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(54) Title: METHOD OF MANAGING AND DISPATCHING YEAST FOR THE PRODUCTION OF FERMENTED BEVERAGES

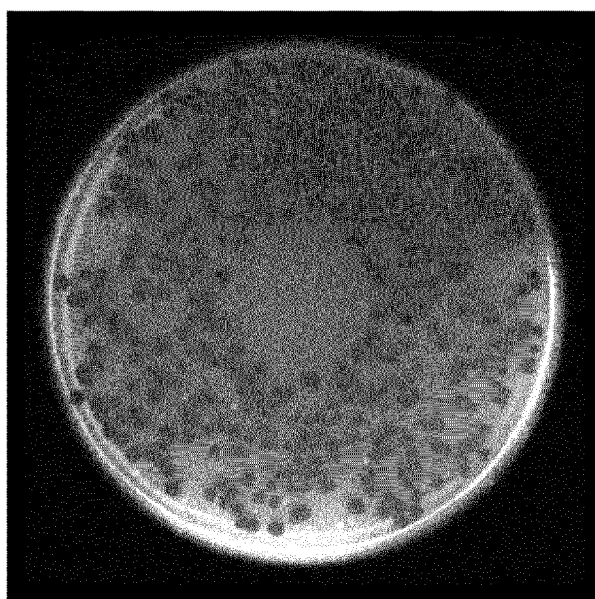


FIG. 1

(57) Abstract: A method of yeast management comprising pre-
serving and propagating yeast cells at a central yeast collection fa-
cility and dispatching yeast cells from that yeast collection facility
to distant yeast processing sites, the yeast management method
comprising: a) Preserving yeast cells at a temperature of minus
80°C or less; b) Thawing said yeast cells under conditions al-
lowing revitalisation of a majority the yeast cells; c) Propagating
the thawed yeast cells; d) Packing at least part of the propagated
yeast cells for transport to yeast processing sites; e) Production
of a fermented beverage by inoculating a fermentable broth with
yeast cells propagated from the packed and transported yeast cells
of step (d); f) Optionally preserving part of the propagated yeast
cells by freezing the yeast cells to a temperature of minus 80°C
or lower, Characterized in that the method comprises a step of:
g) growing the yeast cells on an exclusively nonfermentable car-
bon source to selectively propagate yeast cells having a regularly
functional respiratory chain (non petites).

METHOD OF MANAGING AND DISPATCHING YEAST FOR THE PRODUCTION OF FERMENTED BEVERAGES

The present invention concerns a method of managing yeast and dispatching the yeast to process sites, in particular for the production of fermented beverages.

BACKGROUND TO THE INVENTION

At present, yeast cultures are propagated and transported in the form of so-called yeast slopes. A drawback of the present managing method is that the yeast slopes may contain a rather large number (up to 5% or even more) of yeast cells having defects in their respiratory chain. Pitching to a wort or fermentable broth with a yeast culture comprising such defect yeast cells (petites), may result in a slowed down fermentation or typically may result in a higher production of off-flavours during fermentation, increasing beer maturation time at the end of fermentation and/or result in an undesired flavour profile of the fermented beverage and/or flocculate at an undesired time or rate during the fermentation process.

As the number of petites tend to increase during reuse of yeast batches in subsequent fermentation cycles, yeast slopes with a high number of petites, cannot guarantee high quality brews during a number of consecutive fermentation cycles, which results in an important cost increase for brewing.

It is known that petites are defects in the yeast's mitochondrial or chromosomal DNA that occur when the yeast is grown under elevated stress conditions. The yeast management system in, for example breweries, comprises several process steps wherein yeast cells are under high stress conditions. In particular, in companies producing fermented beverages in a worldwide network of breweries, yeast cultures are typically kept in a central yeast collection that, on a regular basis, dispatches the yeast cultures to the different breweries.

At the central yeast collection, the yeast cultures are preserved by freezing and storage at a temperature of minus 80°C or lower. For dispatch, the yeast cultures are subsequently

thawed, propagated and transferred to a nutrient medium (agar) in a vial or container (the so-called yeast slopes).

During the freezing and thawing of the yeast culture and even subsequent propagation and transportation steps, free oxygen radicals can be formed which are known to cause DNA mutations and may impact the respiration chain of the yeast cells, potentially resulting in yeast slopes having a high amount of yeast cells having deficient functional respiratory chain and negatively impacting the brewing process in the breweries.

