., the methanol oxidase genes (AOX and MOX) from *Hansenula* and *Pichia*, respectively, the glucoamylase (glaA) genes from *A.niger* and *A.awamori*, the *A.oryzae* TAKA-amylase gene, the *A.nidulans* gpdA gene and the *T.reesei* cellobiohydrolase genes.

Examples of strong constitutive and/or inducible promoters which are preferred for use in fungal expression hosts are those which are obtainable from the fungal genes for xylanase (xlnA), phytase, ATP-synthetase subunit 9 (oliC), triose phosphate isomerase (tpi), alcohol dehydrogenase (AdhA), amylase (amy), amyloglucosidase (AG - from the glaA gene), acetamidase (amdS) and glyceraldehyde-3-phosphate dehydrogenase (gpd) promoters.

Examples of strong yeast promoters which may be used include those obtainable from the genes for alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, lactase, 3-phosphoglycerate kinase, plasma membrane ATPase (PMA1) and triosephosphate isomerase.

Examples of strong bacterial promoters which may be used include the amylase and SPo2 promoters as well as promoters from extracellular protease genes.

Promoters suitable for plant cells which may be used include napaline synthase (nos), octopine synthase (ocs), mannopine synthase (mas), ribulose small subunit (rubisco ssu), histone, rice actin, phaseolin, cauliflower mosaic virus (CMV) 35S and 19S and circovirus promoters.

The vector may further include sequences flanking the polynucleotide giving rise to RNA which comprise sequences homologous to ones from eukaryotic genomic sequences, preferably fungal genomic sequences, or yeast genomic sequences. This will allow the introduction of the polynucleotides into the genome of fungi or yeasts by homologous recombination. In particular, a plasmid vector comprising the expression cassette flanked by fungal sequences can be used to prepare a vector suitable for delivering the polynucleotides to a fungal cell. Transformation techniques using these fungal vectors are known to those skilled in the art.

Host Cells and Expression

In a further aspect the invention describes a process for preparing a protein having mutarotase activity which comprises cultivating a host cell transformed or transfected with an expression vector as described above under conditions suitable for expression by the vector of a coding sequence encoding said protein, and recovering the expressed protein. Polynucleotides as described herein can be incorporated into a recombinant replicable vector, such as an expression vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention describes a method of making a polynucleotide as described herein by introducing a polynucleotide as described herein into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about the replication of the vector. Suitable host cells include bacteria such as *E. coli*, yeast, mammalian cell lines and other eukaryotic cell lines, for example insect cells such as Sf9 cells and (e.g. filamentous) fungal cells.

Preferably a protein having mutarotase activity is produced as a secreted protein in which case the DNA sequence encoding a mature form of the polypeptide in the expression construct may be operably linked to a DNA sequence encoding a signal sequence. In the case where the gene encoding the secreted protein has in the wild type strain a signal sequence preferably the signal sequence used will be native (homologous) to the DNA sequence encoding the polypeptide. Alternatively the signal sequence is foreign (heterologous) to the DNA sequence encoding the polypeptide, in which case the signal sequence is preferably endogenous to the host cell in which the DNA sequence is expressed. Examples of suitable signal sequences for yeast host cells are the signal sequences derived from yeast MFalpha genes. Similarly, a suitable signal sequence for filamentous fungal host cells is e.g. a signal sequence derived from a filamentous fungal amyloglucosidase (AG) gene, e.g. the *A.niger* glaA gene. This signal sequence may be used in combination with the amyloglucosidase (also called (gluco) amylase) promoter itself, as well as in combination with other promoters. Hybrid signal sequences may also be used within the context of the present invention.

Preferred heterologous secretion leader sequences are those originating from the fungal amyloglucosidase (AG) gene (glaA - both 18 and 24 amino acid versions e.g.

from *Aspergillus*), the MFalpha gene (yeasts e.g. *Saccharomyces*, *Pichia* and *Kluyveromyces*) or the alpha-amylase gene (*Bacillus*).

The vectors may be transformed or transfected into a suitable host cell as described above to provide for expression of a protein having mutarotase activity. This process may comprise culturing a host cell transformed with an expression vector as described above under conditions suitable for expression of said protein, and optionally recovering the expressed protein.

The invention thus further describes host cells transformed or transfected with or comprising a polynucleotide or vector as described above. Preferably the polynucleotide is carried in a vector which allows the replication and expression of the polynucleotide. The cells will be chosen to be compatible with the said vector and may for example be prokaryotic (for example bacterial), or eukaryotic fungal, yeast or plant cells.

The invention encompasses processes for the production of a protein having mutarotase activity by means of recombinant expression of a DNA sequence encoding said protein. For this purpose the DNA sequence as described herein can be used for gene amplification and/or exchange of expression signals, such as promoters, secretion signal sequences, in order to allow economic production of the polypeptide in a suitable homologous or heterologous host cell. A homologous host cell is herein defined as a host cell which is of the same species or which is a variant within the same species as the species from which the DNA sequence is derived.

Suitable host cells are preferably prokaryotic microorganisms such as bacteria, or more preferably eukaryotic organisms, for example fungi, such as yeasts or filamentous fungi, or plant cells. In general, yeast cells are preferred over filamentous fungal cells because they are easier to manipulate. However, some proteins are either poorly secreted from yeasts, or in some cases are not processed properly (e.g. hyperglycosylation in yeast). In these instances, a filamentous fungal host organism should be selected.

Bacteria from the genus *Bacillus* are very suitable as heterologous hosts because of their capability to secrete proteins into the culture medium. Other bacteria suitable as hosts are those from the genera *Streptomyces* and *Pseudomonas*. A preferred yeast host cell for the expression of the DNA sequence encoding the polypeptide is one of the genus *Saccharomyces*, *Kluyveromyces*, *Hansenula*, *Pichia*,

Yarrowia, or *Schizosaccharomyces*. More preferably, a yeast host cell is selected from the group consisting of the species *Saccharomyces cerevisiae*, *Kluyveromyces lactis* (also known as *Kluyveromyces marxianus* var. *lactis*), *Hansenula polymorpha*, *Pichia pastoris*, *Yarrowia lipolytica*, and *Schizosaccharomyces pombe*.

