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(54) Title: MASHING PROCESS

(57) Abstract: The present invention provides processes for producing a brewers wort comprising forming a mash from a grist, and contacting said mash with a pullulanase.

MASHING PROCESS

REFERENCE TO A SEQUENCE LISTING

This application contains a Sequence Listing in computer readable form. The computer readable form is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to an improved mashing process for production of a brewer's wort and for production of a beer.

BACKGROUND OF THE INVENTION

In modern mashing processes, enzymes are often added as a supplement when mashing malt is low in enzymes or to allow use of all adjunct grists. Enzymes may also be applied in mashing of well modified malts with high enzyme content in order to increase the extract recovery as well as the amount of fermentable sugars. It is thus well known to apply debranching enzymes, *e.g.*, isoamylase or pullulanase to increase the yield fermentable sugars. Debranching enzymes may be applied in processes for production of low calorie beer. Such processes are the subject of Willox et al. (MBAA Technical Quarterly, 1977, 14: 105), U.S. Patent Nos. 4,528,198, 4,666,718, and 4,318,927, and GB 2056484 and GB 2069527.

SUMMARY OF THE INVENTION

The present inventors have now surprisingly discovered that by using a certain pullulanase, mashing can be achieved using a smaller amount of enzyme protein.

Accordingly, in a first aspect the invention provides a process for producing a brewers wort comprising forming a mash from a grist, and contacting said mash with a pullulanase (E.C. 3.2.1.41), wherein said pullulanase has an amino acid sequence which a) is at least 50% identical to the amino acid sequence shown in SEQ ID NO: 4, or b) is encoded by a nucleic acid sequence which hybridizes under low stringency conditions with i) a complementary strand of a nucleic acid sequence encoding the amino acid sequence shown in SEQ ID NO: 4, or ii) a subsequence of (i) of at least 100 nucleotides.

In a second aspect the invention provides a wort produced by the process of the first aspect.

In a third aspect the invention provides concentrated and/or dried wort produced by the process of the first aspect.

In a fourth aspect the invention provides beer produced from the wort of the second and third aspect.

In a fifth aspect the invention provides a composition suitable for use in the process of the first aspect, said composition comprising pullulanase (E.C. 3.2.1.41), glucoamylase and optionally alpha-amylase, wherein the pullulanase has an amino acid sequence which a) is at least 50% identical to the amino acid sequence shown in SEQ ID NO: 4, or b) is encoded by a nucleic acid sequence which hybridizes under low stringency conditions with i) a complementary strand of a nucleic acid sequence encoding the amino acid sequence shown in SEQ ID NO: 4, or ii) a subsequence of (i) of at least 100 nucleotides.

DETAILED DESCRIPTION OF THE INVENTION

Brewing processes are well-known in the art, and generally involve the steps of malting, mashing, and fermentation. Mashing is the process of converting starch from the milled barley malt and solid adjuncts into fermentable and unfermentable sugars to produce wort of the desired composition. Traditional mashing involves mixing milled barley malt and adjuncts with water at a set temperature and volume to continue the biochemical changes initiated during the malting process. The mashing process is conducted over a period of time at various temperatures in order to activate the endogenous enzymes responsible for the degradation of proteins and carbohydrates. By far the most important change brought about in mashing is the conversion of starch molecules into fermentable sugars. The principal enzymes responsible for starch conversion in a traditional mashing process are alpha- and beta-amylases. Alpha-amylase very rapidly reduces insoluble and soluble starch by splitting starch molecules into many shorter chains that can be attacked by beta-amylase. The disaccharide produced is maltose. In addition to the maltose formed during mashing short branched glucose oligomers are produced. The short branched glucose oligomers are non fermentable sugars and add to the taste as well as the amount of calories of the finished beer.

After mashing, when all the starch has been broken down, it is necessary to separate the liquid extract (the wort) from the solids (spent grains). Wort separation, lautering, is important because the solids contain large amounts of protein, poorly modified starch, fatty material, silicates, and polyphenols (tannins). Following the separation of the wort from the spent grains the wort may be fermented with brewers yeast to produce a beer.

Further information on conventional brewing processes may be found in "Technology Brewing and Malting" by Wolfgang Kunze of the Research and Teaching Institute of Brewing, Berlin (VLB), 2nd revised Edition 1999, ISBN 3-921690-39-0.

The short branched glucose oligomers formed during mashing may be further hydrolyzed by addition of exogenous enzymes (enzymes added in addition to the malt). Debranching enzymes such as pullulanase and isoamylase hydrolyze the branching alpha-1-6 glucosidic bonds in these oligomers, thereby releasing glucose or maltose and straight-chained oligomers which are subject to the action of endogenous (malt derived) and/or exogenous enzymes, *e.g.*, alpha-amylases, beta-amylases and glucoamylases.

The present invention provides a new process suitable for producing a wort that is low in non-fermentable sugars. The process applies an expressly selected pullulanase activity.

Definitions

Throughout this disclosure, various terms that are generally understood by those of ordinary skill in the arts, are used. Several terms are used with specific meaning, as defined below.

As used herein the term "**grist**" is understood as the starch or sugar containing material that is the basis for beer production, *e.g.*, the barley malt and the adjunct. Generally, the grist does not contain any added water.

The term "**malt**" is understood as any malted cereal grain, in particular barley.

