



(51) International Patent Classification:

C12P 7/06 (2006.01) *C12N 1/22* (2006.01)
C12P 7/08 (2006.01) *C12N 1/32* (2006.01)
C12P 7/10 (2006.01)

(21) International Application Number:

PCT/US2016/066480

(22) International Filing Date:

14 December 2016 (14.12.2016)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/270,104 21 December 2015 (21.12.2015) US

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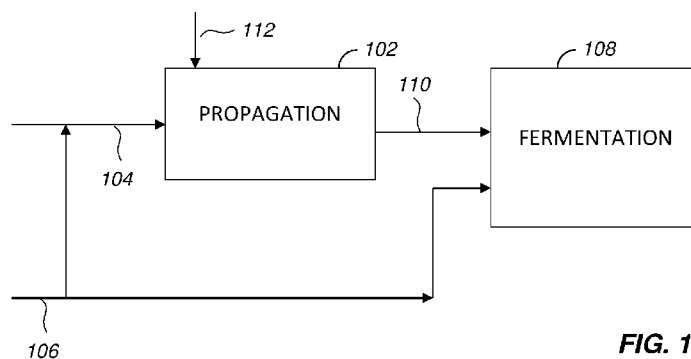
(81) Designated States (unless otherwise indicated, for every
 kind of national protection available): AE, AG, AL, AM,
 AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
 BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM,
 DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
 HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN,
 KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA,
 MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG,
 NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS,
 RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY,
 TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN,
 ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
 kind of regional protection available): ARIPO (BW, GH,
 GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ,
 TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU,
 TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE,
 DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,
 LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
 SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
 GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: METHODS, SYSTEMS, AND COMPOSITIONS FOR PROPAGATION OF A FERMENTATION MICROORGANISM

**FIG. 1**

(57) Abstract: Methods, systems, and compositions for propagating a fermentation microorganism that can have improved product yield during fermentation of a hydrolysate containing at least one growth inhibitor, including ethanol. In some embodiments, the method comprises: (a) providing a hydrolysate for fermentation, wherein the hydrolysate comprises exogenous ethanol in an amount of at least 10 g/L; (b) providing a propagation reactor containing: a propagation medium comprising: the hydrolysate in an amount of at least 25% by volume and glucose in an amount of greater than 14 g/L; and a first cell mass of a fermentation microorganism; and (c) propagating the first cell mass in the propagation medium for a time period to form a second cell mass of the fermentation microorganism that is greater than the first cell mass.

METHODS, SYSTEMS, AND COMPOSITIONS FOR PROPAGATION OF A FERMENTATION MICROORGANISM

TECHNICAL FIELD

5 The present disclosure generally relates to methods, systems, and compositions for propagating a fermentation microorganism for fermentation of a hydrolysate, particularly methods, systems, and compositions for propagating in a medium containing said hydrolysate.

BACKGROUND

10 This section is intended to introduce various aspects of the art, which may be associated with exemplary embodiments described herein. This discussion is believed to assist in providing a framework to facilitate a better understanding of particular aspects of the present disclosure. Accordingly, it should be understood that this section should be read in this light, and not necessarily as admissions of any prior art.

15 Cellulosic biomass offers a promising alternative to petroleum, providing renewable and “carbon neutral” sources of fuels, such as bioethanol, and of other traditionally petroleum-based products such as plastics. Cellulosic biomass can be pretreated to prepare the biomass for enzymatic hydrolysis, which is a process typically known as saccharification, to provide sugars for fermentation by certain fermentation
20 microorganisms. The resulting hydrolysate comprises hexose and/or pentose sugar, which may be converted by a yeast into fermentation product.

 A common fermentation microorganism is yeast, which can typically purchased from a yeast supplier (e.g., in dried form, and/or as a cream liquid suspension). The purchased yeast is typically grown or propagated to generate additional yeast for
25 fermentation. Yeast is typically propagated or grown under aerobic conditions to avoid ethanol formation during propagation while fermentation is carried out under anaerobic conditions. Conventional propagation methods usually are conducted under conditions that minimize the “Crabtree” effect where the yeast switches over from aerobic metabolic pathways which facilitate growth to anaerobic metabolic pathways which produces
30 ethanol, even under highly aerated conditions. Conventional propagation methods typically suppress the ethanol producing anaerobic metabolism by using a low glucose concentration, such as below 5 g/L as disclosed in the background of US9034631 and/or highly aerated conditions to provide sufficient oxygen to the yeast.

Moreover, the presence of inhibitors in hydrolysate can also negatively impact the ethanol production during fermentation. While various propagation techniques are known to address fermentation inhibitors such as acetic acid, including US20150252319, they are insufficient to address yeast growth during fermentation of hydrolysates that contain other
5 inhibitors, including ethanol, as well as the sensitivity of a propagation process to the Crabtree effect.

SUMMARY

Accordingly, it would be desirable to provide for methods, systems, and compositions for propagating a fermentation microorganism that can have improved
10 growth under anaerobic conditions in hydrolysate containing at least one growth inhibitor, including ethanol, where it is not necessary to suppress the Crabtree effect during propagation.

The present disclosure describes a method of propagating a fermentation microorganism comprising: (a) providing a hydrolysate for fermentation, wherein the
15 hydrolysate comprises exogenous ethanol in an amount of at least 10 g/L; (b) providing a propagation reactor containing: a propagation medium comprising: the hydrolysate in an amount of at least 25% by volume and glucose in an amount of greater than 14 g/L; and a first cell mass of a fermentation microorganism; and (c) propagating the first cell mass in the propagation medium for a time period to form a propagated culture comprising a
20 second cell mass of the fermentation microorganism that is greater than the first cell mass.

The propagation medium can further comprise xylose in an amount of at least 5 g/L. The propagation medium can comprise at least 2.5 g/L ethanol at the beginning of propagation. At least 15 g/L of endogenous ethanol can be produced by the fermentation microorganism during propagation.

25 Other features of embodiments of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art
30 from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

These drawings illustrate certain aspects of some of the embodiments of the invention, and should not be used to limit or define the invention.

FIG. 1 illustrates a process for propagating a fermentation microorganism according to aspects of the present disclosure.

FIG. 2 illustrates optional features for a process for propagating a fermentation microorganism according to aspects of the present disclosure.

FIG. 3 illustrates one option for generating a hydrolysate for fermentation using yeast propagated according to aspects of the present disclosure.

FIG. 4 is a chart showing the amount of ethanol produced during propagation in Examples 1 and 2, with Example 1 being the control.

FIG. 5 is a chart showing the ethanol yield from fermentation in Examples 1 and 2, with Example 1 being the control.