5 Most preferred for the expression of the DNA sequence encoding a protein having mutarotase activity, however, are filamentous fungal host cells. Preferred filamentous fungal host cells are selected from the group consisting of the genera *Aspergillus*, *Trichoderma*, *Fusarium*, *Disporotrichum*, *Penicillium*, *Acremonium*, *Neurospora*, *Thermoascus*, *Myceliophthora*, *Sporotrichum*, *Thielavia*, and *Talaromyces*.

10 More preferably a filamentous fungal host cell is of the species *Aspergillus ozyae*, *Aspergillus sojae* or *Aspergillus nidulans* or is of a species from the *Aspergillus niger* Group (as defined by Raper and Fennell, The Genus *Aspergillus*, The Williams & Wilkins Company, Baltimore, pp 293-344, 1965). These include but are not limited to *Aspergillus niger*, *Aspergillus awamori*, *Aspergillus tubigenensis*, *Aspergillus aculeatus*,
15 *Aspergillus foetidus*, *Aspergillus nidulans*, *Aspergillus japonicus*, *Aspergillus oryzae* and *Aspergillus ficuum*, and also those of the species *Trichoderma reesei*, *Fusarium graminearum*, *Penicillium chrysogenum*, *Acremonium alabamense*, *Neurospora crassa*, *Myceliophthora thermophilum*, *Sporotrichum cellulophilum*, *Disporotrichum dimorphosporum* and *Thielavia terrestris*.

20 Examples of preferred expression hosts are fungi such as *Aspergillus* species (in particular those described in EP-A-184,438 and EP-A-284,603) and *Trichoderma* species; bacteria such as *Bacillus* species (in particular those described in EP-A-134,048 and EP-A-253,455), especially *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Pseudomonas* species; and yeasts such as *Kluyveromyces* species
25 (in particular those described in EP-A-096,430 such as *Kluyveromyces lactis* and in EP-A-301,670) and *Saccharomyces* species, such as *Saccharomyces cerevisiae*.

Host cells include plant cells, and the invention therefore further describes transgenic organisms, such as plants and parts thereof, which contain one or more cells as described herein. The cells may heterologously express a protein having
30 mutarotase activity or may heterologously contain one or more of the polynucleotides as described herein. The transgenic (or genetically modified) plant may therefore have inserted (typically stably) into its genome a sequence encoding a protein having mutarotase activity. The transformation of plant cells can be performed using known

techniques, for example using a Ti or a Ri plasmid from *Agrobacterium tumefaciens*. The plasmid (or vector) may thus contain sequences necessary to infect a plant, and derivatives of the Ti and/or Ri plasmids may be employed.

The host cell may overexpress a protein having mutarotase activity, and techniques for engineering over-expression are well known and can be used in the present invention. The host may thus have more than one copy of the polynucleotide as described herein.

Alternatively, direct infection of a part of a plant, such as a leaf, root or stem can be effected. In this technique the plant to be infected can be wounded, for example by cutting the plant with a razor, puncturing the plant with a needle or rubbing the plant with an abrasive. The wound is then inoculated with the *Agrobacterium*. The plant or plant part can then be grown on a suitable culture medium and allowed to develop into a mature plant. Regeneration of transformed cells into genetically modified plants can be achieved by using known techniques, for example by selecting transformed shoots using an antibiotic and by sub-culturing the shoots on a medium containing the appropriate nutrients, plant hormones and the like.

Culture of host cells and recombinant production

The invention also describes cells that have been modified to express the mutarotase or a variant thereof. Such cells include transient, or preferably stably modified higher eukaryotic cell lines, such as mammalian cells or insect cells, lower eukaryotic cells, such as yeast and filamentous fungal cells or prokaryotic cells such as bacterial cells.

It is also possible for a protein having mutarotase activity to be transiently expressed in a cell line or on a membrane, such as for example in a baculovirus expression system.

The production of a protein having mutarotase activity can be effected by the culturing of microbial expression hosts, which have been transformed with one or more polynucleotides as described herein, in a conventional nutrient fermentation medium.

The recombinant host cells as described herein may be cultured using procedures known in the art. For each combination of a promoter and a host cell, culture conditions are available which are conducive for expression the DNA sequence encoding a protein having mutarotase activity. After reaching the desired cell density or

titre of a protein having mutarotase activity the culturing is ceased and the a protein having mutarotase activity is recovered using known procedures.

The fermentation medium can comprise a known culture medium containing a carbon source (e.g. glucose, maltose, molasses, etc.), a nitrogen source (e.g. ammonia, ammonium sulphate, ammonium nitrate, ammonium chloride, etc.), an organic nitrogen source (e.g. yeast extract, malt extract, peptone, etc.) and inorganic nutrient sources (e.g. phosphate, magnesium, potassium, zinc, iron, etc.). Optionally, an inducer (dependent on the expression construct used) may be included or subsequently be added.

The selection of the appropriate medium may be based on the choice of expression host and/or based on the regulatory requirements of the expression construct. Suitable media are well-known to those skilled in the art. The medium may, if desired, contain additional components favoring the transformed expression hosts over other potentially contaminating microorganisms.

The fermentation may be performed over a period of from 0.5-30 days. Fermentation may be a batch, continuous or fed-batch process, at a suitable temperature in the range of between 0°C and 45°C and, for example, at a pH from 2 to 10. Preferred fermentation conditions include a temperature in the range of between 20°C and 37°C and/or a pH between 3 and 9. The appropriate conditions are usually selected based on the choice of the expression host and the protein to be expressed.