The present invention addresses the above problems and allows guaranteeing the quality of yeast cells in terms of viability and fermentation capability at the time of dispatch of the yeast slopes.

SUMMARY OF THE INVENTION

The present invention concerns a method of yeast management comprising preserving and propagating yeast cells at a central yeast collection facility and dispatching yeast cells from that yeast collection facility to distant yeast processing sites, the yeast management method comprising:

- (a) Preserving yeast cells at a temperature of minus 80°C or less;
- (b) Thawing said yeast cells under conditions allowing revitalisation of a majority the yeast cells;
- (c) Propagating the thawed yeast cells;
- (d) Packing at least part of the propagated yeast cells for transport to yeast processing sites;
- (e) Production of a fermented beverage by inoculating a fermentable broth with yeast cells propagated from the packed and transported yeast cells of step (d);
- (f) Optionally preserving part of the propagated yeast cells by freezing the yeast cells to a temperature of minus 80°C or lower,

Characterized in that the method comprises a step of:

(g) growing the yeast cells on an exclusively non-fermentable carbon source to selectively propagate yeast cells having a regularly functional respiratory chain (non-petites).

- 5 Preferably, the invention comprises an additional step of conditioning the yeast cells for preservation.

Method step (f) can be performed at any time during the above method such as after thawing of the yeast cells; during propagation of the thawed yeast cells; before, during or
10 after propagation of the yeast cells; prior to or during the conditioning; or prior to the optional freezing of the yeast cells.

In method step (f) the yeast cells are grown on the exclusively non-fermentable carbon source for a period allowing at least 3 doubling cycles of the yeast cells having a regularly
15 functional respiratory chain. The non-fermentable carbon source is hereby preferably selected from the group comprising: glycerol, lactate, ethanol and/or admixtures thereof.

Prior to freezing of the yeast (culture) for preservation, the yeast is preferably propagated on a carbon source comprising maltose and trehalose and/or may be treated with RNAlater
20 or Allproctect.

During preservation, the yeast cells may be in contact with a medium comprising a non-fermentable carbon source; on a medium comprising zinc; a medium comprising an oxygen scavenger and/or an antioxidant (eg. glutathion); a medium comprising 15 to 80w%,
25 preferably 60 to 80w%, glycerol as cryoprotectant.

The yeast cells are preferably packed in a vial or container containing an agar comprising a carbon source, whereby the yeast cells are not provided as single clone forming colonies (at no point single clones are selected). This is especially the case when the yeast cells form a
30 complex culture. The agar comprising a fermentable carbon source, preferably contains a yeast-malt broth (eg. 3g/L yeast extract, 3g/L malt extract, 5g/L peptone and 10 g/L dextrose)

The vials or containers may be hermetically sealed after introduction of the yeast cells. Optionally an oxygen scavenger is provided in said vial or container.

- 5 The present invention also concerns vials or containers containing a yeast culture for transporting the yeast cells in accordance with the method described supra. The yeast culture in said vial or container comprising at least 99% of yeast cells having a regularly functional respiratory chain (max. 1% petites).
- 10 Preferably, the vial or container according to the invention is made from or coated with a material having a permeability for radiation with a wavelength ranging from 10nm to 400nm of less than 20%.

The method and vials or containers according to the present invention are believed to be particularly suitable for dispatching yeast cultures for the preparation of a fermented beverage at the yeast processing site.

Finally, the yeast culture, preferably comprises yeast of a *Saccharomyces* sp.

20 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1a A culture (control) containing both normal (red, grey in the figures) as well as respiratory deficient (white, small) cells have different phenotypes when plated on a YPD – plate (Yeast extract 1%; Peptone 2%; Dextrose 2%; Agarose 2%) overlaid with TTC (2,3,5-triphenyltetrazolium chloride);

Fig. 1b The culture prepared by a method according to the present invention plated on the same type of plate as before (Yeast extract 1%; Peptone 2%; Dextrose 2%; Agarose 2%) and overlaid with TTC (2,3,5-triphenyltetrazolium chloride).