DETAILED DESCRIPTION

The present disclosure generally relates to methods, systems, and compositions for propagating a fermentation microorganism for fermentation of a hydrolysate, particularly methods, systems, and compositions for propagating a fermentation microorganism in a medium containing said hydrolysate. According to one aspect described herein, there is provided a method of propagating a fermentation microorganism comprising:

(a) providing a hydrolysate for fermentation, wherein the hydrolysate comprises exogenous ethanol in an amount of at least 10 g/L;

(b) providing a reactor containing:

a propagation medium comprising: the hydrolysate in an amount of at least 25% by volume and glucose in an amount of greater than 14 g/L; and

a first cell mass of a fermentation microorganism; and

(c) propagating the first cell mass in the propagation medium for a time period to form a propagated culture comprising a second cell mass of the fermentation microorganism that is greater than the first cell mass.

According to another aspect described herein, there is provided a system for propagating a fermentation microorganism comprising:

(a) a propagation reactor vessel including:

a propagation medium containing glucose in an amount of greater than 14 g/L and at least 25% by volume of a hydrolysate for fermentation, wherein the hydrolysate comprises exogenous ethanol in an amount of at least 10 g/L; and

a first cell mass of a fermentation microorganism, wherein the microorganism can
5 use at least a portion of the glucose to grow for a time period to form a second cell mass of the fermentation microorganism that is greater than the first cell mass; and

(b) an oxygen inlet to provide oxygen to the propagation reactor vessel.

Optionally, the propagation medium further comprises xylose in an amount of at least 5 g/L. Preferably, the hydrolysate in the propagation medium can be provided to the
10 reactor before propagation begins, where the propagation medium comprises exogenous ethanol from the hydrolysate in an amount of at least 2.5 g/L ethanol at the beginning of propagation (e.g., in the first hour of propagation). At least 15 g/L of ethanol can be produced by the fermentation microorganism during propagation. As such, the propagated culture can optionally comprise at least 17.5 g/L ethanol total during, near, and/or at the end
15 of propagation. The hydrolysate can optionally contain at least 20% total solids (TS). The reactor content after propagation can be added to an amount of hydrolysate to initiate fermentation.

Accordingly, there is provided a propagated fermentation microorganism composition for fermentation of a hydrolysate, said composition comprising:

- 20 (a) a fermentation microorganism population that has been propagated for at least 4 hours;
- (b) a propagation medium comprising at least 25% by volume of a hydrolysate for fermentation, wherein the hydrolysate comprises exogenous ethanol not produced by the microorganism in the composition in an amount of at least 10
25 g/L; and
- (c) at least 15 g/L endogenous ethanol produced by the fermentation microorganism population during propagation.

Also, there is provided a composition for propagation of a fermentation microorganism in a reactor comprising:

- 30 (a) a propagation medium comprising glucose in an amount of greater than 14 g/L, at least 25% by volume of a hydrolysate for fermentation, and at least 2.5 g/L exogenous ethanol not produced by the yeast in the composition, wherein at

least a portion of the exogenous ethanol is provided by the hydrolysate in the propagation medium; and

- (b) a first cell mass of a fermentation microorganism, wherein the microorganism can use at least a portion of the glucose to grow for a time period to form a second cell mass of the yeast that is greater than the first cell mass.

Propagation

Propagating a fermentation microorganism according to the present invention includes providing a hydrolysate for fermentation and using that hydrolysate as part of a growth medium in which a first cell mass of a fermentation microorganism is propagated, where the hydrolysate contains exogenous ethanol. As used herein, "exogenous ethanol" refers to the amount of ethanol that is initially present in the hydrolysate prior to propagation or fermentation. That is, exogenous ethanol in the hydrolysate is not produced by yeast in propagation or fermentation. Suitable conventional methods and equipment may be used to implement the propagation process as described herein, where one of ordinary skill can make adjustments as described. The embodiments herein can be used to propagate various suitable types of fermentation microorganisms, which are further discussed below. The preferred fermentation microorganism is a yeast, so reference to a yeast occurs throughout the disclosure. It is understood such references do not limit the scope of the claims.

Various illustrative embodiments will now be described in detail with reference to the accompanying drawings, which are not intended to limit the scope of the methods, systems, and compositions for propagating yeast described herein. Although the following description provides numerous specific details are set forth for a thorough understanding of illustrative embodiments, it will be apparent to one skilled in the art, that embodiments may be practiced without some or all of these specific details. In other instances, well known process steps and/or structures have not been described in detail in order to not unnecessarily obscure the present invention. The features and advantages of embodiments may be better understood with reference to the drawings and discussions that follow.

In addition, when like elements are used in one or more figures, identical reference characters will be used in each figure, and a detailed description of the element will be provided only at its first occurrence. Some features of the systems described herein may be omitted in certain depicted configurations in the interest of clarity. Moreover, certain features such as, but not limited to pumps, valves, gas bleeds, gas inlets, fluid inlets, fluid

outlets and the like have not necessarily been depicted in the figures, but their presence and function will be understood by one having ordinary skill in the art. In the figures, arrows have been drawn to depict the direction of material flow (liquid, gas, and/or solids).

Referring to FIG. 1, system 100 comprises propagation reactor vessel 102, to which
5 propagation medium 104 is provided. Propagation medium 104 contains an amount of hydrolysate 106, which is subsequently fermented in fermentation reactor vessel 108 using propagated yeast 110 from propagation reactor vessel 102. In addition to propagation medium 104, a first cell mass amount of yeast 112 is also provided to propagation vessel 102. Yeast 112 can be provided to propagation medium 104 while the medium is being
10 formed, after the medium is formed, or both. Additionally or alternatively, yeast 112 can be provided to propagation reactor vessel 102 separate from medium 104, as shown.

Hydrolysate 106 in propagation medium 104 can serve as a carbon source supporting yeast growth in propagation vessel 102. By using the hydrolysate itself in the medium during propagation, the propagated yeast is conditioned to grow in an environment
15 that contains inhibitors the yeast would encounter subsequently during fermentation. In particular, propagation medium 104 preferably comprises hydrolysate 106 in an amount of at least 25% by volume, at least 50% by volume, or at least 75% by volume. The amount of hydrolysate in the propagation medium can be in a range of greater than 25% to 75% by volume, including 25% to 50% by volume, 25% to 75% by volume, or 50% to 75% by
20 volume, such as about 25% by volume, about 50% by volume, or about 75% by volume. For instance, the propagation medium can comprise hydrolysate in an amount of greater than 25% by volume, such as at least 30% by volume, at least 40% by volume, at least 50% by volume, at least 60% by volume, at least 70% by volume, or at least 75% by volume.