After fermentation, if necessary, the cells can be removed from the fermentation broth by means of centrifugation or filtration. After fermentation has stopped or after removal of the cells, a protein having mutarotase activity may then be recovered and, if desired, purified and isolated by conventional means. The protein having mutarotase activity can be purified from fungal mycelium or from the culture broth into which the mutarotase is released by the cultured fungal cells.

Preferably, a protein having mutarotase activity is produced from a fungus, more preferably from an *Aspergillus*, most preferably from *Aspergillus niger*.

Modifications

A protein having mutarotase activity may be chemically modified, e.g. post-translationally modified. For example, they may be glycosylated (one or more times) or comprise modified amino acid residues. They may also be modified by the addition of

histidine residues to assist their purification or by the addition of a signal sequence to promote secretion from the cell. The protein having mutarotase activity may have amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification, such as a poly-histidine tract, an antigenic epitope or a binding domain.

A protein having mutarotase activity may be labelled with a revealing label. The revealing label may be any suitable label which allows the polypeptide to be detected. Suitable labels include radioisotopes, e.g. ^{125}I , ^{35}S , enzymes, antibodies, polynucleotides and linkers such as biotin.

A protein having mutarotase activity may be modified to include non-naturally occurring amino acids or to increase the stability of the polypeptide. When the proteins or peptides are produced by synthetic means, such amino acids may be introduced during production. The proteins or peptides may also be modified following either synthetic or recombinant production.

A protein having mutarotase activity may also be produced using D-amino acids. In such cases the amino acids will be linked in reverse sequence in the C to N orientation. This is conventional in the art for producing such proteins or peptides.

A number of side chain modifications are known in the art and may be made to the side chains of a protein having mutarotase activity. Such modifications include, for example, modifications of amino acids by reductive alkylation by reaction with an aldehyde followed by reduction with NaBH_4 , amidination with methylacetimidate or acylation with acetic anhydride.

The sequences as described herein may also be used as starting materials for the construction of "second generation" enzymes. "Second generation" mutarotases are mutarotases, altered by mutagenesis techniques (e.g. site-directed mutagenesis or gene shuffling techniques), which have properties that differ from those of wild-type mutarotase or recombinant mutarotase such as those produced by the present invention. For example, their temperature or pH optimum, specific activity, substrate affinity or thermostability may be altered so as to be better suited for use in a particular process.

Amino acids essential to the activity of the mutarotase of the invention, and therefore preferably subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis.

In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (e.g. mutarotase activity) to identify amino acid residues that are critical to the activity of the molecule. Sites of enzyme-substrate interaction can also be determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance, crystallography or photo-affinity labeling.

Gene shuffling techniques provide a random way to introduce mutations in a polynucleotide sequence. After expression the isolates with the best properties are re-isolated, combined and shuffled again to increase the genetic diversity. By repeating this procedure a number of times, genes that code for fastly improved proteins can be isolated. Preferably the gene shuffling procedure is started with a family of genes that code for proteins with a similar function. The family of polynucleotide sequences as described herein would be well suited for gene shuffling to improve the properties of secreted mutarotases.

Alternatively classical random mutagenesis techniques and selection, such as mutagenesis with NTG treatment or UV mutagenesis, can be used to improve the properties of a protein having mutarotase activity. Mutagenesis can be performed directly on isolated DNA, or on cells transformed with the DNA of interest. Alternatively, mutations can be introduced in isolated DNA by a number of techniques that are known to the person skilled in the art. Examples of these methods are error-prone PCR, amplification of plasmid DNA in a repair-deficient host cell, etc.

The use of yeast and filamentous fungal host cells is expected to provide for post-translational modifications (e.g. proteolytic processing, myristilation, glycosylation, truncation, and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant produced protein having mutarotase activity.

Preparations

A protein having mutarotase activity may be in an isolated form. It will be understood that the protein may be mixed with carriers or diluents which will not interfere with the intended purpose of the protein and still be regarded as isolated. A protein having mutarotase activity may also be in a substantially purified form, in which case it will generally comprise the protein in a preparation in which more than 70%, e.g.

more than 80%, 90%, 95%, 98% or 99% of the proteins in the preparation is a protein having mutarotase activity.

A protein having mutarotase activity may be provided in a form such that they are outside their natural cellular environment. Thus, they may be substantially isolated or purified, as discussed above, or in a cell in which they do not occur in nature, for example a cell of other fungal species, animals, plants or bacteria.

In a most preferred embodiment, the invention provides a method for improving carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate and wherein said protein having mutarotase activity is active during crystallization, wherein said protein having mutarotase activity comprises the amino acid sequence as depicted in SEQ ID NO:1 and more preferably said protein having mutarotase activity comprises the amino acids 23-403 or 24-403 as depicted in SEQ ID NO:1 .

The amount of enzyme (i.e. protein having mutarotase activity) added before or during crystallization can easily be determined by the skilled person and is for example within the range of 0.1 to 100 U/ml.

Crystallization

This invention describes the use of mutarotase (aldose-1-epimerase) in a process for carbohydrate crystallization. Preferably the carbohydrate is a reducing sugar, more preferably the carbohydrate is lactose. Carbohydrate crystallization can be performed from a pure, partly purified or a crude solution of carbohydrate. In general, the steps of a crystallization process can be described as:

- (i) concentration of carbohydrates into a supersaturated solution,
- (ii) transfer of the obtained concentrated carbohydrate solution to crystallization tanks,
- (iii) after which temperature decrease and optional seeding with carbohydrate crystals initiates crystallization,
- (iv) after crystallization has been allowed to proceed for a certain amount of time, the obtained slurry is transferred to a decanter or a centrifuge to separate the formed crystals from the remains

(v) the obtained crystals are washed and optionally subjected to a drying step

The phrase "adding a protein having mutarotase activity before or during crystallization" is used to indicate that the protein having mutarotase activity is added during concentration of the carbohydrates into a supersaturated solution or during or after transfer of the obtained concentrated carbohydrate solution to crystallization tanks.