The term "**adjunct**" is understood as the part of the grist which is not barley malt. The adjunct may comprise any starch rich plant material, *e.g.*, unmalted grain, such as barley, rice, corn, wheat, rye, sorghum and readily fermentable sugar and/or syrup.

The term "**mash**" is understood as a starch containing slurry comprising grist steeped in water.

The term "**wort**" is understood as the unfermented liquor run-off following extracting the grist during mashing.

The term "**spent grains**" is understood as the drained solids remaining when the grist has been extracted and the wort separated.

The term "**beer**" is understood as fermented wort, *i.e.*, an alcoholic beverage brewed from barley malt, optionally adjunct and hops.

The term "**homologous sequence**" is used to characterize a sequence having an amino acid sequence that is at least 70%, preferably at least 75%, or at least 80%, or at least 85%, or

90%, or at least 95%, at least 96%, at least 97%, at least 98% at least 99% or even at least 100% identical to a known sequence. The relevant part of the amino acid sequence for the homology determination is the mature polypeptide, *i.e.*, without the signal peptide. The term "**homologous sequence**" is also used to characterize DNA sequences which hybridize at low stringency, medium stringency, medium/high stringency, high stringency, or even very high stringency with a known sequence. Suitable experimental conditions for determining hybridization at low, medium, or high stringency between a nucleotide probe and a homologous DNA or RNA sequence involves presoaking of the filter containing the DNA fragments or RNA to hybridize in 5 x SSC (Sodium chloride/Sodium citrate, Sambrook et al., 1989) for 10 min, and prehybridization of the filter in a solution of 5 x SSC, 5 x Denhardt's solution (Sambrook et al., 1989), 0.5% SDS and 100 micrograms/ml of denatured sonicated salmon sperm DNA (Sambrook et al., 1989), followed by hybridization in the same solution containing a concentration of 10ng/ml of a random-primed (Feinberg and Vogelstein, 1983, *Anal. Biochem.* 132:6-13), 32P-dCTP-labeled (specific activity > 1 x 109 cpm/microgram) probe for 12 hours at about 45°C. The filter is then washed twice for 30 minutes in 2 x SSC, 0.5% SDS at about 55°C (low stringency), more preferably at about 60°C (medium stringency), still more preferably at about 65°C (medium/high stringency), even more preferably at about 70°C (high stringency), and even more preferably at about 75°C (very high stringency). Molecules to which the oligonucleotide probe hybridizes under these conditions are detected using an x-ray film.

The term "**identity**" when used about polypeptide or DNA sequences and referred to in this disclosure is understood as the degree of identity between two sequences indicating a derivation of the first sequence from the second. The identity may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman and Wunsch, 1970, *Journal of Molecular Biology* 48: 443-453. The following settings for polypeptide sequence comparison are used: GAP creation penalty of 3.0 and GAP extension penalty of 0.1. The degree of identity between an amino acid sequence of the present invention and a different amino acid sequence ("foreign sequence") is calculated as the number of exact matches in an alignment of the two sequences, divided by the length of the "invention sequence" or the length of the "foreign sequence", whichever is the shortest. The result is expressed in percent identity.

Wort production

In accordance with the first aspect the invention provides a process for producing a brewer's wort comprising forming a mash from a grist, and contacting said mash with a pullulanase (E.C. 3.2.1.41), wherein said pullulanase has an amino acid sequence which a) is at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, or at least 85%, or 90%, or at least 95%, at least 96%, at least 97%, at least 98% or even at least 99% identical to the amino acid sequence shown in SEQ ID NO: 4, or b) is encoded by a nucleic acid sequence which hybridizes under low stringency, medium stringency, medium/high stringency, high stringency, or even very high stringency with i) a complementary strand of a nucleic acid sequence encoding the amino acid sequence shown in SEQ ID NO: 4, or ii) a subsequence of (i) of at least 100 nucleotides, at least 200 nucleotides, at least 300 nucleotides, at least 500 nucleotides, at least 1000 nucleotides, or even at least 1500 nucleotides. In a preferred embodiment, the pullulanase has an amino acid sequence which differs by no more than 100 amino acids, preferably by no more than 80 amino acids, more preferred by no more than 50 amino acids, more preferably by no more than 30 amino acids, even more preferably by no more than 20 amino acids, and most preferably by no more than 10 amino acids from the amino acid sequence of SEQ ID NO: 3.

The grist of the first aspect comprises starch containing malted grain and/or adjunct. The grist may preferably comprise from 0% to 100%, preferably from 20% to 100%, preferably from 30% to 100%, more preferably from 40% to 100%, even more preferably from 50% to 100%, yet more preferably from 60% to 100%, such as from 80% to 100% or even most preferably from 90% to 100% adjunct, unmalted grain and/or unmalted barley. In a particular embodiment the adjunct is composed of 100% unmalted barley. Furthermore, the grist preferably comprises from 0% to 100%, preferably from 20% to 100%, preferably from 30% to 100%, more preferably from 40% to 100%, even more preferably from 50% to 100%, yet more preferably from 60% to 100%, or most preferably from 70% to 100%, or even most preferably from 90% to 100% malted grain and/or malted barley. In a particular embodiment the grist comprises approximately 50% malted grain, *e.g.*, malted barley, and approximately 50% adjunct, *e.g.*, unmalted grain, such as unmalted barley.