Propagation medium 104 can comprise glucose in an amount of greater than 14
25 g/L, and glucose can serve as a carbon source as well. The glucose in the propagation medium can come from hydrolysate 106 provided to propagation medium 104 and/or glucose in addition to that in hydrolysate 106 can be added to propagation medium 104. For instance, if hydrolysate 106 contains glucose in an amount of about 100 g/L, then an amount of at least 25% by volume provides propagation medium 104 about 25 g/L glucose, and propagation medium 104 may contain 75% by volume a YPD (yeast extract peptone
30 dextrose) that contains 20 g/L. In that instance, propagation medium 104 would have about 40 g/L glucose. Preferably, glucose is provided to the propagation medium and not

generated via enzymatic hydrolysis of polysaccharides and/or oligosaccharides during propagation.

As mentioned, the propagation medium preferably comprises glucose in an amount of greater than 14 g/L, such as about 40 g/L or greater, about 60 g/L or greater, or about 80 g/L or greater. The amount of glucose in the propagation medium can be in a range of greater than 14 g/L to about 90 g/L, including at least 15 g/L, at least 20 g/L, at least 25 g/L, at least 30 g/L, at least 35 g/L, at least 40 g/L, at least 45 g/L, at least 50 g/L, at least 60 g/L, at least 70 g/L, at least 80 g/L, or at least 90 g/L. As such, the propagation medium can comprise glucose in an amount in a range of 25 g/L to 80 g/L, such as 40 g/L to 70 g/L.

In addition to glucose, yeast can also be propagated in a propagation medium that further comprises xylose in an amount of at least 5 g/L, such as at least 10 g/L, at least 15 g/L, at least 20 g/L, at least 25 g/L at least 30 g/L, at least 35 g/L, at least 40 g/L, at least 45 g/L, or at least 50 g/L. The amount of xylose in the propagation medium can be in a range of about 5 g/L to about 50 g/L, such as 20 g/L to 40 g/L. The xylose in the propagation medium can come from hydrolysate 106 provided to propagation medium 104. For instance, if hydrolysate 106 contains xylose in an amount of about 60 g/L, then an amount of at least 25% by volume provides propagation medium 104 about 15 g/L xylose.

The presence of glucose in the propagation medium can cause the Crabtree effect where the yeast produces ethanol during propagation. Conventional propagation methods that aim to suppress the Crabtree effect provide propagation conditions to keep the level of ethanol present in the culture during, near, and/or at the end of the propagation step low (e.g., FIGS. 1 and 5 of US20150252319).

It had been discovered that improved fermentation in an exogenous-ethanol-containing hydrolysate can be improved if propagation is conducted using the hydrolysate, where the propagation medium can contain a high glucose concentration (e.g., greater than 14 g/L) and exogenous ethanol (e.g., at least 2.5 g/L), even if the Crabtree effect takes place during propagation. For instance, the yeast propagated according to aspects described herein can provide improved ethanol yield during fermentation of an exogenous-ethanol-containing hydrolysate as compared to yeast propagated a medium that does not contain a high amount of glucose and exogenous ethanol. While improved ethanol yield during fermentation can be observed with fermentation of hydrolysate containing greater than 10 g/L exogenous ethanol (e.g., at least about 14 g/L), the improvement in yields over

yeasts not propagated according to aspects described herein increases as the amount of exogenous ethanol in the hydrolysate increases (such as at least 20 g/L, at least 25 g/L, at least 30 g/L, or at least 35 g/L). Example 1 below demonstrates yeast propagated in a propagation medium comprising the exogenous-ethanol-containing hydrolysate in an amount of 50% by volume has greater ethanol yield than the same yeast propagated in a propagation medium that does not contain said hydrolysate. There can be a correlation of improvement in ethanol yields and initial ethanol amount or concentration (exogenous ethanol) in the hydrolysate. For instance, the difference between the fermentation product yield by yeast propagated according to aspects described and the control can increase as the amount of exogenous ethanol in the hydrolysate increases.

Accordingly, referring to FIG. 1, at least 15 g/L of ethanol can be produced by the yeast during propagation in vessel 102, where this ethanol can be referred to as "endogenous ethanol" or "produced ethanol." For instance, the endogenous ethanol amount can be at least 15 g/L, at least 20 g/L, at least 25 g/L, at least 30 g/L, at least 35 g/L, at least 40 g/L, at least 45 g/L, or at least 50 g/L. The amount of ethanol produced during propagation can be in a range of 15 g/L to 50 g/L, such as 25 g/L to 35 g/L. Also, the ethanol produced during propagation can be in a range of 20 g/L to 45 g/L.

The concentration of exogenous and endogenous ethanol can be determined by any suitable method known to one of ordinary skill. For instance, the ethanol concentration at the start of propagation is measured by taking a 1 mL sample of the propagation medium with the yeast already added, where the sample is centrifuged, filtered, and then run on an Aminex HPX 87-H HPLC column to measure ethanol against a set of standards. This ethanol concentration at the start of propagation is considered exogenous ethanol. At the end of propagation, the ethanol concentration is then again measured in the same fashion. The difference between the ethanol concentration measured at the end of propagation and the exogenous ethanol concentration from the beginning constitutes the ethanol produced during propagation or endogenous ethanol.

Even though the present disclosure describes allowing for ethanol production during propagation, propagation is still primarily an aerobic process. As such, the propagation reaction vessel to which the propagation medium and yeast are provided and in which yeast is propagated comprises an oxygen inlet to provide oxygen to the propagation reactor vessel for growth during propagation. The oxygen amount provided to the propagation reactor vessel may be controlled using suitable equipment known to one of

ordinary skill. A suitable amount of oxygen for respiration is well known and can be provided in a propagation reactor vessel by any well-known aerator apparatus such as an air sparging system. Further, sufficient aeration can be promoted by agitating the propagation medium. Agitation is well known and can be provided by, e.g., mechanical stirring.

Accordingly, there is provided a method of propagating yeast comprising:

(a) providing a hydrolysate for fermentation,

(b) providing a reactor containing:

a propagation medium comprising: the hydrolysate in an amount of at least 25% by volume; and

a first cell mass of a yeast; and

(c) propagating the first cell mass in the composition in the reactor for a time period to form a propagated culture comprising a second cell mass of the yeast that is greater than the first cell mass, wherein at least 15 g/L of ethanol is produced by the yeast during propagation.

Because the propagated culture as provided herein can still provide improved fermentation performance despite the Crabtree effect during propagation, the present disclosure may enable the elimination of providing a glucose stream during propagation and/or metering of a glucose stream to ensure suppression of the Crabtree effect, where careful monitoring of a glucose stream is often a necessity in conventional propagation methods, as described in US20150252319. US9034631 proposes to eliminate a glucose stream, thereby eliminating associated technical challenges, and use enzymes to hydrolyze polysaccharides as a glucose replacement. This proposal unfortunately increases operation costs through the use of expensive enzymes to generate sugars (including glucose), where the present disclosure provides methods that would not incur such enzyme costs.