Alternatively, the protein having mutarotase activity can be added at the same time the seed crystals are added. The protein having mutarotase activity may also be added when crystallization has already started. For example, the protein having mutarotase activity is added when a certain amount of crystals is already formed.

The use of a mutarotase during carbohydrate crystallization has advantages over the prior art in that it can speed up crystallization thereby decreasing residence time in the crystallization tank. A shorter crystallization process will also minimize risks for spoilage of the crystallization solution. Additional benefits are an increase in purity, quality and/or uniformity of the carbohydrate crystals. Additionally, the use of mutarotase during crystallization may lead to a productivity or yield increase of the carbohydrate.

The process of carbohydrate crystallization generally involves concentration of the carbohydrate in an aqueous solution by evaporation at high temperature or low pressure. By decreasing the temperature again, more water evaporation or the addition of alcohols to the solution, the solution can become supersaturated. In such a supersaturated solution the different anomers of the reducing sugar will be in equilibrium. Crystallization may then be initiated by seeding crystals to the supersaturated solution, preferably with seeding-crystals of one of the anomers of the reducing sugar. Additionally, continuous crystallization methods have been described in the art. By careful adjustment of the crystallization conditions a regular crystal growth can be obtained.

It was realized as part of this invention that mutarotase will have a positive effect on the crystallization of reducing sugars by means of one or more mechanisms.

Since only one of the anomers of a reducing sugar will crystallize, the supersaturation of this anomer will decrease. A decrease in supersaturation will slow down the crystallization rate. Maintaining high crystal growth rate will therefore require again an increase in supersaturation which can be achieved by e.g. cooling the solution, further evaporation or extra addition of alcohols. All these treatments are costly. Additionally, a decrease in temperature or evaporation will increase the viscosity

of the solution, thereby decreasing crystal growth rate by reduced mass transfer of the anomer in solution to the growing crystal surface. Addition of mutarotase can prevent a drastic decrease in supersaturation of the crystallizing anomer of the reducing sugar, by replenishing the anomer that crystallizes, by mutarotation of the other anomer(s).
5 Consequently, in the presence of mutarotase, crystal growth can occur at sufficient rate without additional measures such as excessive cooling or at an increased rate when the solution is cooled or otherwise treated to increase supersaturation.

Crystal growth of reducing sugars is known to be negatively influenced by molecules that resemble the crystallizing anomer. E.g. it was shown that the presence
10 of β -lactose has a strong negative effect on the growth of crystals of α -lactose. It is thought that β -lactose competes with α -lactose, and is build into growing α -lactose crystals and thereby prevents further growth of the crystals (Michaels and van Kreveland (1966) *Neth. Milk Dairy J.* 20, 163). Reducing sugars are crystallized from supersaturated solutions where the different anomers are in equilibrium. Crystallization
15 of one of the anomers will lead to change in the equilibrium, increasing the relative concentration of the competing anomer. Due to this, this change in equilibrium of the anomers will further decrease crystal growth rate. Addition of mutarotase during crystallization will restore the equilibrium between the anomers and decrease the concentration of the non-crystallizing anomer, thereby speeding up crystal growth.

20 As a result, adjustment of the process conditions in the presence of mutarotase allows steering at an increased crystallization rate, an increased productivity, an increased yield or increased quality of the crystals.

Carbohydrates relevant for this invention are preferably reducing sugars where
25 two or more anomeric forms can exist, more preferably reducing sugars where only one anomeric form is crystallized. Even more preferred are reducing sugars such as glucose (dextrose), fructose, galactose, xylose or lactose. The invention therefore provides a method for improving carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate and wherein said protein having mutarotase activity is active during crystallization,
30 wherein said carbohydrate is a reducing sugar such as glucose, fructose, galactose, xylose or lactose.

The use of mutarotase in the crystallization of such sugars is part of this embodiment and can have a positive effect on velocity, productivity and yield of the process, and quality of the formed crystals. Use of mutarotase may lead to adaptation of the process conditions to gain the optimal effect of the enzyme activity.

5 Glucose crystallization at lower temperatures leads to the specific crystallization of α -D-glucose-monohydrate or anhydrous α -D-glucose, dependent on the conditions used. Crystallization of β -D-glucose only occurs at temperature between 115 and 150 °C and is industrially less relevant. The use of mutarotase in the crystallization of α -D-glucose is part of this embodiment.

10 Aqueous crystallisation of fructose is difficult due to the very high solubility of fructose in water. Crystallisation processes have used the addition of lower alcohols, including ethanol, to concentrated fructose syrups to decrease fructose solubility and solution viscosity, thus enhancing the crystallisation process of fructose in water. Crystalline fructose consists solely of the anhydrous β -D-fructopyranose form. In
15 solution, however, fructose can exist as any of five tautomeric forms, including α and β forms of fructopyranose and fructofuranose. The use of mutarotase in the crystallization of fructose is part of this embodiment.

Also galactose can exist in two anomeric forms in solution. Crystallization of β -D-galactose leads to unstable β -D-galactose crystals while crystallization of α -D-galactose
20 leads to stable α -D-galactose crystals. In practice this means that galactose specifically crystallizes as α -D-galactose. The use of mutarotase in the crystallization of galactose is part of this embodiment.

In solution lactose can also occur in two anomeric forms. At lower temperature specifically α -hydrate lactose crystals are formed, while at temperatures >93.5 °C β -
25 anhydrate crystals are formed. The α -lactose crystals are industrially most important. In a preferred embodiment, the invention therefore provides a method for improving carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate and wherein said protein having mutarotase activity is active during crystallization, wherein said carbohydrate is lactose.

