Nonetheless, barley mutants may be prepared by random mutagenesis using irradiation or chemical treatment, such as incubating kernels for 12 h to over night with a solution of sodium azide (NaN_3). An example concerns barley kernels mutagenized through the use of NaN_3, and subsequently screened for high levels of free phosphate in an effort to screen for low-phytate mutants (Rasmussen and Hatzack, 1998); a total of 10 mutants out of 2,000 screened kernels were identified. Although far from always possible, finding a particular mutant after NaN_3 treatment is dependent on persistence and an effective screening method.

Sustainability

In a world seeking solutions to its energy, environmental and food challenges, one focus of society is to limit or reduce atmospheric CO_2 concentrations – especially focusing on CO_2 emissions from industrial systems. The principal reason is that an increase in the concentration of a greenhouse gas causes a change in Earth's energy balance, with CO_2 being the largest single contributor. As a consequence of the widespread concern about climate change, and also based on economic rationales and constraints, breweries may play an active part by using energy as efficiently as possible, and by reducing greenhouse gas emissions from operations more effectively. Until now, the focal point has been on technological means to solve the above-mentioned issues on sustainability.

SUMMARY ~~OF INVENTION~~

Heat treatment methods have been described for reduction of LOX activity and DMS levels of DMS. Said treatment was generally undertaken during malting and/or preparation of wort, meaning that products of LOX activity were allowed to accumulate in barley until undertaking of the heat treatment. Analysis of barley revealed that significant amounts of products of LOX activity were present in barley, even prior to malting (Wackerbauer and Meyna, 2002). It is apparent that heat treatment methods are highly energy consuming.

The present disclosure~~invention~~ provides methods for preparing a cereal based beverage with low levels of one or more off-flavours and precursors thereof (notably low levels of

DMS and T2N and precursors thereof), wherein the method involves reduced energy input, the method comprising the steps of:

(i) providing a cereal plant, or part thereof, wherein said cereal plant comprises:
  (a) a first mutation that results in a total loss of functional LOX-1; and
  (b) a second mutation resulting in a total loss of functional LOX-2; and
  (c) a third mutation resulting in a total loss of functional MMT;
(ii) optionally malting at least part of said cereal, thereby obtaining malted cereal;
(iii) mashing said cereal and/or malted cereal and optionally additional adjuncts, thereby obtaining a wort;
(iv) heating said wort optionally in the presence of additional ingredient(s), wherein at the most 4% of the wort volume is evaporated, thereby obtaining heated wort;
(v) processing said heated wort into a beverage;

thereby preparing a cereal derived beverage with low levels of one or more off-flavours and precursors thereof.

In particular, the disclosure~~invention~~ provides methods for preparing a barley based beverage with low levels of one or more off-flavours and precursors thereof (notably low levels of DMS and T2N and precursors thereof), wherein the method involves reduced energy input, the method comprising the steps of:

(i) providing a barley plant, or part thereof, wherein said barley plant comprises:
  (a) a first mutation that results in a total loss of functional LOX-1; and
  (b) a second mutation resulting in a total loss of functional LOX-2; and
  (c) a third mutation resulting in a total loss of functional MMT;
(ii) optionally malting at least part of said barley, thereby obtaining malted barley;
(iii) mashing said barley and/or malted barley and optionally additional adjuncts, thereby obtaining a wort;
(iv) heating said wort optionally in the presence of additional ingredient(s), wherein at the most 4% of the wort volume is evaporated, thereby obtaining heated wort;

(v) processing said heated wort into a beverage;

thereby preparing a barley derived beverage with low levels of one or more off-flavours and precursors thereof.