In addition to nutrients already provided in the hydrolysate, the propagation medium may include other optional nutrients or agents to optimize growth, which can include, for example, antibiotics, supplemental or accessory enzymes, materials for adjusting and maintaining pH, nutrients or other components providing nutritional or other benefits to the yeast. For instance, the propagation medium may, but need not, further comprise one or more of the following: yeast extract, nitrogen (often in the form of urea), diammonium phosphate, magnesium sulfate, zinc sulfate or other salts, and the like.

Yeast can be introduced into the reaction vessel for propagation in any initial amount. That is, referring to FIG. 1, the first cell mass of yeast 112 can be any suitable amount known by one of ordinary skill in the art. As mentioned, yeast 112 can be provided to propagation vessel 102 independently and/or as part of propagation medium 104. Typically, the initial amount is determined based on considerations known to one of ordinary skill, such as the desired time period for completing propagation and the desired cell count at the end of propagation, the nutrient(s) in the propagation medium, the carbon source including glucose, temperature, pH, the volume of the propagation medium, and the like.

The temperature and/or the pH of propagation medium 104 in vessel 102 can be any temperature that permits the contents of the propagation medium to function properly such as permitting the enzymes to break down the feedstock material into sugars and the yeast to reproduce., as known by one of ordinary skill in the art. Exemplary temperatures include a temperature in the range from 15° C. to 50° C., preferably from 20° C. to 40° C., and even more preferably from 25° C. to 37° C. Exemplary pH values include a pH in the range from 2-8, preferably from 3 to 7.5, and even more preferably from 3.5 to 6.5.

The yeast used in the propagation process as initial yeast population (e.g., the first cell mass of yeast 112) may be wild-type and/or genetically engineered yeast. Yeasts are herein defined as eukaryotic microorganisms and include all species of the subdivision Eumycotina (Alexopoulos, C. J., 1962, In: Introductory Mycology, John Wiley & Sons, Inc. , New York) that predominantly grow in unicellular form.

Yeasts may either grow by budding of a unicellular thallus or may grow by fission of the organism. A preferred yeast can be a yeast may belong to the genera *Saccharomyces*, *Kluyveromyces*, *Candida*, *Pichia*, *Schizosaccharomyces*, *Hansenula*, *Kloeckera*, *Schwanniomyces*, or *Yarrowia*. Preferably, the yeast can be one capable of anaerobic fermentation, more preferably one capable of anaerobic alcoholic fermentation. Optionally, the yeast can be *Saccharomyces cerevisiae*.

The yeast can be an industrial yeast. An industrial yeast cell may be defined as follows. The living environments of yeast cells in industrial processes are significantly different from that in the laboratory. Industrial yeast cells must be able to perform well under multiple environmental conditions which may vary during the process. Such variations include change in nutrient sources, pH, ethanol concentration, temperature, oxygen concentration, etc., which together have potential impact on the cellular growth and

ethanol production by *Saccharomyces cerevisiae*. Under adverse industrial conditions, the environmental tolerant strains should allow robust growth and production. Industrial yeast strains are generally more robust towards these changes in environmental conditions which may occur in the applications they are used, such as in the baking industry, brewing
5 industry, wine making and the ethanol industry. Examples of industrial yeast (*S. cerevisiae*) are genetically engineered Ethanol Red® (Fermentis) Fermiol® (DSM) and Thermosacc® (Lallemand). The yeast can be inhibitor tolerant. Inhibitor tolerant yeast cells may be selected by screening strains for growth on inhibitors containing materials, such as illustrated in Kadar et al, Appl. Biochem. Biotechnol. (2007), Vol. 136-140, page
10 847-858, wherein an inhibitor tolerant *S. cerevisiae* strain ATCC 26602 was selected. RN1016 is a xylose and glucose fermenting *S. cerevisiae* strain from DSM, Bergen op Zoom, the Netherlands.

The yeast can be capable of converting hexose (C6) sugars and/or pentose (C5) sugars. Optionally, the yeast can anaerobically ferment at least one C6 sugar and at least
15 one C5 sugar. For example the yeast can be capable of using L-arabinose and xylose in addition to glucose anaerobically. The yeast can be capable of converting L-arabinose into L-ribulose and/or xylulose 5-phosphate and/or into a desired fermentation product, for example into ethanol. Organisms, for example *S. cerevisiae* strains, able to produce ethanol from L-arabinose may be produced by modifying a host yeast introducing the araA (L-
20 arabinose isomerase), araB (L-ribuloglyoxalate) and araD (L-ribulose-5-P4-epimerase) genes from a suitable source. Such genes may be introduced into a host cell in order that it is capable of using arabinose. Such an approach is given is described in WO2003/095627. araA, araB and araD genes from *Lactobacillus plantarum* may be used and are disclosed in WO2008/041840. The araA gene from *Bacillus subtilis* and the araB and araD genes from
25 *Escherichia coli* may be used and are disclosed in EP1499708. Optionally, the araA, araB and araD genes may derived from of at least one of the genus *Clavibacter*, *Arthrobacter* and/or *Gramella*, in particular one of *Clavibacter michiganensis*, *Arthrobacter aureus*, and/or *Gramella forsetii*, as disclosed in WO 2009011591. The yeast may also comprise one or more copies of xylose isomerase gene and/or one or more copies of xylose reductase
30 and/or xylitol dehydrogenase.

The yeast may comprise one or more genetic modifications to allow the yeast to ferment xylose. Examples of genetic modifications are introduction of one or more xylA-gene, XYL1 gene and XYL2 gene and/or XKS1-gene; deletion of the aldose reductase

(GRE3) gene; overexpression of PPP-genes TAD, TKL1, RPE1 and RKI1 to allow the increase of the flux through the pentose phosphate pathway in the cell. Examples of genetically engineered yeast is described in EP1468093 and/or WO2006009434.

The fermentation product of the propagated fermentation microorganism herein
5 may be any useful product. It can be a product selected from the group consisting of ethanol, n-butanol, isobutanol, lactic acid, 3-hydroxy-propionic acid, acrylic acid, acetic acid, succinic acid, fumaric acid, malic acid, itaconic acid, maleic acid, citric acid, adipic acid, an amino acid, such as lysine, methionine, tryptophan, threonine, and aspartic acid, 1,3-propane-diol, ethylene, glycerol, a β -lactam antibiotic and a cephalosporin, vitamins,
10 pharmaceuticals, animal feed supplements, specialty chemicals, chemical feedstock, plastics, solvents, fuels, including biofuels and biogas or organic polymers, and an industrial enzyme, such as a protease, a cellulase, an amylase, a glucanase, a lactase, a lipase, a lyase, an oxidoreductases, a transferase or a xylanase. For example the fermentation products may be produced by yeast propagated according to aspects described herein, which examples however should herein not be construed as limiting. For instance,
15 n-butanol may be produced by cells as described in WO2008121701 or WO2008086124; lactic acid as described in US2011053231 or US2010137551; 3-hydroxy-propionic acid as described in WO2010010291; or acrylic acid as described in WO2009153047. Also, yeasts modified to convert compounds other than sugars to ethanol can also be propagated
20 according to aspects described herein, which examples however should herein not be construed as limiting. For example, acetate can be converted to ethanol as described in WO2014074895 or ethanol can be produced via a pathway that has reduced glycerol production, as such that described in WO2014081803.