DETAILED DESCRIPTION OF THE INVENTION

The viability of yeast (eg. *Saccharomyces cerevisiae*) in a beverage fermentation process is a key feature for optimization of the conversion of sugars into ethanol and for optimizing production of desired flavour compounds essential for the organoleptic signature of the resulting beverage.

In accordance with the present invention, yeast viability and yeast quality can be ensured. In beer production, yeasts are typically stored (preserved) for long term in a freezer at temperatures of -80°C or lower. The yeast preservation can be achieved by first conditioning the yeast cells and subsequently freezing the conditioned yeast cells. An example of yeast preservation comprises the steps of, aseptically inoculating a yeast in a sterile brewers wort and subsequently propagating the yeast in aseptic conditions.

After propagation a, preferably small, sample of the yeast is transferred to a cryovial and mixed with a cryoprotectant solution, such as a 15-80w%, preferably 60-80w% glycerol solution. The cryovial is subsequently closed and stored in a freezer at -80°C or lower.

As freezing of the yeast cells and later thawing of the yeast cells are best performed at high rate, it is preferred to use cryovials of limited volume (eg. 4ml).

An example of yeast preservation comprises aseptically inoculating yeast (eg. from an agar medium, optionally comprising trehalose and/or maltose) in an autoclaved 500 ml Schott bottle filled with 90ml of sterile, 13,5°Plato brewers wort. The bottle is closed with a waterlock and incubated on a shaking platform at 105 rpm in an incubator at 19°C. After 42 hours of incubation (yeast propagation), a 2.1 ml sample of the yeast is added to a 4 ml sterile cryovial (eg. CryoclearTM by Globe Scientific Inc.) to which 0,9 ml of a sterile 73%v/v glycerol-solution is added after which both are mixed. After mixing, the cryovial is stored at a temperature of -80°C for preservation.

As yeast cultures used in beverage fermentation processes will evolve during subsequent fermentation cycles, it is necessary to start from a 'fresh' yeast culture for fermentation

after a given number of brewing cycles (typically after 4, 5 or 6 fermentation cycles) to limit the chance of brewing a batch of fermented beverage that has a non-desired organoleptic profile.

- 5 The 'fresh' yeast can either be propagated starting from a thawed yeast removed from the freezer or starting from a yeast culture propagated from a sample of propagated thawed yeast removed from the freezer.

10 As in larger breweries, yeast preservation is centralized, fresh yeast samples need to be dispatched to several breweries at regular time intervals and yeast viability in these samples needs to be ensured throughout the entire yeast management system from preservation until fermentation.

15 In order to ensure or even improve yeast viability during the subsequent parts of the yeast management system, the present invention provides a method including:

- i. Thawing said yeast cells under conditions allowing revitalisation of a majority the yeast cells;
- ii. Propagating the thawed yeast cells;
- iii. Packing at least part of the propagated yeast cells for transport to yeast processing
20 sites;
- iv. Production of a fermented beverage by inoculating a fermentable broth with yeast cells propagated from the packed and transported yeast cells of step (iii);
- v. Optionally preserving part of the propagated yeast cells by freezing the yeast cells to a temperature of minus 80°C or lower,

25 whereby in accordance with the present invention, the method comprises a step of:

- vi. growing the yeast cells on an exclusively non-fermentable carbon source to selectively propagate yeast cells having a regularly functional respiratory chain (non-petites).

30 Growing the yeast cells on an exclusively non-fermentable carbon source such as glycerol, lactate or ethanol, allows propagating only those yeast cells that have a regularly functional respiratory chain and hence can use non-fermentable sugars as carbon source, which is not the case for yeast cells having defects in their mitochondrial DNA.

As an example of the above method of the invention, a cryovial stored at -80°C is removed (starting from the storage and preservation example described supra) from the freezer and thawed by placing it in a waterbath at 31°C. As the cryovial preferably only contains a limited volume of yeast solution (eg. 3 ml), thawing at high rates is achieved.