30 An aqueous solution of xylose contains five tautomeric forms: α - and β -pyranose forms, α - and β -furanose forms, and an aldehyde, or open, form. The proportion of these forms depends on the temperature and on the concentration of the solution. Only

the α -pyranose form is a crystallizing form. The use of mutarotase in the crystallization of xylose is part of the invention as well.

For the purpose of this invention, the source of the reduced sugar used in the crystallization process may be diverse. In a preferred embodiment, the invention provides a method for improving carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate and wherein said protein having mutarotase activity is active during crystallization, wherein said crystallization of said carbohydrate is performed on a pure, partly purified or a crude solution of said carbohydrate.

Crude material that may be used as source for the crystallization of lactose will commonly be whey permeate, but may also be whey, or milk, or partially treated or purified forms thereof. Source material might also be previously crystallized or amorphous dried lactose, or any other source containing a sufficient quantity of lactose. In a further preferred embodiment, the invention provides a method for improving carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate and wherein said protein having mutarotase activity is active during crystallization, wherein said crude solution of said carbohydrate is whey, whey powder, milk or milk powder.

Crude material that may be used as source for the crystallization of glucose may be invert sugar, hydrolyzed starch from corn, wheat, rice or another agricultural product, glucose syrup or any other source containing a sufficient quantity of glucose.

Crude material that may be used as source for the crystallization of fructose may be invert sugar, high-fructose (corn) syrup, honey, or any other source containing a sufficient quantity of fructose.

Crude material that may be used as source for the crystallization of galactose may be whey permeate or another lactose source treated with lactase, or any other source containing a sufficient quantity of galactose.

Crude material that may be used as source for the crystallization of xylose may be hydrolyzed agricultural waste such as birch wood, corn cobs or cotton seed hulls, among others. Hydrolyzed xylan or any other source containing a sufficient quantity of xylose may be used as source material.

Part of the invention concerns the improvement of the dissolution of crystalline carbohydrates. In the production of α -lactose monohydrate crystals for pharmaceutical applications it is essential that the product is as pure as possible, and no remnants of the milk or whey are present. Since a single crystallization step is often not sufficient to obtain pure α -lactose crystals, the α -lactose is generally dissolved again and re-crystallized one or more times. The dissolution of α -lactose crystals in water is however a slow process due to the poor solubility of α -lactose. It is part of the invention that the addition of mutarotase prior to or during dissolution of α -lactose crystals increases the rate of dissolution. Since the enzyme increases the rate of conversion of the solubilised α -lactose into β -lactose, the velocity of α -lactose dissolution will increase.

In yet another preferred embodiment, the invention provides a method for improving carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate and wherein said protein having mutarotase activity is active during crystallization, further comprising dissolving the produced carbohydrate crystals (preferably in the presence of a protein having mutarotase activity) resulting in a dissolved carbohydrate solution. In a further preferred embodiment, the invention provides a method for improving carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate and wherein said protein having mutarotase activity is active during crystallization, further comprising dissolving the produced carbohydrate crystals (preferably in the presence of a protein having mutarotase activity) resulting in a dissolved carbohydrate solution, further comprising subjecting the obtained dissolved carbohydrate solution to a further (for example a second or even a third) crystallization process, which is optionally performed in the presence of a protein having mutarotase activity.

In yet another embodiment, the invention provides use of mutarotase for improving carbohydrate crystallization.

In another embodiment, the invention provides a product obtainable (preferably obtained) by any of the herein claimed methods. Preferably, said product is a crystallized carbohydrate and even more preferably, said product is crystallized lactose.

The obtained product typically differs from the crystallized products of the prior art in respect of purity or uniformity.

Hereafter the invention is illustrated by the following non-limiting examples.

5

EXAMPLES

Example 1

Cloning and expression of the *Aspergillus niger* mutarotase gene

10 *Aspergillus niger* CBS513.88 was grown for 3 days at 30 degrees Celsius in PDB (Potato dextrose broth, Difco) and chromosomal DNA was isolated from the mycelium using the Q-Biogene kit (catalog nr. 6540-600; Omnilabo International BV, Breda, the Netherlands), using the instructions of the supplier. This chromosomal DNA was used for the amplification of the coding sequence of the mutarotase gene using PCR.

15 To specifically amplify the mutarotase gene MUT from the chromosomal DNA of *Aspergillus niger* CBS513.88, two PCR oligonucleotide primers were designed (SEQ ID NO: 4 and SEQ ID NO: 5). Primer sequences were partly obtained from a sequence that was found in the genomic DNA of *Aspergillus niger* CBS513.88 and published under number AM270022.1 in Genbank (SEQ ID NO: 2). The MUT protein sequence
20 can be found back under the number An02g09090 (SEQ ID NO: 1). We describe here for the first time the efficient expression and characterization of a secreted *Aspergillus* mutarotase, which can be easily over-expressed and secreted in amounts that are relevant for applications in the food industry.

25 SEQ ID NO: 4: 5'-CCCTTAATTAACATAGGCATCATGCATGTCAAAAGCTACCTGTC-3'
SEQ ID NO: 5: 5'-TTAGGCGCGCCTTACACAGTACCGAACTGGTAAG-3'