Malted grain used in the process of the first aspect may comprise any malted grain, and preferably malted grain selected from malted barley, wheat, rye, sorghum, millet, corn, and rice, and most preferably malted barley.

The adjunct used in the process of the first aspect may be obtained from tubers, roots, stems, leaves, legumes, cereals and/or whole grain. The adjunct may comprise raw and/or refined starch and/or sugar containing material derived from plants like wheat, rye, oat, corn, rice, milo, millet, sorghum, potato, sweet potato, cassava, tapioca, sago, banana, sugar beet and/or sugar cane. Preferably, the adjunct comprises unmalted grain, *e.g.*, unmalted grain selected from the list consisting of barley, wheat, rye, sorghum, millet, corn, and rice, and most preferably unmalted barley. Adjunct comprising readily fermentable carbohydrates such as sugars or syrups may be added to the barley malt mash before, during or after mashing process of the invention but is preferably added after the mashing process.

According to the invention, a pullulanase (E.C. 3.2.1.41) enzyme activity is exogenously supplied and present in the mash. The pullulanase may be added to the mash ingredients, *e.g.*, the water and/or the grist before, during or after forming the mash. In a particularly preferred embodiment an alpha-amylase (E.C. 3.2.1.1) and/or a glucoamylase (E.C. 3.2.1.3), is added and present in the mash together with the pullulanase.

In another preferred embodiment, a further enzyme is added to the mash, said enzyme being selected from the group consisting of isoamylase, protease, laccase, xylanase, lipase, phospholipolase, phytase, phytin and esterase.

During the mashing process, starch extracted from the grist is gradually hydrolyzed into fermentable sugars and smaller dextrins. Preferably, the mash is starch negative to iodine testing, before extracting the wort.

The mashing process generally applies a controlled stepwise increase in temperature, where each step favors one enzymatic action over the other, eventually degrading proteins, cell walls and starch. Mashing temperature profiles are generally known in the art. In the present invention the saccharification (starch degradation) step in the mashing process is preferably performed between 60°C and 66°C, more preferably between 61°C and 65°C, even more preferably between 62°C and 64°C, and most preferably between 63°C and 64°C. In a particular embodiment of the present invention the saccharification temperature is 64°C.

Obtaining the wort from the mash typically includes straining the wort from the spent grains, *i.e.*, the insoluble grain and husk material forming part of grist. Hot water may be run through the spent grains to rinse out, or sparge, any remaining extract from the grist. The application of a thermostable cellulase in the process of the present invention results in efficient reduction of beta-glucan level facilitating wort straining thus ensuring reduced cycle time and high extract recovery. Preferably the extract recovery is at least 80%, preferably at least 81%,

more preferably at least 82%, even more preferably at least 83%, such as at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, and most preferably at least 91%.

Following the separation of the wort from the spent grains of the grist of any of the aforementioned embodiments of the first aspect, the wort may be used as it is or it may be dewatered to provide a concentrated and/or dried wort. The concentrated and/or dried wort may be used as brewing extract, as malt extract flavoring, for non-alcoholic malt beverages, malt vinegar, breakfast cereals, for confectionary etc.

In a preferred embodiment, the wort is fermented to produce an alcoholic beverage, preferably a beer, *e.g.*, ale, strong ale, bitter, stout, porter, lager, export beer, malt liquor, barley wine, happoushu, high-alcohol beer, low-alcohol beer, low-calorie beer or light beer. Fermentation of the wort may include pitching the wort with a yeast slurry comprising fresh yeast, *i.e.*, yeast not previously used for the invention or the yeast may be recycled yeast. The yeast applied may be any yeast suitable for beer brewing, especially yeasts selected from *Saccharomyces* spp. such as *S. cerevisiae* and *S. uvarum*, including natural or artificially produced variants of these organisms. The methods for fermentation of wort for production of beer are well known to the person skilled in the arts.

The process of the invention may include adding silica hydrogel to the fermented wort to increase the colloidal stability of the beer. The processes may further include adding kieselguhr to the fermented wort and filtering to render the beer bright.

According to an aspect of the invention is provided beer produced from the wort of the second or third aspect, such as a beer produced by fermenting the wort to produce a beer. The beer may be any type of beer, *e.g.*, ales, strong ales, stouts, porters, lagers, bitters, export beers, malt liquors, happoushu, high-alcohol beer, low-alcohol beer, low-calorie beer or light beer.

Enzymes

The enzymes to be applied in the present invention should be selected for their ability to retain sufficient activity at the process temperature of the processes of the invention, as well as under the pH regime in the mash and should be added in effective amounts. The enzymes may be derived from any source, preferably from a plant or an alga, and more preferably from a microorganism, such as from a bacterium or a fungus.

Pullulanase (E.C. 3.2.1.41)

A preferred pullulanase enzyme to be used in the processes and/or compositions of the invention is a pullulanase having an amino acid sequence which is at least 50%, such as at least 55%, such as at least 60%, such as at least 65%, such as at least 66%, such as at least 70%, such as at least 75%, such as at least 80%, such as at least 85%, such as at least 86%, such as at least 87%, such as at least 88%, such as at least 89%, such as at least 90%, such as at least 95%, such as at least 98% or even 100% identical to the sequence shown in SEQ ID NO: 4; ion particular when aligned using the Program Needle using Matrix: BLOSUM62; Gap initiation penalty: 10.0; Gap extension penalty: 0.5; Gapless Identity Matrix.