Next a sample (eg. 900 microliter) of the cryovial content is transferred to an autoclaved Schott bottle filled with 90ml of sterile, 13,5°Plato brewers wort. The bottle is closed with a waterlock and incubated on a shaking platform at 105 rpm in an incubator at 19°C. After 42 hours of incubation, 900 microliter of the yeast culture is transferred to an autoclaved 500 ml Schott bottle filled with 90 ml ml of sterile YPGlycerol medium (yeast extract 1% w/w; Peptone 2% w/w; glycerol 2,5% v/v and water). The bottle is closed by a waterlock and incubated on a shaking platform at 105 rpm in an incubator at 19°C for a time allowing at least 3 doubling cycles of the yeast cells (eg. 66 hours). As the YPGlycerol medium does not contain fermentable sugars, only yeast cells able to retrieve carbon from glycerol will propagate. After 66 hours of incubation the mixture is homogenized thoroughly and incubated on a YM-agar (yeast extract 1% w/w; malt extract 3% w/w; agar 2% w/w and water) in a tube. The tube is then closed with a cotton plug and labelled.

The tube (also addressed to as yeast slope) is incubated horizontally for 48 to 72 hours at 24°C, subsequently cooled to 4°C and then shipped in a safe container in a cooled transport to a yeast processing site.

Part of the yeast incubated on the YM-agar is typically used as a starting point for preservation at -80°C or lower as disclosed supra.

In support of the invention, figure 1 shows a yeast culture prepared by method steps i) and ii) as identified above, whilst figure 2 shows a yeast culture starting from the same preservation sample as the culture in Figure 1, but prepared by a method according to the present invention as exemplified here above. It is clear that no respiratory deficient colonies remain after the growth step on YPGlycerol, a medium that as its sole carbon source has glycerol, an exclusively non-fermentable carbon source

The yeast processing site can either be a brewery or a laboratory, where the yeast is propagated and subsequently inoculated in a fermentable broth for beverage production or
5 shipped to a brewery for doing the same.

Apart from yeast extract, malt extract, agar and water, the YM-agar may also comprise trehalose and/or cereal rootlets.

10 During transport, the yeast slopes may be closed by a cotton plug or hermetically sealed. Additionally an oxygen scavenger and/or antioxidant such as glutathion may be provided in the tube or agar.

Beer yeast cultures may be well defined yeast cultures or undefined yeast cultures, yet as
15 during the entire yeast management system and during brewing the yeast cells are subjected to several stress factors, it is preferred that during the entire yeast management systems working with single clones (point single clones) is avoided.

CLAIMS

1. A method of yeast management comprising preserving and propagating yeast cells at a central yeast collection facility and dispatching yeast cells from that yeast collection
5 facility to distant yeast processing sites, the yeast management method comprising:

- a) Preserving yeast cells at a temperature of minus 80°C or less;
- b) Thawing said yeast cells under conditions allowing revitalisation of a majority the yeast cells;
- c) Propagating the thawed yeast cells;
- 10 d) Packing at least part of the propagated yeast cells for transport to yeast processing sites;
- e) Production of a fermented beverage by inoculating a fermentable broth with yeast cells propagated from the packed and transported yeast cells of step (d);
- f) Optionally preserving part of the propagated yeast cells by freezing the yeast cells to
15 a temperature of minus 80°C or lower,

Characterized in that the method comprises a step of:

- g) growing the yeast cells on an exclusively non-fermentable carbon source to selectively propagate yeast cells having a regularly functional respiratory chain (non-petites).

2. The method according to claim 1, comprising a step of conditioning the yeast cells for preservation.

3. The method according to claim 2, comprising performing method step (f) after
25 thawing of the yeast cells; during propagation of the thawed yeast cells; before, during or after propagation of the yeast cells; prior to or during the conditioning of the yeast cells; or prior to the optional freezing of the yeast cells.

4. The method according to any of the preceding claims, the non-fermentable carbon
30 source is selected from the group comprising: glycerol, lactate, ethanol and/or admixtures thereof.

5. The method according any of the preceding claims, wherein the yeast cells are grown on the exclusively non-fermentable carbon source for a period allowing at least 3 doubling cycles of the yeast cells having a functional respiratory chain.
- 5 6. The method according to any of the preceding claims, wherein the yeast cells are packed in a vial or container containing an agar comprising a carbon source, optionally including cereal rootlets and/or trehalose.
7. The method according to claim 6, comprising providing an oxygen scavenger in said
10 vial or container.
8. The method according to any of the preceding claims, wherein prior to preservation, the yeast is propagated on a carbon source comprising maltose and/or trehalose.
- 15 9. The method according to any of the preceding claims, wherein during preservation, the yeast cells are in contact with a medium comprising a non-fermentable carbon source, other than ethanol.
10. The method according to any of the preceding claims, wherein during any of the
20 steps a) to f), the yeast cells are in contact with a medium comprising an oxygen scavenger and/or an antioxidant (eg. glutathion).
11. The method according to any of the preceding claims, wherein during preservation, the yeast cells are in contact with a medium comprising 15 to 80w% glycerol as
25 cryoprotectant, preferably at 60-80w%.
12. The method according to any of the preceding claims, not working with single clones (no point single clones are selected).
- 30 13. The method or packaging according to any of the preceding claims, the yeast cells is a *Saccharomyces sp.*