The first, direct PCR primer (SEQ ID NO: 4) contains 23 nucleotides MUT coding sequence starting at the ATG start codon, preceded by a 23 nucleotides sequence
30 including a Pad restriction site. The second, reverse primer (SEQ ID NO: 5) contains 23 nucleotides complementary to the reverse strand of the region at the MUT stop codon, preceded by an *Asc*I restriction site. Using these primers we were able to amplify a 1.2 kb sized fragment with chromosomal DNA from *Aspergillus niger* CBS513.88 as

template. The thus obtained 1.2 kb sized fragment was isolated, digested with *Pad* and *AscI* and purified. The *Pad* /*AscI* fragment comprising the MUT coding sequences was exchanged with the *Pad* / *AscI* *phyA* fragment from pGBFIN-5 (WO 99/32617). Resulting plasmid is the MUT expression vector named pGBFINMUT. The expression
5 vector pGBFINMUT was linearized by digestion with *NotI*, which removes all *E. coli* derived sequences from the expression vector. The digested DNA was purified using phenol: chloroform: isoamylalcohol (24:23:1) extraction and precipitation with ethanol. These vectors were used to transform *Aspergillus niger* CBS513.88. An *Aspergillus niger* transformation procedure is extensively described in WO 98/46772. It is also
10 described how to select for transformants on agar plates containing acetamide, and to select targeted multicopy integrants. Preferably, *A. niger* transformants containing multiple copies of the expression cassette are selected for further generation of sample material. For the pGBFINMUT expression vector, 30 *A. niger* transformants were purified; first by plating individual transformants on selective medium plates followed by
15 plating a single colony on PDA (potato dextrose agar: PDB + 1.5% agar) plates. Spores of two individual transformants were collected after growth for 1 week at 30 degrees Celsius. Spores were stored refrigerated and were used for the inoculation of liquid media.

The two *A. niger* transformant strains were used for generation of sample material
20 by cultivation of the strains in shake flask cultures. A useful method for cultivation of *A. niger* strains and separation of the mycelium from the culture broth is described in WO 98/46772. Cultivation medium was in CSM-MES (150 g maltose, 60 g Soytone (Difco), 15 g $(\text{NH}_4)_2\text{SO}_4$, 1 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g L-arginine, 80 mg Tween-80, 20 g MES pH6.2 per liter medium). 5 ml samples were taken from duplicate cultivations
25 on day 4-8 of the fermentation, centrifuged for 10 min at 5000 rpm in a Hereaus labofuge RF and supernatants were stored at -20°C until further analyses.

It became clear that the transformants containing the pGBFINMUT vector had a surprisingly efficient secretion of a protein of apparent molecular weight of approximately 60 kDa when analyzed with SDS-PAGE. This protein is named MUT
30 hereafter. The size of the expressed protein is much larger than the molecular weight that is predicted from the protein sequence, which is approximately 45 kDa. We presume that after removal of the signal sequence, MUT is heavily glycosylated before secretion from *Aspergillus niger*. The size of the MUT protein is completely different

from an aldose-1-epimerase from *Aspergillus niger* mycelium that has been described before (Kinoshita et al (1981) Biochimica et Biophysica Acta 662, 285-290). The molecular weight of this enzyme, that was isolated from a cell lysate instead of secreted, was determined to be 260 kDa, being a homo-dimer with individual subunits of 130 kDa.

Selected strains can be used for isolation and purification of a larger amount of secreted *Aspergillus* mutarotase, when fermentation and down-stream processing is scaled up. This enzyme can then be used for further analysis, and for the use in diverse industrial applications.

Example 2

Fermentation, purification, and characterization of fungal mutarotase

Fresh *Aspergillus niger* MUT transformant #1 spores were used to inoculate shake flasks with 2 liters modified CSM medium (8% maltose, 3% bactosoytone, pH 5.1). After three days cultivation at 30°C, the cells were killed off by adding 3.5 g/l (final concentration) sodium benzoate and keeping at 30 °C for 6 hours. 10 g/l CaCl₂ and 45 g/l filter-aid Dicalite BF were added to the culture broth, and filtration was carried out in one step using filter cloth and filters Z-2000 and Z-200 (Pall). The filter cake remaining on the filter was washed with 1.1 liters of sterile milliQ water. The culture filtrate including the wash was sterile-filtered using 0.22 µm GP Express PLUS Membrane (Millipore). The sterile filtrate was concentrated by ultrafiltration on a Pellicon-2 mini Cassette Biomax 5k (Millipore). The ultra-filtrate was purified with ALEX chromatography using a Q-sepharose FF XK 16/20 column (gel volume approx 30 ml). The following buffer system was used: Buffer A: 20 mM Na-phosphate, pH 7, Buffer B: 20 mM Na-phosphate, pH 7 + 1M NaCl. The purification cycle was: equilibration with buffer A (5 column volumes), loading of the sample, washing with buffer A (3 column volumes), gradient elution from buffer A to buffer B in 20 column volumes. Flow rate 5 ml/min. The eluate was retrieved in several fractions. The fractions with minor contaminations were pooled, washed with 20 mM Na-phosphate pH 8.2, and loaded on the same column equilibrated with 20 mM Na-phosphate pH 8.2. MUT was bound to the column and eluted with 20 mM Na-phosphate pH 8.2, 1M NaCl. The fractions with minor contaminations were pooled and used for subsequent activities determinations. The purity of the MUT sample was checked by SDS-gel electrophoresis and was found to

be >90% pure. This enzyme solution was stabilized using the addition of glycerol up to 50% and L-methionine up to 0.1%, and stored at -20 °C until use.

Enzymatic characterization.

5 The specific activity of MUT was measured using the standard NAD and glucose dehydrogenase coupled assay. When α -D-glucose is converted to β -D-glucose by mutarotase in the presence of β -D-glucose dehydrogenase and NAD, β -D-glucose is oxidized to D-glucono[^]-lactone with equimolar reduction of NAD to NADH. Therefore, optical density measurement of NADH at 340 nm ($\epsilon_{340} = 6220 \text{ mol}^{-1} \text{ cm}^{-1}$) was used
10 for indirect determination of mutarotase activity.

The reaction mixture contained the following components: 0.465 ml of 50 mM Tris-HCl buffer (pH 7.5) with 3 mM NAD and 10 units of glucose dehydrogenase; 0.025 ml MUT solution (0.004 mg/ml). The reaction was initiated by the addition 0.010 ml of freshly prepared 100 mM D-glucose to the reaction mix at the ambient temperature.