Most preferably the pullulanase is derived from *Bacillus acidopullulyticus*. The pullulanase may have the amino acid sequence disclosed by Kelly et al., 1994 (*FEMS Microbiol. Letters* 115: 97-106) (SEQ ID NO: 6) or a homologous sequence.

Isoamylase (E.C. 3.2.1.68)

Another enzyme applied in the processes and/or compositions of the invention may be an alternative debranching enzyme, such as an isoamylase (E.C. 3.2.1.68). Isoamylase hydrolyzes alpha-1,6-D-glucosidic branch linkages in amylopectin and beta-limit dextrins and can be distinguished from pullulanases by the inability of isoamylase to attack pullulan, and by the limited action on alpha-limit dextrins. Isoamylase may be added in effective amounts well known to the person skilled in the art. Isoamylase may be added alone or together with a pullulanase.

Alpha-amylase (EC 3.2.1.1)

A particular alpha-amylase enzyme to be used in the processes and/or compositions of the invention may be a *Bacillus* alpha-amylase. Well-known *Bacillus* alpha-amylases include alpha-amylase derived from a strain of *B. licheniformis*, *B. amyloliquefaciens*, and *B. stearothermophilus*. In the context of the present invention, a contemplated *Bacillus* alpha-amylase is an alpha-amylase as defined in WO 99/19467 on page 3, line 18 to page 6, line 27. A preferred alpha-amylase has an amino acid sequence having at least 90% identity to SEQ ID NO: 4 in WO 99/19467 (herein disclosed as SEQ ID NO: 7), such as at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or particularly at least 99%. Most preferred maltogenic alpha-amylase is SEQ ID NO: 9 or comprise the variants thereof disclosed in WO 99/43794. Contemplated variants and hybrids are described in WO 96/23874, WO 97/41213, and WO

99/19467. Specifically contemplated is an alpha-amylase (E.C. 3.2.1.1) from *B. stearothermophilus* having the amino acid sequence disclosed as SEQ ID NO: 3 in WO 99/19467 (herein disclosed as SEQ ID NO: 10) with the mutations: I181* + G182* + N193F.

Bacillus alpha-amylases may be added in the amounts of 1.0-1000 NU/kg DS, preferably from 2.0-500 NU/kg DS, preferably 10-200 NU/kg DS.

Another particular alpha-amylase to be used in the processes of the invention may be any fungal alpha-amylase, *e.g.*, an alpha-amylase derived from a species within *Aspergillus*, and preferably from a strain of *Aspergillus niger.* Especially contemplated are fungal alpha-amylases which exhibit a high identity, *i.e.*, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85% or even at least 90% identity to the amino acid sequences shown SEQ ID NO: 1 in WO 2002/038787 (herein disclosed as SEQ ID NO: 11). Fungal alpha-amylases may be added in an amount of 1-1000 AFAU/kg DS, preferably from 2-500 AFAU/kg DS, preferably 20-100 AFAU/kg DS.

Glucoamylases (E.C.3.2.1.3)

A further particular enzyme to be used in the processes and/or compositions of the invention may be a glucoamylase (E.C.3.2.1.3) derived from a microorganism or a plant. Preferred are glucoamylases of fungal or bacterial origin selected from the group consisting of *Aspergillus* glucoamylases, in particular *A. niger* G1 or G2 glucoamylase (Boel et al., 1984, EMBO J. 3(5): 1097-1102), or variants thereof, such as disclosed in WO 92/00381 and WO 00/04136; the *A. awamori* glucoamylase (WO 84/02921), *A. oryzae* (*Agric. Biol. Chem.*, 1991, 55(4): 941-949), or variants or fragments thereof.

Other contemplated glucoamylases include *Talaromyces* glucoamylases, in particular derived from *Talaromyces emersonii* (WO 99/28448), *Talaromyces leycettanus* (U.S. Patent No. Re. 32,153), *Talaromyces duponti*, and *Talaromyces thermophilus* (U.S. Patent No. 4,587,215). Preferred glucoamylases include the glucoamylases derived from *Aspergillus oryzae*, such as a glucoamylase having at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or particularly at least 99% or even at least 90% identity to the amino acid sequence shown in SEQ ID NO:2 in WO00/04136. Other preferred glucoamylases include the glucoamylases derived from *Talaromyces emersonii* such as a glucoamylase having at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or particularly at least 99% or even at least 90% identity to the amino acid sequence of *Talaromyces emersonii* (WO 99/28448).

Bacterial glucoamylases contemplated include glucoamylases from the genus *Clostridium*, in particular *C. thermoamylolyticum* (EP 135,138), and *C. thermohydrosulfuricum* (WO 86/01831).

Also contemplated are the commercial products AMG 200L; AMG 300 L; SAN™ SUPER and AMG™ E (from Novozymes); OPTIDEX™ 300 (from Genencor Int.); AMIGASE™ and AMIGASE™ PLUS (from DSM); G-ZYME™ G900 (from Enzyme Bio-Systems); G-ZYME™ G990 ZR (*A. niger* glucoamylase and low protease content). Glucoamylases may be added in effective amounts well known to the person skilled in the art.