In an aspect, there is provided a method for preparing a barley based beverage with low levels of one or more off-flavours and/or precursors thereof, wherein the method involves reduced energy input, the method comprising the steps of: (i) providing a barley plant or part thereof, wherein said barley plant comprises: (a) a first mutation that results in a total loss of functional lipoxygenase (LOX)-1; and (b) a second mutation resulting in a total loss of functional LOX-2; and (c) a third mutation resulting in a total loss of functional S-adenosylmethionine:methionine S-methyltransferase (MMT); (ii) optionally malting at least part of said barley, thereby obtaining malted barley; (iii) mashing said barley and/or malted barley and optionally additional adjuncts, thereby obtaining a wort; (iv) heating said wort optionally in the presence of additional ingredient(s), wherein at the most 4% of the wort volume is evaporated, thereby obtained heated wort; (v) processing said heated wort into a beverage; thereby preparing the barley based beverage with low levels of one or more off-flavours and/or precursors thereof.

It is also an objective of the present disclosure~~invention~~ to provide barley plants suitable for use in the disclosed methods. Thus, it is an objective of the present disclosure~~invention~~ to provide agronomically useful barley plants comprising all of the three different traits, i.e. a null-LOX-1–null-LOX-2–null-MMT barley plant (herein also denoted "double-null-LOX–null-MMT"). In another aspect, there is provided a barley plant, or a part thereof, wherein said barley plant comprises: a) a first mutation that results in a total loss of functional LOX-1, and b) a second mutation resulting in a total loss of functional LOX-2 and c) a third mutation resulting in a total loss of functional MMT. Selection of a useful barley plant concerns not only plant vigour, but also the combined lack of LOX-1, LOX-2 and MMT enzymic activities, utilizing biochemical assays as described in detail herein below. The barley plants according to the present disclosure~~invention~~ may be introduced into any suitable breeding scheme, such as selfing, backcrossing, crossing to populations, and the like.

A way to accelerate the process of plant breeding comprises the initial multiplication of generated mutants by application of tissue culture and regeneration techniques. As described in Example 3 and schematically shown in **FIG. 3**, a traditional barley breeding scheme was employed to generate a barley plant with the double-null-LOX–null-MMT trait from a double-null-LOX barley plant and a null-MMT plant. Thus, another embodimentaspect of the present disclosureinvention is to provide cells, which upon growth and differentiation produce barley plants having the double-null-LOX–null-MMT trait. For example, breeding may involve traditional crossings, preparing fertile anther-derived, doubled haploid plants using tissue culturing such as anther culture or microspore culturings.

The present disclosureinvention discloses that reduced energy input for kiln drying is achievable through application of null-LOX grains, because there is no need to inactivate endogenous LOX enzyme. The double-null-LOX–null-MMT mutant described by the present invention disclosure can be utilized for production of a raw material lacking the corresponding enzyme activities, making the mutant of interest to achieve lower energy consumption during kiln drying in the malting facility, but also in the brewhouse because of reduced heat input during wort boiling.

From a beer or beverage quality standpoint, there is also a need for a double-null-LOX–null-MMT raw material, in order to functionally eliminate or drastically reduce T2N and DMS levels in the products.

In addition to the above-mentioned, potential properties of a double-null-LOX–null-MMT malt, a corresponding barley could be useful for the production of off-flavour–low barley beer, here defined as beer produced through omission of the malting process, but instead providing a mash consisting of numerous external enzymes (e.g. Ondea Pro, an enzyme mixture produced by Novozymes). As addressed herein below in Example 7, it was surprising by the present inventors to find that wort produced by mashing unmalted double-null-LOX–null-MMT barley raw material contained very low levels of T2N and DMS off-flavours, while that from wild-type barley was surprisingly high in DMS levels despite that the barley kernels had not undergone germination. Accordingly, some DMS precursors (DMSP) must be present in the dry, mature barley kernel – a new, not-yet described property, which is explored in beer products of the instant application. Accordingly, double-null-LOX–null-MMT grains are useful in the production of barley-brewed wort and beer in order to minimize levels of T2N and DMS in fresh and aged beer products.

In another aspect, there is provided a malt composition comprising a processed barley plant, or a part thereof, wherein said barley plant is the barley plant as defined herein.