Once the yeast is present in the propagation medium, the yeast can grow for any
25 desired time period. Typically, the yeast will be grown under conditions to provide a sufficient amount of yeast cells to produce fermentation products during fermentation. Also, the yeast cells are typically grown for an economically efficient time period and preferably where the yeast population is still sufficiently viable as known to one of ordinary skill. Referring to FIG. 1, illustrative time periods of propagation in vessel 102
30 include from 30 minutes to 100 hours, such as at least 6 hours, at least 12 hours, at least 24 hours, at least 36 hours, at least 48 hours, at least 60 hours, or at least 72 hours. Preferably, the time period for propagation is in a range from 10 hours to 24 hours, to 48 hours, or to 72 hours.

The propagation can be conducted until the first cell mass of yeast has grown to form a propagated culture comprising a second cell mass that comprises at least two generations. For instance, the propagation can be conducted until the second cell mass of yeast comprises at least two generations, such as, at least three, at least four, at least five, or
 5 at least six generations, compared to the first cell mass. A generation of growth herein means a doubling of yeast cell mass in weight (g).

The definition of a generation here is a doubling of yeast cell mass. The doubling of the amount of cell mass can be determined by any suitable methods known to one of ordinary skill. For example, the amount of cell mass can be described by C_x (cell mass
 10 concentration) at given time to be given by the following equation:

$$C_x(t) = C_x(0) * e^{(\mu * t)} \quad (\text{eq. 1})$$

The doubling time (T_d in h) or generation time (T_g h) can be derived from the is equation by substituting $C_x(t) = 2 * C_x(0)$.

$$T_d = \ln(2) / \mu(\text{hr}) \quad (\text{eq. 2})$$

15 Where μ = specific growth rate in g biomass/g biomass/h or 1/h).

The cell mass growth rate can be measured by various means known to one of ordinary skill. For instance, the increase of cell mass amount can be analyzed by determining the amount of cells per weight or volume unit of a culture using any of the following method or a suitable alternative method:

20 Turbidity

Optical Density in the visible light spectrum (usual range: 600 nm to 700 nm) of a culture

A pellet volume after centrifugation,

The dry weight content after drying at constant weight at 105° C.

25 Cell count per volume (microscopically),

Colony Forming Unit (CFU/ml) after plating on a solid agar medium and growing colonies on a plate from single cells

Alternatively one can derive the amount of biomass from a metabolic activity measured in a closed reactor system such as:

30 The rate of carbon dioxide production (CPR carbon dioxide production rate or CER Carbon Dioxide Evolution Rate generally expressed as mmol CO₂/L/hr)

The rate of oxygen consumption (OUR Oxygen Uptake Rate mmol O₂/L.hr)

Substrate uptake rate (r_s =substrate uptake rate in g /L.hr uptake rate of glucose, xylose, arabinose or ammonia)

When $\ln(C_x)$ or $\ln(CPR)$, $\ln(OUR)$ or $\ln(r_s)$ or is plotted versus time in an exponential growth experiment (no nutrient limitations and no toxic products formed) a straight line is obtained with the slope being the specific growth rate μ . With μ and eq. 2 one can calculate the doubling time and with the growth time one can calculate the amount of doublings or the number of generations

Capacitive measurement that uses the dielectric properties of living cells, which has the advantage of measuring only viable cell density. Unlike optical techniques, the system is not sensitive to gas bubbles, micro-carrier, cell debris and other particles in suspension.

Referring to FIG. 1, after sufficient propagation in vessel 102 to form a propagated culture, the cell mass of propagated yeast 110 may be isolated (such as filtration and/or centrifuge) from the propagated culture of vessel 102 prior to being provided to fermentation reactor vessel 108, and/or the propagated culture containing propagated yeast 110 can be fed as a whole broth to fermentation reactor 108. These steps may be executed in conventional manners known to one of ordinary skill in the art. Optionally, part of the propagated yeast or culture can be recycled to the propagator.

The propagation process described herein can also further include an optional pre-propagation conditioning step to generate an amount of yeast to use as the initial yeast amount or first cell mass of yeast in the propagation reactor vessel. Referring to FIG. 1, the first cell mass of yeast 112 shown in FIG. 1 can comprise yeast grown in an optional pre-propagation conditional step generated as described in FIG. 2. Referring to FIG. 2, propagation medium 104A and an initial cell mass of yeast 114 are provided to optional pre-propagation conditioning reactor vessel 116. The initial cell mass of yeast 114 is grown for a time period under conditions described for the propagation step until the growth provides a sufficient amount of cell mass for inoculation of the propagation step. In particular, the descriptions of the propagation medium of the present disclosure, including descriptions of propagation medium 104 in FIG. 1, are applicable to propagation medium 104A of FIG. 2. Propagation medium 104A can comprise hydrolysate 106 in an amount of at least 25% by volume and glucose in an amount of greater than 14 g/L. At least a portion of the glucose may be provided by hydrolysate 106. Similarly, the descriptions of the yeast selected for fermentation and growth conditions are described above for the propagation process, including those related to propagation vessel 102. Cell

mass growth or amount of yeast 114 grown in pre-propagation vessel 116 can be determined as described above. The yeast cells can be grown in pre-propagation vessel 116 for a similar time period as that described above for propagation in propagation vessel 102. For instance, illustrative time periods in pre-propagation vessel 116 include from 30
5 minutes to 100 hours, such as at least 6 hours, at least 12 hours, at least 24 hours, at least 36 hours, at least 48 hours, at least 60 hours, or at least 72 hours. Preferably, the time period for propagation is in a range from 10 hours to 24 hours, to 48 hours, or to 72 hours.

Referring to FIG. 2, the cell mass of yeast 112 grown after this time period may be isolated (e.g., centrifuged or filtered) from pre-propagation conditioning reactor vessel 116
10 prior to being provided to propagation reactor vessel 102, and/or the pre-propagation conditioned yeast 112 can be fed in whole or in part as a whole broth to propagation reactor vessel 102.