Protease

Suitable proteases include microbial proteases, such as fungal and bacterial proteases. Preferred proteases are acidic proteases, *i.e.*, proteases characterized by the ability to hydrolyze proteins under acidic conditions below pH 7.

The proteases are responsible for reducing the overall length of high-molecular-weight proteins to low-molecular-weight proteins in the mash. The low-molecular-weight proteins are a necessity for yeast nutrition and the high-molecular-weight-proteins ensure foam stability. Thus it is well-known to the skilled person that protease should be added in a balanced amount which at the same time allows amble free amino acids for the yeast and leaves enough high-molecular-weight-proteins to stabilize the foam. Proteases may be added in the amounts of 0.1-1000 AU/kg DS, preferably 1-100 AU/kg DS and most preferably 5-25 AU/kg DS.

Cellulase (E.C. 3.2.1.4)

The cellulase may be of microbial origin, such as derivable from a strain of a filamentous fungus (*e.g.*, *Aspergillus*, *Trichoderma*, *Humicola*, *Fusarium*). Specific examples of cellulases include the endoglucanase (endoglucanase I) obtainable from *H. insolens* and further defined by the amino acid sequence of fig. 14 in WO 91/17244 (herein disclosed as SEQ ID NO: 12) and the 43 kD *H. insolens* endoglucanase described in WO 91/17243.

A particular cellulase to be used in the processes of the invention may be an endo-glucanase, such as an endo-1,4-beta-glucanase. Especially contemplated is the beta-glucanase shown in SEQ.ID.NO: 2 in WO 2003/062409 (herein disclosed as SEQ ID NO: 14) and homologous sequences. Commercially available cellulase preparations which may be used include CELLUCLAST®, CELLUZYME®, CEREFLO® and ULTRAFLO® (available from

Novozymes A/S), LAMINEX™ and SPEZYME® CP (available from Genencor Int.) and ROHAMENT® 7069 W (available from Röhm, Germany).

Beta-glucanases may be added in the amounts of 1.0-10000 BGU/kg DS, preferably from 10-5000 BGU/kg DS, preferably from 50-1000 BGU/kg DS and most preferably from 100-500 BGU/kg DS.

MATERIALS AND METHODS

Enzymes

Pullulanase 1 derived from *Bacillus acidopullulyticus* and having the sequence showed in SEQ ID NO: 1. Pullulanase 1 is available from Novozymes as Promozyme 400L.

Pullulanase 2 derived from *Bacillus deramificans* (U.S. Patent No. 5,736,375) and having the sequence showed in SEQ ID NO: 2. Pullulanase 2 is available from Novozymes as Promozyme D2.

Pullulanase 3 derived from *Bacillus acidopullulyticus* and having the sequence showed in SEQ ID NO: 4.

Acid fungal alpha-amylase derived from *Aspergillus niger* and having the sequence showed in SEQ ID NO: 11.

Glucoamylase G1 derived from *Aspergillus niger* (Boel et al., supra).

Methods

Alpha-amylase activity (NU)

Alpha-amylase activity may be determined using potato starch as substrate. This method is based on the break-down of modified potato starch by the enzyme, and the reaction is followed by mixing samples of the starch/enzyme solution with an iodine solution. Initially, a blackish-blue color is formed, but during the break-down of the starch the blue color gets weaker and gradually turns into a reddish-brown, which is compared to a colored glass standard.

One Kilo Novo alpha amylase Unit (KNU) equals 1000 NU. One KNU is defined as the amount of enzyme which, under standard conditions (*i.e.*, at 37°C +/- 0.05; 0.0003 M Ca^{2+}; and pH 5.6) degrades 5.26 g starch dry matter (Merck Amylum solubile).

Acid alpha-amylase activity (AFAU)

Acid alpha-amylase activity may be measured in AFAU (Acid Fungal Alpha-amylase Units), which are determined relative to an enzyme standard. 1 FAU is defined as the amount of enzyme which degrades 5.260 mg starch dry matter per hour under the below mentioned standard conditions.

Acid alpha-amylase, an endo-alpha-amylase (1,4-alpha-D-glucan-glucanohydrolase, E.C. 3.2.1.1) hydrolyzes alpha-1,4-glucosidic bonds in the inner regions of the starch molecule to form dextrins and oligosaccharides with different chain lengths. The intensity of color formed with iodine is directly proportional to the concentration of starch. Amylase activity is determined using reverse colorimetry as a reduction in the concentration of starch under the specified analytical conditions.

$$\text{STARCH} + \text{IODINE} \xrightarrow[40^\circ,\ \text{pH}\ 2.5]{\text{ALPHA-AMYLASE}} \text{DEXTRINS} + \text{OLIGOSACCHARIDES}$$

$\lambda = 590$ nm

blue/violet t = 23 sec. decoloration

Standard conditions/reaction conditions:

Substrate:	Soluble starch, approx. 0.17 g/L
Buffer:	Citrate, approx. 0.03 M
Iodine (I2):	0.03 g/L
CaCl2:	1.85 mM
pH:	2.50 ± 0.05
Incubation temperature:	40°C
Reaction time:	23 seconds
Wavelength:	590 nm
Enzyme concentration:	0.025 AFAU/mL
Enzyme working range:	0.01-0.04 AFAU/mL

Glucoamylase activity (AGU)

The Novo Glucoamylase Unit (AGU) is defined as the amount of enzyme, which hydrolyzes 1 micromole maltose per minute at 37°C and pH 4.3.