15 The increase in absorption at 340 nm was measured for several minutes. A blank was determined by using the same mixture without MUT solution, and subtracted from the value obtained with MUT. The amount of mutarotase in the assay was adjusted to give an absorption change between 0.01 and 0.06 per min under these conditions. One unit of the specific mutarotase activity was defined as μmol substrate converted/min/mg
20 protein at 22 °C, pH 7.5, after correction for the nonenzymatic rate.

When the specific activity of the isolated and purified MUT from *Aspergillus niger* was measured using this assay, it turned out to be approximately 50 U/mg.

In order to test the thermostability of the isolated mutarotase, the enzyme solution was incubated for 2, 4, 6 and 8 hours at 55 °C before measuring the activity at 22 °C
25 using the assay described above. No decrease in activity was noticed after this pre-incubation, showing that the mutarotase was stable under these conditions.

Example 3

Use of mutarotase in the crystallization of lactose

Monitor crystallization of α -lactose in MTP-plate

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A supersaturated aqueous solution of lactose (50% w/w) was prepared from α -lactose monohydrate powder by dissolution in demineralised water, ensured through occasional shaking and heating to -80 degrees Celsius in a microwave for approximately 30 seconds

in a closed vessel to prevent evaporation of the solvent. The solution was then placed in a water bath at 40°C with shaking for 30 min to ensure that mutarotation equilibrium was achieved. In the crystallization experiment the lactose solution was buffered at pH 6.9 by the addition of a 0.1 M sodium phosphate buffer solution until 5.3 mM.

5

60 U or 120 U of MUT was added per ml of the supersaturated lactose solution in a 96 well microtiter plate (MTP), mixed and incubated at 25 degrees Celsius. Five glass beads (diameter 1 mm) were added per well and the solutions were incubated under shaking. The development of lactose crystals was followed in time for 5 hours using a TECAN Genius apparatus, by measuring the optical density (OD) of the solution at 600 nm every 35 seconds. A similar experiment was performed with the addition of demineralised water instead of MUT, to follow spontaneous crystallization under these conditions. All incubations were performed in triplicate to correct for experimental error. Time required to obtain an optical density of 1 and 2 were monitored, and is depicted in Table 1. Especially the time to reach OD2 is significantly shorter in all the samples that used mutarotase. This shows that mutarotase will stimulate the crystallization of lactose under these conditions.

10

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	Time required to reach OD1 (sec)			Average	SD
120 U/ml MUT	2135	2275	2310	2240	93
60 U/ml MUT	2625	2555	2625	2602	40
no MUT	2730	2555	2800	2695	126
	Time required to reach OD2 (sec)			Average	SD
120 U/ml MUT	3045	3150	3185	3127	73
60 U/ml MUT	3430	3325	3465	3407	73
no MUT	7280	5740	7245	6755	879

Table 1: Time required to reach the indicated OD in the presence or absence of mutarotase.

Average gives the average time required to reach the indicated OD. SD gives the standard deviation of the obtained time points in triplicate measurements.

20

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The presence of glass beads in the incubation stimulates the nucleation of lactose and enhances the rate of crystallization in this experiment. To monitor the crystallization in the absence of agitation by glass beads, the experiment was repeated with and without 60 U/ml mutarotase in a 48-well MTP plate. Also in this experiment a clear effect of mutarotase on the rate of lactose crystallization could be detected. On average the time to reach an OD of 0.5 was 1.5 hours in the presence of mutarotase, while OD0.5 was only reached after 5

hours in the absence of mutarotase. OD1 was reached after 3.5 hours with, and 5.5 hours without added mutarotase.

Analysis of sugars by HPLC-RI

5 The amount of lactose in the soluble phase is analyzed using a HPLC system equipped with an Aminex HPX-87P column (BioRad). Separation of the various sugars is based on a combination of size exclusion, ion-exchange and reversed phase mechanisms. Elution is performed with MQ water at a flow of 0.6 ml/min for 22 min, while column temperature is 85°C. A refractive index (RI) detector (internal oven at 35°C, sensitivity 32) is used for
10 monitoring the sugars.

Before analysis the crystal suspension is centrifuged for 3 min at 10.000 rpm. The supernatant is analyzed after proper dilution. Lactose is quantified using standard solutions. The lactose content of the supernatant is determined after 6 hours incubation of a supersaturated lactose solution in the presence or absence of mutarotase.

Example 4

Use of mutarotase in the dissolution of crystalline α -lactose

Two gram of crystalline α -lactose (Sigma) was mixed with 10 ml 5 mM potassium phosphate buffer pH6.8. The slurry was divided over two beakers and to one of the beakers
20 75 U of MUT was added. The dissolution of α -lactose was followed in time under constant stirring of the slurry in both beakers, at 20 degrees Celsius. The lactose in the beaker that had obtained the mutarotase was dissolved significantly faster than the control beaker where no mutarotase had been added.

Example 5

Use of porcine kidney mutarotase in the crystallization of lactose

A similar experiment as described in Example 3 is performed. The only relevant difference between these experiments is the use of porcine kidney mutarotase instead of *Aspergillus niger* mutarotase for these experiments. Porcine kidney mutarotase can be purchased from
30 Biozyme Laboratories Ltd (product code MUR1F). The activity of MUR1F can be determined using the method described in Example 2. A similar amount of mutarotase activity can be used to stimulate the crystallization of α -lactose, exactly as described in Example 3.

Example 6

Use of mutarotase in the crystallization of glucose

The effect of the crystallization of α -glucose is tested under similar conditions as described for lactose in Example 3. Supersaturated solutions of D-glucose are prepared and used in a crystallization experiment by decreasing temperature. Since glucose is much more soluble than lactose (approximately 91 g of glucose per 100 g of water at 25 °C), ethanol (or another alcohol) can be added to increase supersaturation of the glucose solution before decreasing the temperature. Additionally, seed crystals of α -D-glucose may be added to stimulate crystal growth. Crystallization time is compared between samples with or without added mutarotase, essentially as described under Example 3.