Accordingly, the present disclosure provides a method comprising:

- (a) providing a hydrolysate for fermentation,
- 15 (b) providing a first reactor containing:
 - a pre-propagation medium comprising: the hydrolysate in an amount of at least 25% by volume and glucose in an amount of greater than 14 g/L; and
 - a first cell mass of a yeast;
- (c) growing the first cell mass in the pre-propagation medium in the first reactor for
20 a time period to form a propagated culture comprising a second cell mass of the yeast that is greater than the first cell mass;
- (d) providing at least a portion of the second cell mass from step (c) to a second reactor containing a propagation medium comprising: the hydrolysate in an amount of at least 25% by volume and glucose in an amount of greater than 14 g/L; and
- 25 (e) propagating said portion of the second cell mass in the propagation medium in the second reactor for a time period to form a propagated culture comprising a third cell mass of the yeast that is greater than said portion of the second cell mass.

The present disclosure provides methods that are particularly applicable for propagating yeast that provides improved yields from fermentation of a hydrolysate that
30 contains exogenous ethanol. As mentioned above, various methods have been developed to address acetic acid in conventional hydrolysates used in fermentation processes but not other inhibitors, including ethanol. This is because conventional hydrolysates typically do not contain ethanol since the typical process of pretreatment to generate the hydrolysate

does not involve generating ethanol in the hydrolysate prior to production of ethanol by the yeast added during the fermentation process. For instance, conventional processes typically involve providing cellulosic biomass material, such as corn stover, to a pretreatment process to provide a pretreated material that is more susceptible to hydrolysis
5 by enzymes. This enzymatic hydrolysis step generates a hydrolysate for fermentation. The biomass of the conventional process has not been treated to generate or contain ethanol when it enters pretreatment, and the pretreatment process typically does result in generation or addition of ethanol to the pretreated material. Similarly, the enzymatic hydrolysis step also does not result in generation or addition of ethanol to the hydrolysate.
10 The lack of ethanol in conventional hydrolysate is demonstrated by Table 1 in US20150252319, showing the composition of conventional lignocellulosic hydrolysate, which has zero vol% ethanol.

Exogenous ethanol in a hydrolysate can be an inhibitor to yeast growth, which can result in reduced ethanol production. Thus, fermentation of a hydrolysate containing
15 exogenous ethanol using a method, system, or composition according to some aspects of present disclosure can result in improved the ethanol production compared to fermentation that employs yeast not propagated according to aspects disclosed herein.

Accordingly, the present disclosure provides an initial yeast composition for propagation in a reactor, where propagation can be carried out for a time period using this
20 initial propagation composition. The initial propagation composition comprises:

- (a) a propagation medium comprising glucose in an amount of greater than 14 g/L, at least 25% by volume of a hydrolysate for fermentation, and at least 2.5 g/L exogenous ethanol; and
- (b) a first cell mass of a fermentation microorganism, wherein the fermentation
25 microorganism can use at least a portion of the glucose to grow for a time period to form a propagated culture comprising a second cell mass of the microorganism that is greater than the first cell mass;
wherein the exogenous ethanol is not produced by the microorganism in the composition, wherein at least a portion of the exogenous ethanol is provided by
30 the hydrolysate in the propagation medium.

Accordingly, there is provided a propagated yeast composition for fermentation of a hydrolysate, where the composition comprises:

- (a) a propagated population of a fermentation microorganism; and

- (b) a propagation medium comprising at least 25% by volume of a hydrolysate for fermentation and at least 2.5 g/L exogenous ethanol not produced by the fermentation microorganism in the composition, wherein at least a portion of the exogenous ethanol is provided by the hydrolysate in the propagation medium; and
- (c) at least 15 g/L endogenous ethanol produced by the microorganism during propagation.

Fermentation

Referring to FIGS. 1 and 2, the yeast propagated in reactor vessel 102 are provided to fermentation reactor vessel 108 for fermentation. It is preferred that enzymatic hydrolysis and fermentation are conducted in separate vessels so that each biological reaction can occur at its respective optimal temperature. Fermentation of hydrolysate 106 may produce one or more of the fermentation products selected from an alcohol, a sugar alcohol, an organic acid and a combination thereof. Hydrolysate 106 can be provided to fermentation reactor vessel 108 as a whole broth from hydrolysis and is not diluted.

The fermentation in vessel 108 is typically conducted at a pH between about 4.0 and about 6.0, or between about 4.5 and about 6.0. To attain the foregoing pH range for fermentation, it may be necessary to add alkali to the hydrolysate stream.

The fermentation product(s) depend on the fermentation microorganism selected for fermentation, as known to one of ordinary skill, including the descriptions provided above. Illustrative fermentation microorganisms include a fungi, such as yeast, or a bacteria. For example, the fermentation product is preferably an alcohol, such as ethanol. For ethanol production, the fermentation is typically carried out with a *Saccharomyces* spp. yeast. Glucose and any other hexoses present in the sugar stream may be fermented to ethanol by wild-type *Saccharomyces cerevisiae*, although genetically modified yeasts may be employed as well, as discussed below. The ethanol may then be distilled to obtain a concentrated ethanol solution.

Xylose and arabinose that are derived from the hemicelluloses may also be fermented to ethanol by a yeast strain that naturally contains, or has been engineered to contain, the ability to ferment these sugars to ethanol. Examples of microbes that have been genetically modified to ferment xylose include recombinant *Saccharomyces* strains into which has been inserted either (a) the xylose reductase (XR) and xylitol

dehydrogenase (XDH) genes from *Pichia stipitis* (U.S. Pat. Nos. 5,789,210, 5,866,382, 6,582,944 and 7,527,927 and European Patent No. 450530) or (b) fungal or bacterial xylose isomerase (XI) gene (U.S. Pat. Nos. 6,475,768 and 7,622,284). Examples of yeasts that have been genetically modified to ferment L-arabinose include, but are not limited to, recombinant *Saccharomyces* strains into which genes from either fungal (U.S. Pat. No. 7,527,951) or bacterial (WO2008/041840) arabinose metabolic pathways have been inserted.

Organic acids that may be produced during the fermentation include lactic acid, citric acid, ascorbic acid, malic acid, succinic acid, pyruvic acid, hydroxypropanoic acid, itaconoic acid and acetic acid. In a non-limiting example, lactic acid is the fermentation product of interest. The most well-known industrial microorganisms for lactic acid production from glucose are species of the genera *Lactobacillus*, *Bacillus* and *Rhizopus*.

Moreover, xylose and other pentose sugars may be fermented to xylitol by yeast strains selected from the group consisting of *Candida*, *Pichia*, *Pachysolen*, *Hansenula*, *Debaryomyces*, *Kluyveromyces* and *Saccharomyces*. Bacteria are also known to produce xylitol, including *Corynebacterium* sp., *Enterobacter liquefaciens* and *Mycobacterium smegmatis*.