In another aspect, there is provided a barley based beverage prepared from a barley plant, or a part thereof, as defined herein, wherein said beverage contains a level of dimethyl sulfide (DMS) below 10 ppb.

DESCRIPTION OF DRAWINGS

FIG. 1 presents simplified overviews of the barley biochemical pathways leading to formation of T2N (**A**), and SMM (**B**). Enzymes absent in Triple-Null barley are highlighted in black boxes. Enzyme abbreviations are those defined in the instant application. **FIG. 1B** shows selected components of the SMM cycle in which SMM is synthesized by methyl transfer from S-adenosylmethionine (SAM) to methionine (Met), catalyzed by the enzyme Met-*S*-methyltransferase (MMT). SMM can in turn serve as methyl donor for Met synthesis from homocysteine (Hcy), in a reaction catalyzed by the enzyme Hcy-S-methyltransferase (HMT). The illustration shows how the essentially irreversible reactions are connected. Each turn of the cycle is futile as it consumes and then regenerates two Mets while converting ATP to adenosine, PPi and Pi (not shown).

FIG. 2 shows results of HPLC experiments to verify the null-MMT phenotype of Mutant 8063 and Mutant 14018. (**A**) An example on HPLC-based separation of an extract from shoots of cv. Prestige, showing elution of aspartic acid (Asp), glutamic acid (Glu),

CLAIMS

1. A method for preparing a barley based beverage with low levels of one or more off-flavours and/or precursors thereof, wherein the method involves reduced energy input, the method comprising the steps of:

(i) providing a barley plant or part thereof, wherein said barley plant comprises:
(a) a first mutation that results in a total loss of functional lipoxygenase (LOX)-1; and
(b) a second mutation resulting in a total loss of functional LOX-2; and
(c) a third mutation resulting in a total loss of functional S-adenosylmethionine:methionine S-methyltransferase (MMT);

(ii) optionally malting at least part of said barley, thereby obtaining malted barley;
(iii) mashing said barley and/or malted barley and optionally additional adjuncts, thereby obtaining a wort;
(iv) heating said wort optionally in the presence of additional ingredient(s), wherein at the most 4% of the wort volume is evaporated, thereby obtained heated wort;
(v) processing said heated wort into a beverage;

thereby preparing the barley based beverage with low levels of one or more off-flavours and/or precursors thereof.

2. The method according to claim 1, wherein step (i) comprises providing barley kernels.

3. The method according to anyone of the preceding claims, wherein said off-flavours are dimethyl sulphide (DMS) and trans-2-nonenal (T2N).

4. The method according to claim 3, wherein the low levels of DMS are levels of DMS below 30 ppb, preferably less than 25 ppb, more preferably less than 20 ppb and levels of T2N below 0.025 ppb, such as below 0.024 ppb.

5. The method according to anyone of the preceding claims, wherein said precursors of off-flavours are S-methyl-methionine (SMM) and T2N potential.

6. The method according to claim 5, wherein the low levels of SMM and T2N potential are levels of SMM less than 50 ppb, preferably less than 40 ppb, more preferably less than 30 ppb, even more preferably less than 20 ppb and levels of T2N potential is less than 2, preferably less than 1.5 ppb.

7. The method according to claim 5, wherein the low levels of SMM are levels of SMM less than 50 ppb, preferably less than 40 ppb, more preferably less than 30 ppb, even more preferably less than 20 ppb and the low levels of T2N potential are levels of T2N potential less than 60%, preferably less than 50% of the level of T2N potential in a beverage prepared in the same manner from wild-type barley, preferably from cv. Quench.

8. The method according to anyone of the preceding claims, wherein all of said provided barley is malted.

9. The method according to anyone of the preceding claims, wherein said barley is malted using a method comprising the steps of

a) steeping said barley;
b) germinating said barley;
c) kiln drying said barley

10. The method according to claim 9, wherein kiln drying of said barley is performed at a temperature of at the most 80°C, yet more preferably at a temperature of at the most 75°C, such as at temperature of at the most 70°C, for example at a temperature of at the most 65°C, such as at temperature of at the most 60°C, for example at a temperature of at the most 55°C, such as at a temperature of at the most 50°C, for example at a temperature of at the most 45°C, such as at a temperature of at the most 40°C.