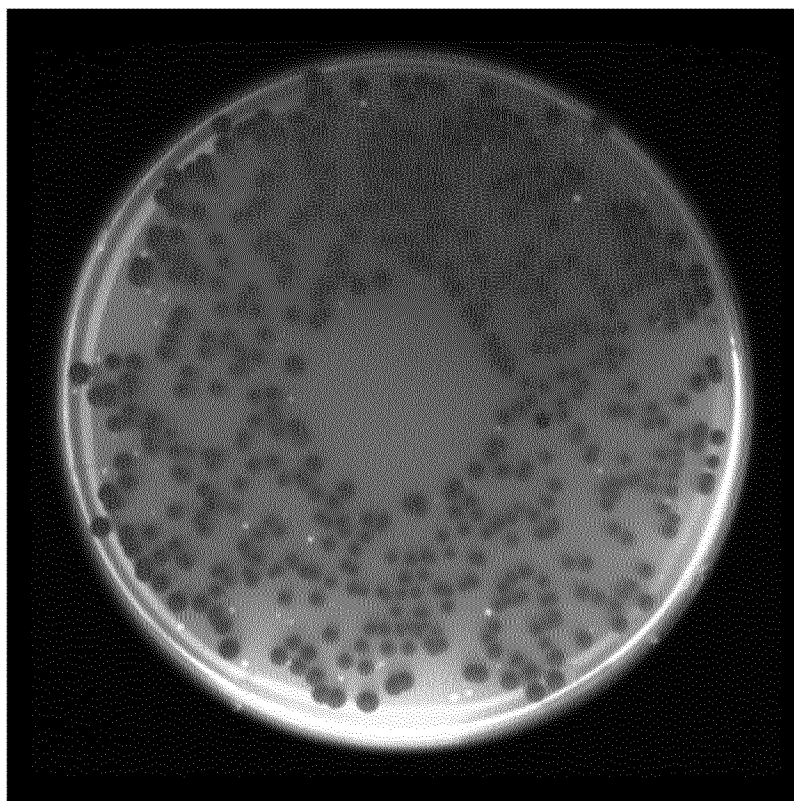


FIG. 1

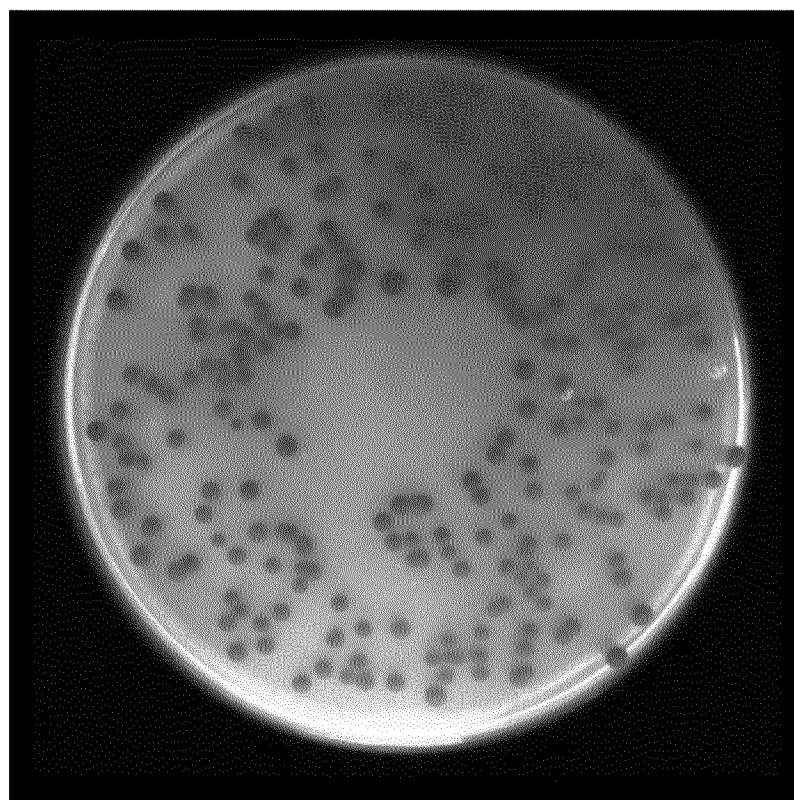


FIG. 2

INTERNATIONAL SEARCH REPORT

International application No
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A. CLASSIFICATION OF SUBJECT MATTER INV. C12N1/18 C12N1/04 C12C11/00 C12C12/00 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) C12N C12C		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, BIOSIS, FSTA, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LAWRENCE S. J. ET AL: "Incidence and Characterization of Petites Isolated from Lager Brewing Yeast Saccharomyces cerevisiae Populations", JOURNAL OF THE AMERICAN SOCIETY OF BREWING CHEMISTS, vol. 70, no. 4, 2012, pages 268-274, XP009502374, ISSN: 0361-0470, DOI: 10.1094/ASBCJ-2012-0917-01 the whole document abstract ----- -/-	1-13
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "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 10 October 2018		Date of mailing of the international search report 18/10/2018
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer van de Kamp, Mart

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2018/070988

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GIBSON B. R. ET AL: "Petite mutation in aged and oxidatively stressed ale and lager brewing yeast", LETTERS IN APPLIED MICROBIOLOGY, vol. 46, no. 6, 1 June 2008 (2008-06-01), pages 636-642, XP055434749, GB ISSN: 0266-8254, DOI: 10.1111/j.1472-765X.2008.02360.x the whole document abstract -----	1-13
Y	BRIGGS D. E. ET AL: "Brewing: Science and Practice", 2004, XP055435010, Retrieved from the Internet: URL:http://fars.itvhe.ac.ir/_fars/Document s/99ae7cc8-c9a8-4356-8d24-f6c208322cb7.pdf [retrieved on 2017-12-13] the whole document 13.5 Yeast propagation -----	1-13