In practice, the fermentation is typically performed at or near the temperature and pH optimum of the fermentation microorganism (i.e., yeast). A typical temperature range for the fermentation of glucose to ethanol using *Saccharomyces cerevisiae* is between about 25° C and about 35° C, although the temperature may be higher if the yeast is naturally or genetically modified to be thermostable. The dose of the fermentation microorganism will depend on other factors, such as the activity of the fermentation microorganism, the desired fermentation time, the volume of the reactor and other parameters. It should be appreciated that these parameters may be adjusted as desired by one of skill in the art to achieve optimal fermentation conditions.

The fermentation may also be supplemented with additional nutrients required for the growth of the fermentation microorganism. For example, yeast extract, specific amino acids, phosphate, nitrogen sources, salts, trace elements and vitamins may be added to the hydrolysate slurry to support their growth.

The fermentation may be conducted in batch, continuous or fed-batch modes with or without agitation. Preferably, the fermentation reactors are agitated lightly with mechanical agitation. A typical, commercial-scale fermentation may be conducted using

multiple reactors. The fermentation microorganisms may be recycled back to the fermenter or may be sent to distillation without recycle.

If ethanol or butanol is the fermentation product, the recovery is carried out by distillation, typically with further concentration by molecular sieves or membrane
5 extraction.

The fermentation broth that is sent to distillation is a dilute alcohol solution containing solids, including unconverted cellulose, and any components added during the fermentation to support growth of the fermentation microorganisms.

Fermentation microorganisms are potentially present during the distillation
10 depending upon whether or not they are recycled during the fermentation. The broth is preferably degassed to remove carbon dioxide and then pumped through one or more distillation columns to separate the alcohol from the other components in the broth. The mode of operation of the distillation system depends on whether the alcohol has a lower or a higher boiling point than water. Most often, the alcohol has a lower boiling point than
15 water, as is the case when ethanol is distilled.

In those embodiments where ethanol is concentrated, the column(s) in the distillation unit is preferably operated in a continuous mode, although it should be understood that batch processes are also encompassed by the present invention. Heat for the distillation process may be introduced at one or more points either by direct steam
20 injection or indirectly via heat exchangers. The distillation unit may contain one or more separate beer and rectifying columns, in which case dilute beer is sent to the beer column where it is partially concentrated. From the beer column, the vapour goes to a rectification column for further purification. Alternatively, a distillation column is employed that comprises an integral enriching or rectification section.

25 After distillation, the water remaining may be removed from the vapour by a molecular sieve resin, by membrane extraction, or other methods known to those of skill in the art for concentration of ethanol beyond the 95% that is typically achieved by distillation. The vapour may then be condensed and denatured.

An aqueous stream(s) remaining after ethanol distillation and containing solids,
30 referred to herein as "still bottoms", is withdrawn from the bottom of one or more of the column(s) of the distillation unit. This stream will contain inorganic salts, unfermented sugars and organic salts.

When the alcohol has a higher boiling point than water, such as butanol, the distillation is run to remove the water and other volatile compounds from the alcohol. The water vapor exits the top of the distillation column and is known as the “overhead stream”.

Hydrolysate

5 The hydrolysate that is fermented and used for propagation as described herein refers to any hydrolysed cellulosic biomass. “Biomass,” “lignocellulosic biomass,” or “cellulosic biomass” refers to any cellulosic or lignocellulosic material and includes materials comprising cellulose, and optionally further comprising hemicellulose, lignin, starch, oligosaccharides and/or monosaccharides.

10 Biomass may also comprise additional components, such as protein and/or lipid. Biomass may be derived from a single source, or biomass can comprise a mixture derived from more than one source; for example, biomass could comprise a mixture of corn cobs and corn stover, or a mixture of grass and leaves. Biomass includes, but is not limited to, bioenergy crops (e.g., sorghum, poplars, willows, switch grass, alfalfa, prairie bluestream, 15 corn, soybean, algae and seaweed), agricultural residues (e.g., corn stalks, straw, seed hulls, sugarcane leavings, bagasse, nutshells, and manure from cattle, poultry, and hogs), municipal solid waste (e.g., waste paper), industrial solid waste, sludge from paper manufacture, yard waste, wood and forestry waste (e.g., wood or bark, sawdust, timber slash, and mill scrap). Examples of biomass include, but are not limited to, corn cobs, crop 20 residues such as corn husks, corn stover, grasses, wheat, wheat straw, barley straw, hay, rice straw, switchgrass, waste paper, sugarcane bagasse, sorghum, miscanthus, hemp, tropical poplar, willow, sugar beet, any energy cane, components obtained from milling of grains, trees, branches, roots, leaves, wood chips, sawdust, shrubs and bushes, vegetables, fruits, flowers and animal manure.

25 Biomass can comprise cellulose in an amount greater than about 5%, greater than about 30%, or greater than about 40% (w/w) . For example, biomass may comprise from about 10% to about 50% (w/w) cellulose, or any amount in between. Optionally, biomass can comprise lignin in an amount greater than about 10%, more typically in an amount greater than about 15% (w/w).

30 Conventional hydrolysates, including lignocellulosic or cellulosic hydrolysates as described in US9034631 and US20150252319, typically do not contain ethanol prior to fermentation. Accordingly, methods for propagating yeasts to ferment conventional hydrolysates do not produce propagated yeast suitable for fermentation of hydrolysates that

contain exogenous ethanol. Unlike conventional propagation methods, embodiments described herein are particularly applicable for propagating yeast that provides improved yields from fermentation of a hydrolysate that contains exogenous ethanol.

Illustrative hydrolysates for propagation and fermentation according to aspects
5 described herein can contain at least 10 g/L of exogenous ethanol, including in a range of at least 5 g/L to 80 g/L, such as at least 10 g/L, at least 15 g/L, at least 20 g/L, at least 25 g/L, at least 30 g/L, at least 35 g/L, at least 40 g/L, at least 50 g/L, at least 60 g/L, or at least 70 g/L. For instance, the amount of exogenous ethanol in the hydrolysate can be in a range of about 5 g/L and up to 20 g/L. The amount of exogenous ethanol in the
10 hydrolysate can also be in a range of about 40 g/L – 70 g/L. The hydrolysate can comprise exogenous ethanol in an amount in a range of 10 g/L to 40 g/L.