11. The method according to any one of claims 9 to 10, wherein the kiln drying temperature does not rise above 80°C, preferably does not rise above 75°C at any time during kiln drying.

12. The method according to anyone of claims 1 to 6, wherein none of the provided barley is malted.

13. The method according to anyone of the preceding claims, wherein said barley and/or said malted barley is milled prior to said mashing.

14. The method according to anyone of the preceding claims, wherein the wort composition has been produced by a method involving a mashing step using a mashing in temperature not exceeding 69°C.

15. The method according to anyone of the preceding claims, wherein the temperature during mashing at no time exceeds 80°C, preferably 78°C.

16. The method according to anyone of the preceding claims, wherein additional adjunct(s) are added in step (iii), and said additional adjuncts are selected from the group consisting of maize, rice and unmalted cereal other than barley.

17. The method according to anyone of the preceding claims, wherein additional ingredient(s) are added in step (iv) and said additional ingredient is hops or an extract thereof.

18. The method according to anyone of the preceding claims, wherein said wort is heated to a temperature of at the most 99.8°C, such as at the most 99.5°C, for example at the most 99°C.

19. The method according to anyone of the preceding claims, wherein said wort is heated to a temperature above 80°C for at the most 30 min, preferably at the most 20 min.

20. The method according to anyone of the preceding claims, wherein at the most 3%, such as at the most 2%, for example at the most 1%, such as at the most 0.5%, for example at the most 0.1%, such as at the most 0.01% of the wort volume is evaporated.

21. The method according to anyone of the preceding claims, wherein step v) comprises cooling said heated wort.

22. The method according to anyone of the preceding claims, wherein step v) comprises fermentation of the wort.

23. The method according to anyone of the preceding claims, wherein the method does not involve heating to a temperature of more than 99°C at any step.

24. The method according to anyone of the preceding claims, wherein the method does not involve heating to a temperature above 80°C for more than 30 min, preferably for more than 20 min.

25. A barley plant, or a part thereof, wherein said barley plant comprises:

a) a first mutation that results in a total loss of functional LOX-1, and

b) a second mutation resulting in a total loss of functional LOX-2 and

c) a third mutation resulting in a total loss of functional MMT.

26. The barley plant according to claim 25, wherein the gene encoding LOX-1 of said plant comprises a mutation selected from the group consisting of a frame shift mutation, a deletion, a non-sense mutation, an insertion and a splice site mutation.

27. The barley plant, or a part thereof, according to claim 25, wherein the gene encoding LOX-1 of said plant comprises a premature stop codon.

28. The barley plant, or a part thereof, according to any one of claims to 25 to 27, wherein the gene encoding LOX-1 of said plant comprises a premature stop codon situated at the most 705, preferably at the most 665 codons downstream of the start codon.

29. The barley plant, or a part thereof, according to claim 28, wherein the gene encoding LOX-1 of said plant comprises a nonsense codon, said codon corresponding to base nos. 3572–3574 of SEQ ID NO:2.

30. The barley plant according to any one of claims 25 to 29, wherein the gene encoding LOX-2 of said plant comprises a mutation selected from the group consisting of a frame shift mutation, a deletion, a non-sense mutation, an insertion and a splice site mutation.

31. The barley plant, or a part thereof, according to any one of claims 25 to 30, wherein the gene encoding LOX-2 of said plant comprises a premature stop codon.

32. The barley plant, or a part thereof, according to any one of claims 25 to 31, wherein the gene encoding LOX-2 of said plant comprises a premature stop codon situated at the most 707, preferably at the most 684 codons downstream of the start codon.

33. The barley plant, or a part thereof, according to claim 32, wherein the gene encoding LOX-2 of said plant comprises a mutation at nucleotide position 2689 of SEQ ID NO:5, leading to formation of a stop codon.