Example 7

Use of mutarotase in the crystallization of fructose

The effect of the crystallization of fructose is tested under similar conditions as described for lactose in Example 3. Supersaturated solutions of fructose are prepared and used in a crystallization experiment by decreasing temperature. Since fructose is much more soluble than lactose (approximately 430 g of fructose per 100 g of water at 30 °C), ethanol (or another alcohol) may be added to increase supersaturation of the fructose solution before decreasing the temperature. Additionally, seed crystals of β -D-fructopyranose may be added to stimulate crystal growth. Crystallization time is compared between samples with or without added mutarotase, essentially as described under Example 3.

Example 8

Use of mutarotase in the crystallization of galactose

The effect of the crystallization of galactose is tested under similar conditions as described for lactose in Example 3. Supersaturated solutions of galactose are prepared and used in a crystallization experiment by decreasing temperature. Since galactose is more soluble than lactose (68.30 grams of galactose per 100 grams of water at 20-25° C), ethanol (or another alcohol) may be added to increase supersaturation of the galactose solution before decreasing the temperature. Additionally, seed crystals of α -D-galactose may be added to stimulate crystal growth. Crystallization time is compared between samples with or without added mutarotase, essentially as described under Example 3.

Example 9**Use of mutarotase in the crystallization of xylose**

The effect of the crystallization of xylose is tested under similar conditions as described for
5 lactose in Example 3. Supersaturated solutions of xylose are prepared and used in a
crystallization experiment by decreasing temperature. Since xylose is more soluble than
lactose (122 grams of xylose per 100 grams of water at 25°C), ethanol (or another alcohol)
may be added to increase supersaturation of the xylose solution before decreasing the
temperature. Additionally, seed crystals of the α -pyranose form of D-xylose may be added
10 to stimulate crystal growth. Crystallization time is compared between samples with or
without added mutarotase, essentially as described under Example 3.

CLAIMS

1. A method for improving carbohydrate crystallization comprising adding a protein having mutarotase activity before or during crystallization of said carbohydrate and wherein said protein having mutarotase activity is active during crystallization.

2. A method according to claim 1, wherein said protein having mutarotase activity is a protein having at least 50% identity with the protein of SEQ ID NO: 1.

3. A method according to claim 1 or 2, wherein said protein having mutarotase activity comprises the amino acid sequence as depicted in SEQ ID NO: 1.

4. A method according to any one of claims 1 to 3, wherein said carbohydrate is a reducing sugar such as glucose, fructose, galactose, xylose or lactose.

5. A method according to any one of claims 1 to 4, wherein said carbohydrate is lactose.

6. A method according to any one of claims 1 to 5, wherein said crystallization of said carbohydrate is performed on a pure, partly purified or a crude solution of said carbohydrate.

7. A method according to claim 6, wherein said crude solution of said carbohydrate is whey, whey permeate, milk or partially treated or purified forms thereof

8. A method according to any one of claims 1 to 7, further comprising dissolving the produced carbohydrate crystals resulting in a dissolved carbohydrate solution.

9. A method according to claim 8, wherein said produced carbohydrate crystals are dissolved in the presence of a protein having mutarotase activity.

10. A method according to claim 8 or 9, further comprising subjecting the obtained dissolved carbohydrate solution to a further crystallization process.

11. A method according to claim 10, wherein said further crystallization process is performed in the presence of a protein having mutarotase activity.
- 5 12. Use of mutarotase for improving carbohydrate crystallization.
- 13 A product obtainable by a method according to any one of claims 1 to 11.

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2011/061813

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12P19/12 C12P41/00 C13K5/00 C12P19/02
 ADD. C12N9/90

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)

C12P C12N C13K

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 practical, search terms used)

EPO-Internal , BIOSIS, COMPENDEX, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BENTLEY R ET AL: "Mutarotase from <i>Penicillium notatum</i> . II. The mechanism of the mutarotation reaction.", THE JOURNAL OF BIOLOGICAL CHEMISTRY MAY 1960 LNKD- PUBMED: 13799038, vol . 235, May 1960 (1960-05) , pages 1225-1233 , XP002611784, ISSN: 0021-9258 page 1227, right-hand column -----	12 , 13
X	HYND J: "Drying of whey. ", JOURNAL OF THE SOCIETY OF DAIRY TECHNOLOGY, vol . 33, no. 2, 1980, pages 52-54, XP002611785 ,	13
Y	the whole document ----- -/- .	1-12



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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INTERNATIONAL SEARCH REPORT

International application No

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MIMOUNI A ET AL: "Isothermal batch crystallization of alpha-lactose: A kinetic model combining mutarotation, nucleation and growth steps", INTERNATIONAL DAIRY JOURNAL, vol. 19, no. 3, March 2009 (2009-03), pages 129-136, XP002611786, ISSN: 0958-6946	13
Y	the whole document	1-12
Y	----- BENTLEY R ET AL: "Mutarotase from Penicillium notatum. I. Purification, assay, and general properties of the enzyme.", THE JOURNAL OF BIOLOGICAL CHEMISTRY MAY 1960 LNKD- PUBMED: 13799037, vol. 235, May 1960 (1960-05), pages 1219-1224, XP002611787, ISSN: 0021-9258 the whole document	1-12
Y	----- KINOSHITA S ET AL: "PURIFICATION AND PROPERTIES OF ALDOSE 1 EPIMERASE EC-5.1.3.3 FROM ASPERGILLUS-NIGER", BIOCHIMICA ET BIOPHYSICA ACTA, vol. 662, no. 2, 1981, pages 285-290, XP002611788, ISSN: 0006-3002 the whole document	1-12