The activity is determined as AGU/ml by a method modified after (AEL-SM-0131, available on request from Novozymes) using the Glucose GOD-Perid kit from Boehringer Mannheim, 124036. Standard: AMG-standard, batch 7-1195, 195 AGU/ml. 375 microL substrate (1% maltose in 50 mM Sodium acetate, pH 4.3) is incubated 5 minutes at 37°C. 25 microL enzyme diluted in sodium acetate is added. The reaction is stopped after 10 minutes by adding 100 microL 0.25 M NaOH. 20 microL is transferred to a 96 well microtitre plate and 200 microL GOD-Perid solution (124036, Boehringer Mannheim) is added. After 30 minutes at room temperature, the absorbance is measured at 650 nm and the activity calculated in AGU/ml from the AMG-standard. A detailed description of the analytical method (AEL-SM-0131) is available on request from Novozymes.

Pullulanase activity (PUN):

One pullulanase unit (PUN) is defined as the amount of enzyme, which is capable of forming 1 micromole glucose from pullulan substrate per minute at 50°C in a pH 5 citrate buffer.

Pullulanase samples are incubated with substrate (red pullulan). Endo-pullulanase hydrolyses the alpha-1,6-glycosidic bonds in red pullulan, releasing red substrate degradation products. Non-degraded substrate is precipitated using ethanol. The amount of color released is measured spectrophotometrically at 510 nm and is proportional to the endo-pullulanase activity in the sample. The color formation of samples is compared to the color formation produced by samples with known pullulanase activity.

Pullulanase is a pullulan 6-glucano-hydrolase with the enzyme classification number E.C.3.2.1.41.

Reaction conditions

Temperature	50°C ± 2°C
pH	5.0
Substrate concentration	0.67 % red pullulan
Enzyme concentration	0.04 – 0.13 PUN/ml
Reaction time	30 min.
Wavelength	510 nm

Reagents/ Substrates

Potassium chloride solution 0.5 M

Red pullulan substrate 2%. Supplier Megazyme, Australia

Citrate buffer 0.05 M pH 5.0

Citrate buffer 0.05 M pH 5.0 with 25 mM cysteine

Ethanol 99.8%

Pullulanase Standard preparation of 904 PUN/g diluted into citrate buffer 0.05 M to a standard dilution series from 0.05 – 0.20 PUN/ml

Blank Citrate buffer 0.05 M

Enzyme samples are diluted in citrate buffer 0.05 M to an activity between 0.06 – 0.20 PUN/ml and compared to the standard dilution series.

Example 1

In this example, the ability of different pullulanases to reduce the amount of non-fermentable carbohydrates (dextrin/DP4/4+) in a wort was analyzed.

100% well modified malt was mashed using a mashing temperature profile comprising 46°C for 26 minutes, followed by a 1°C/minute increase till 64°C after which the temperature was held constant. Samples were collected at 98, 128 and 158 minutes.

Enzymes were added at 0 minutes. Glucoamylase and alpha-amylase were added to all treatments in amounts of 1000 AGU/kg DS and 250 AFAU/kg DS respectively. Pullulanase was added according to table 1.

The samples were boiled 10 minutes and filtered (Pore size 0.20 micro-m). The samples were analyzed by HPLC and % non fermentable carbohydrate (DP4/4+) was calculated.

Table 1: % non fermentable carbohydrate after mashing times of 98, 128 and 158 minutes.				
Type of pullulanase	Amount in mg/kg DS	Mashing time		
		98 minutes	128 minutes	158 minutes
None	0	28.87	25.16	22.94
Pullulanase 1	0.74	27.64	24.55	21.96
Pullulanase 1	3.65	23.96	21.35	18.92
Pullulanase 1	7.25	20.71	17.74	16.11
Pullulanase 1	14.39	16.36	14.47	13.22
Pullulanase 2	1.86	28.34	25.21	22.79
Pullulanase 2	9.26	26.81	23.28	21.36
Pullulanase 2	18.40	25.68	22.33	20.17
Pullulanase 2	36.59	23.56	20.58	18.35
Pullulanase 3	1.37	27.11	23.38	20.51
Pullulanase 3	2.74	24.46	20.93	18.18

The data in table 1 was used to calculate, by regression, the enzyme dosages of pullulanase 1 and pullulanase 2 needed to get the same effect as 2.74 mg enzyme protein/kg of pullulanase 3 (see table 2).

Table 2. Pullulanase enzyme protein mg/kg DS needed to achieve the same effect as with 2.74 mg Pullulanase 3 and mashing times of 98, 128 and 158 minutes.		
Mashing time	Pullulanase 1	Pullulanase 2
98 minutes	3.38	27.33
128 minutes	4.04	31.06
158 minutes	4.56	40.37

5

From these results it can be seen that Pullulanase 3 is the most efficient enzyme. Consequently, less Pullulanase 3 enzyme protein is needed to reduce the amount of non-fermentable carbohydrates (dextrin/DP4/4+) and thereby increase attenuation of the wort.

Example 2

The pH profile and temperature profile of different pullulanases were analyzed in the present example.

The pH and temperature profile investigations were based on relative enzyme activity analysis with the conditions described below.