34. The barley plant according to any one of claims 25 to 33, wherein the gene encoding MMT of said plant comprises a mutation selected from the group consisting of a frame shift mutation, a deletion, a non-sense mutation, an insertion and a splice site mutation.

35. The barley plant, or part thereof, according to any one of claim 25 to 34, wherein the mutation in the gene encoding MMT is within a splice site.

36. The barley plant, or part thereof, according to any one of claims 25 to 35, wherein the mutation in the gene encoding MMT is within a splice site of an intron.

37. The barley plant, or a part thereof, according to any one of claims 25 to 36, wherein the mutation in the gene encoding MMT is a mutation within a 5' splice site of an intron, such as a G→A mutation of the terminal 5' base of an intron.

38. The barley plant, or a part thereof, according to any one of claims 25 to 37, wherein the mutation in the gene encoding MMT is a mutation within a 5' splice site of an intron selected from the group consisting of introns 2 and 5.

39. The barley plant, or a part thereof, according to claim 38, wherein the mutation in the gene encoding MMT is a G→A mutation of base no. 3076 of SEQ ID NO:9.

40. The barley plant, or a part thereof, according to claim 38, wherein the mutation, in the gene encoding MMT is a G→A mutation of base no. 1462 of SEQ ID NO:11.

41. The barley plant, or a part thereof, according to anyone of claims 25 to 40, wherein the mutation results in a gene encoding a truncated form of MMT comprising an N-terminal fragment of wild-type MMT and optionally additional C-terminal sequences not found in wild-type MMT, wherein said N-terminal fragment comprises at the most the 500, more preferably at the most the 450, even more preferably at the most the 400, yet more preferably at the most the 350, even more preferably at the most the 320, yet more preferably at the most 311, or at the most 288 N-terminal amino acid residues of SEQ ID NO:13.

42. The barley plant, or a part thereof, according to any one of claims 25 to 41, wherein said plant is progeny of the barley plant designated "Barley, Hordeum vulgare: Line 8063" deposited at ATCC 13 October 2008 with the designation PTA-9543 and the barley plant designated "Barley, Hordeum vulgare L.: Line A689", deposited with ATCC with the deposit number PTA-9640.

43. A malt composition comprising a processed barley plant, or a part thereof, wherein said barley plant is the barley plant according to any of claims 25 to 42.

44. The malt composition according to claim 43, wherein said part of said barley plant is kernel(s).

45. The malt composition according to anyone of claims 43 to 44, wherein the malt composition is selected from the group consisting of green malt and kiln dried malt.

46. The malt composition according to anyone of claims 43 to 45, wherein the malt composition is milled malt.

47. The malt composition according to any of claims 43 to 46, wherein the malt composition comprises at the most 2, preferably at the most 1, more preferably at the most 0.5 µg/g, even more preferably at the most 0.2 µg/g SMM.

48. A barley based beverage prepared from a barley plant, or a part thereof, according to any one of claims 25 to 42, wherein said beverage contains a level of dimethyl sulfide (DMS) below 10 ppb.

49. The beverage according to claim 48, wherein said beverage is prepared by the method according to anyone of claims 1 to 24.

50. The beverage according to anyone of claims 48 to 49, wherein the beverage is a malt beverage.

51. The beverage according to any one of claims 48 to 50, wherein the beverage is a fermented malt beverage.

52. The beverage according to any one of claims 48 to 51, wherein the beverage is beer.

53. The beverage according to any one of claims 48 to 49 and 52, wherein said beverage is barley beer.

54. The beverage according to any one of claims 48 to 53, wherein the beverage is a non-alcoholic malt beverage.

55. The beverages according to any one of claims 48 to 54, wherein said beverage produce at least 1.5 times more, preferably at least 2 times more, yet more preferably at least 3 times more foam in 60 to 80 min, compared to a beverage prepared in the same manner from wild-type barley, preferably from cv. Quench.