Y	US 2012/142067 A1 (DESFOUGERES THOMAS [FR] ET AL) 7 June 2012 (2012-06-07) the whole document example 2 -----	1-13
Y	RAINES M. B.: "Yeast Propagation and Maintenance: Principles and Practices", 18 August 2009 (2009-08-18), XP055435007, Retrieved from the Internet: URL:https://www.maltosefalcons.com/tech/ye ast-propagation-and-maintenance-principles -and-practices [retrieved on 2017-12-13] the whole document page 5, lines 3-6 table 2 page 10, lines 55,56 -----	1-13
Y	EGILMEZ N. K. ET AL: "Preparation and partial characterization of old yeast cells", JOURNALS OF GERONTOLOGY, vol. 45, 1990, pages B9-B17, XP009502340, ISSN: 0022-1422 the whole document page B11, right-hand column, lines 9-20 ----- -/--	1-13

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/070988

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DAY M.: "Yeast Petites and Small Colony Variants: For Everything There Is a Season", ADVANCES IN APPLIED MICROBIOLOGY, vol. 85, 2013, pages 1-41, XP009502321, ISSN: 0065-2164, DOI: 10.1016/B978-0-12-407672-3.00001-0 the whole document abstract 3. The Petite and Its Physiology -----	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2012142067	A1	07-06-2012	AR 084087 A1 17-04-2013
			AU 2011334846 A1 13-06-2013
			BR 112013013696 A2 06-06-2017
			CA 2819323 A1 07-06-2012
			CL 2013001569 A1 28-03-2014
			DK 2646544 T3 16-01-2017
			EP 2646544 A1 09-10-2013
			ES 2608784 T3 17-04-2017
			FR 2968313 A1 08-06-2012
			HR P20161752 T1 24-02-2017
			HU E031826 T2 28-08-2017
			MX 347050 B 07-04-2017
			PL 2646544 T3 28-04-2017
			PT 2646544 T 30-12-2016
			US 2012142067 A1 07-06-2012
			WO 2012072793 A1 07-06-2012