Principals of the analytical method:

The alpha-1,6-glycosidic bonds in pullulan were hydrolyzed by a pullulanase enzyme and the increased reducing sugar capacity was detected by a modified Somogyi-Nelson method.

In the present experiment the activity is assessed as relative activity, where the most active sample is given as 100%. The assay conditions are as follows:

Buffer: citrate 0.1 M + 0.2 M phosphate (adjusted in the pH profile, pH 5 in temperature profile)

Substrate: 0.2% pullulan Sigma (p-4516)

Temperature: 60°C in pH profile, adjusted in temperature profile

Reaction time: 30 minutes

The reducing sugars released by pullulanases were detected according to the principle described in Nelson, 1944, *J. Biol. Chem.* 153: 375-380 and Somogyi, 1945, *J. Biol. Chem.* 160: 61-68. In brief, the hydrolysis reaction is stopped by adding Somogyi's cobber reagent in a volume corresponding to the sample volume (*e.g.*, 2 ml to a sample of 2 ml). The samples are boiled for 20 minutes and cooled down prior to the color reaction. This reaction is performed by adding Nelson's reagent corresponding to ½ the volume of the sample (*e.g.*, 2 ml to 4 ml sample+ Somogyi's cobber reagent). The samples are mixed for 2 minutes followed by addition of water in the same amount as Nelson's reagent. The samples are incubated 30 minutes in the dark and measured in a spectrophotometer at 540 nm.

Reagents can be prepared as follows:

Somogyi's cobber reagent:

Dissolve 70.2 g $Na_2HPO_4x2H_2O$ and 80.0 g $KNAC_4H_4O_6x4H_2O$ (kaliumsodiumtartrat) in 1000 ml H_2O (heat slightly). Furthermore add 60 g NaOH; 16.0 g $CuSO_4x5\ H_2O$ and 360.0 g Na_2SO_4 and fill to 2000 ml. Adjust pH to 10.8 with NaOH

Nelson's reagent:

Dissolve 100.0 g $(NH_4)_6Mo_7O_{24}x7\ H_2O$ in 1200 ml H_2O. Add 84.0 ml H_2SO_4 carefully. Additionally, dissolve 12.00 g $Na_2HAsO_4x7H_2O$ (disodiumhydrogenarsenate) in 100 ml H_2O, and add this solution slowly to the first solution and fill to 2000 ml.

The pH and temperature profiles for the three pullulanases are given in table 3 and 4 below.

Table 3: pH profile given in relative (%) enzyme activity at 60°C

pH	Pullulanase 1	Pullulanase 2	Pullulanase 3
2.4	2.2	1	0.2
2.8	6	2.4	30.7
3.7	10.8	68.5	90
4.2	23.1	100	100
5	94	77.4	92.4
5.8	92.8	31.7	74.2
6.3	43.1	4.4	45.3
6.7	3.7	0	1.8
7.3	0	0	0

Table 4: Temperature profile given in relative (%) enzyme activity at pH 5.0			
Temp. °C	**Pullulanase 1**	**Pullulanase 2**	**Pullulanase 3**
30	22.8	36.7	19.7
45	64.4	72.5	47.3
55	100	100	76.8
60	99.6	85.2	92.6
62.5	87.1	70	101.2
65	42.1	54.4	100
70	9.4	12.7	75.6

These results show that pullulanase 3 has a broad pH profile and activity at high temperatures when compared to the other two pullulanases. These properties make pullulanase 3 a very robust enzyme in brewing (mashing conditions), in particular for saccharification temperatures between 62°C and 65°C.

Example 3

Infusion mashing test were made with Pullulanase 1 and 3 (SEQ ID NOS: 1 and 4). 6 mashing samples were prepared with 100% barley as substrate (grist).

Barley (DS%: 86.73) was milled and for each sample 50.0 g (total DS 43.34 g) were mixed with 200 g tap water at 50°C and 3.0 ml 1 M H_3PO_4.

All samples were added an identical enzyme mix with no pullulanase.

Samples 1-6 were then added pullulanase according to the following table:

Enzyme dose Activity/g

Sample no.	Pullulanase 1 PUN/g	Pullulanase 3 PUN/g
1	0.1	-
2	0.2	-
3	0.5	-
4	-	0.1
5	-	0.2
6	-	0.5

The samples were then tested in automated mashing equipment running the following program.

Time in minutes	Temp. °C
0-30	50
30-44	rising to 64
44-104	64
104-120	rising to 80
120-140	80
140-155	falling to 20

After mashing all samples were added tap water to a total of 300 g and filtered. The filtered samples were then boiled for 10 minutes and diluted 1:1 with deionised water. Subsamples of 50 ml were centrifuged and subjected to standard density analysis for calculation of RDF % (real degree of fermentation). The results are given as RDF % and wort sugar (DP2 and DP4+) in %.

PUN/g	Pullulanase 1	Pullulanase 3
0	0	0
0.1	64	64.6
0.2	65	66.1
0.5	67.3	69.5

	Pullulanase 1		Pullulanase 3	
PUN/g	DP2	DP4+	DP2	DP4+
0	48.5	31.5	48.5	31.5
0.1	48.5	30	49	29
0.2	49.5	29	50	27.5
0.5	50.5	36.5	n.d.	n.d.

The results show that pullulanase 3 is clearly better than pullulanase 1 for making a high % RDF, and still pullulanase 3 is capable of producing maltose levels (DP2 levels) even better than pullulanase 1.

Example 4

Infusion mashing test were made with Pullulanase 3 (SEQ ID NO: 4) to evaluate the maltose generating properties of pullulanase 3. 6 mashing samples were prepared with 100% barley as substrate (grist).

Barley (DS%: 86.73) was milled and for each sample 50.0 g (total DS 43.34 g) were mixed with 200 g tap water at 50°C and 5% Na_2SO_3 and 1 M H_3PO_4

All samples were added an identical enzyme mix with no pullulanase.

Samples 1-6 were then added pullulanase 3 according to the following table:

Sample no.	Pullulanse 3 PUN/g
1	-
2	0.1
3	0.3
4	0.5
5	1.0
6	2.0

The samples were then tested in automated mashing equipment running the following program.

Time in minutes	Temp. °C
0-30	50
30-44	rising to 64
44-104	64
104-120	rising to 80
120-140	80
140-155	falling to 20

5

After mashing all samples were added tap water to a total of 300 g and filtered. The filtered samples were then boiled for 10 minutes and diluted 1:1 with deionized water. Subsamples of 50 ml were centrifuged and subjected to analysis. The results were as follows:

PUN/g	% glucose	% maltose	% dextrin	RDF %
0	3.8	47.5	34	61.2
0.1	3.8	48.2	32	63.2
0.3	3.8	49.8	28.8	65.7
0.5	3.7	51.2	26.4	68.6
1	3.7	52.6	23.9	71
2	3.6	55.6	20.1	74.3

The results show that maltose concentration is increasing with increasing dosage of pullulanase 3, and the increase in maltose % is followed by the increase in attenuation (RDF%). The dextrin fraction (HPLC analysis DP4/4+) is at the same time decreasing.

Only barley beta-amylase can produce maltose in this reaction, and pullulanase 3 is facilitating the action of barley beta-amylase.

Glucose concentration is low and not effected by the action of pullulanase 3 which is an advantage when fermenting the produced wort. Pullulanase 3 help degrading dextrin and facilitate formation of maltose, by the barley beta-amylase, and by this increase attenuation (RDF%) of the wort.

CLAIMS

1. A process for producing a brewers wort comprising forming a mash from a grist, and contacting said mash with a pullulanase, wherein said pullulanase has an amino acid sequence which

a) is at least 86% identical to the amino acid sequence shown in SEQ ID NO: 4; or

b) is encoded by a nucleic acid sequence which hybridizes under low stringency conditions with

i) a complementary strand of a nucleic acid sequence encoding the amino acid sequence shown in SEQ ID NO: 4; or

ii) a subsequence of (i) of at least 100 nucleotides.

2. The process according to claim 1, wherein the pullulanase is derived from *Bacillus acidopullulyticus*.

3. The process according to claim 1 or 2, further comprising contacting said mash with a glucoamylase and/or alpha-amylase.

4. The process according to any of the preceding claims, wherein the glucoamylase and/or alpha-amylase is derived from *Aspergillus niger* or *Talaromyces emersonii*.

5. The process according to any of the preceding claims, further comprising contacting the mash with an enzyme selected from the group consisting of cellulase, isoamylase, xylanase and protease.

6. The process according to any of the preceding claims, wherein the grist comprises malted and/or unmalted grain.

7. The process according to any of the preceding claims, wherein the unmalted grain and/or the malted grain is selected from the list consisting of barley, wheat, rye, sorghum, millet, corn and rice.

8. The process according to any of the preceding claims, wherein the malted grain comprises malted grain selected from malted barley, wheat, rye, sorghum, millet, corn, and rice.

9. The process according to any of the preceding claims, wherein the wort is concentrated and/or dried.

10. The process according to any of the preceding claims, further comprising fermenting the wort to obtain an alcoholic beverage.

11. The process according to claim 10, wherein the alcoholic beverage is a beer.

12. The process according to claim 11, wherein the beer is ale, strong ale, bitter, stout, porter, lager, export beer, malt liquor, barley wine, happoushu, high-alcohol beer, low-alcohol beer, low-calorie beer or light beer.

13. A wort produced by the process according to any of the preceding claims.

14. A concentrated and/or dried wort according to any of the preceding claims.

15. A beer produced from the wort according to any of the preceding claims.

16. A composition suitable for use in the process according to any of the preceding claims, said composition comprising a pullulanase, a glucoamylase and optionally an alpha-amylase, wherein said pullulanase has an amino acid sequence which

a) is at least 50% identical to the amino acid sequence shown in SEQ ID NO: 4; or

b) is encoded by a nucleic acid sequence which hybridizes under low stringency conditions with

i) a complementary strand of a nucleic acid sequence encoding the amino acid sequence shown in SEQ ID NO: 4. or

ii) a subsequence of (i) of at least 100 nucleotides.

17. The composition according to claim 16, wherein the glucoamylase and/or the alpha-amylase is derived from *Aspergillus niger* or *Talaromyces emersonii*.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2007/087209

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12C5/00 C12C7/00 C12C11/00 C12N9/44

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)
C12C C12N

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, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	the whole document	1-17
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 October 2008	17/11/2008

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

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2007/087209

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

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