As mentioned, the propagation medium preferably comprises glucose in an amount of greater than 14 g/L, such as about 40 g/L or greater, about 60 g/L or greater, or about 80 g/L or greater. The amount of glucose in the propagation medium can be in a range of
15 greater than 14 g/L to about 90 g/L, including at least 15 g/L, at least 20 g/L, at least 25 g/L, at least 30 g/L, at least 35 g/L, at least 40 g/L, at least 45 g/L, at least 50 g/L, at least 60 g/L, at least 70 g/L, at least 80 g/L, or at least 90 g/L. The propagation medium can comprise glucose in an amount in a range of 25 g/L to 80 g/L, such as 40 g/L to 70 g/L.

In addition to glucose, the propagation medium can further comprise xylose in an
20 amount of at least 5 g/L, such as at least 10 g/L, at least 15 g/L, at least 20 g/L, at least 25 g/L at least 30 g/L, at least 35 g/L, at least 40 g/L, at least 45 g/L, or at least 50 g/L. The amount of xylose in the propagation medium can be in a range of about 5 g/L to about 50 g/L, such as 20 g/L to 40 g/L. The xylose in the propagation medium can come from hydrolysate 106 provided to propagation medium 104. For instance, if hydrolysate 106
25 contains xylose in an amount of about 60 g/L, then an amount of at least 25% by volume provides propagation medium 104 can be about 15 g/L xylose or higher if more xylose is added.

The hydrolysate can further comprise total solids is at least 20%, such as at least 22%, at least 24%, at least 26%, at least 28%, or at least 30%. The total solids amount in
30 the hydrolysate can be in a range of 20% to 30%, such as in a range of about 21% to 27%. It is understood that one of ordinary skill can adjust the process described herein to produce a hydrolysate with a certain amount of total solids suitable for fermentation. The total solids concentration can be determined using methods known to one of ordinary skill.

An illustrative method to measure total solids is provided by the American Association of Cereal Chemistry, particularly the AACC Method 44-01.01 Calculation of Percent Moisture, Am. Assoc. Cereal Chem. Inc., St. Paul, Minnesota, 2000. Generally, the AACC Method 44-01.01 method defines moisture as 100% - TS%, so the equation for % total solids is as follows:

$$\%Total\ Solids = \frac{(Weight_{dry\ pan\ plus\ dry\ sample} - Weight_{dry\ pan})}{weight_{sample\ as\ received}} \times 100$$

As such, a sample can be weighed as the “sample as received” then placed in a vacuum oven overnight to generate the “dry sample,” which can be weighed to determine the % total solids according to the equation above.

One illustrative process to generate a hydrolysate comprising exogenous ethanol is described in US8946491, the disclosure of which is incorporated herein by reference. A general description of illustrative embodiments of ensiling cellulosic biomass material for hydrolysis to generate a hydrolysate containing exogenous ethanol is further described below.

15 **Ensiling of Cellulosic Biomass**

Cellulosic biomass can be ensiled until it comprises ethanol before it is provided to enzymatic hydrolysis. Referring to FIG. 3, cellulosic biomass 302 is provided to ensiling step 304. At least one of a microbe 306 and an acid 308 is further provided to ensiling step 304 to facilitate production of ethanol in cellulosic biomass 302 in ensiling step 304. Preferably, the cellulosic biomass comprises at least one fermentable sugar-producing plant. The cellulosic biomass can comprise two or more different plant types, including fermentable sugar-producing plant. Optionally, sorghum can be selected, due to its high-yield on less productive lands and high sugar content.

The term “fermentable sugar” refers to oligosaccharides and monosaccharides that can be used as a carbon source (e.g., pentoses and hexoses) by a microorganism (e.g., yeast) in a fermentation process or other processes that convert carbohydrates to an organic product such as alcohols, organic acids, esters, and aldehydes using a microorganism under anaerobic and/or aerobic conditions. The at least one fermentable sugar-producing plant contains fermentable sugars dissolved in the water phase of the plant material at one point in time during its growth cycle. Non-limiting examples of fermentable sugar-producing plants include sorghum, sugarcane, sugar beet, and energy cane. In particular, sugarcane,

energy cane, and sorghum typically contain from about 5% to about 25% soluble sugar w/w in the water phase and have moisture content between about 60% and about 80% on a wet basis when they are near or at their maximum potential fermentable sugar production (e.g., maximum fermentable sugar concentration).

5 The term “wet basis” refers at least to the mass percentage that includes water as part of the mass. If sorghum is used, the sorghum can include any variety or combination of varieties that may be harvested with higher concentrations of fermentable sugar. Certain varieties of sorghum with preferred properties are sometimes referred to as “sweet sorghum.” The sorghum can include a variety that may or may not contain enough
10 moisture to support the juicing process in a sugar cane mill operation. The solid biomass can include a Sugar T sorghum variety commercially produced by Advanta and/or a male parent of Sugar T, which is also a commercially available product of Advanta. The crop used can have from about 5 to about 25 brix, preferably from about 10 to about 20 brix, and more preferably from about 12 to about 18 brix. The term “brix” herein refers at least
15 to the content of glucose, fructose, and sucrose in an aqueous solution where one degree brix is 1 gram of glucose, fructose, and/or sucrose in 100 grams of solution and represents the strength of the solution as percentage by weight (% w/w). The moisture content of the crop used can be from about 50% to 80%, preferably at least 60%.

 Optionally, the crop can be a male parent of Sugar T with a brix value of about 18
20 and a moisture content of about 67%. Additionally or alternatively, the crop can be Sugar T with a brix value of about 12 at a moisture content of about 73%. The brix and moisture content values can be determined by handheld refractometer. Other suitable methods to determine the moisture content can be employed, for instance, a desktop moisture analyzer.

 At least one additive selected from a microbe and an acid can be added to the
25 cellulosic biomass to facilitate the conversion of fermentable sugar into ethanol. The cellulosic biomass with the at least one additive can be stored for a period of time to allow sufficient production of ethanol in the cellulosic biomass.

 If plants are used, the plants can be collected or harvested from the field using any suitable means known to those skilled in the art, such as a forage or silage harvester (a
30 forage or silage chopper). A silage or forage harvester refers to farm equipment used to make silage, which is grass, corn or other plant that has been chopped into small pieces, and compacted together in a storage silo, silage bunker, or in silage bags. A silage or forage harvester has a cutting mechanism, such as either a drum (cutterhead) or a flywheel

with a number of knives fixed to it, which chops and transfers the chopped material into a receptacle that is either connected to the harvester or to another vehicle driving alongside. The plants can be harvested and cut into any length. The chop lengths of the harvester can be to a range of about 3 mm to about 80 mm, preferably about 3mm to about 20 mm, with
 5 examples of about 3 mm to about 13 mm chop lengths being most preferred.

The at least one additive can be added at any point during and/or after the harvest process. If a forage harvester is used, additives can be added to the cellulosic biomass during the harvest process. In particular, forage harvesters are designed for efficiently adding both solid and liquid additives during harvest. Optionally, the selected additive(s)
 10 can be added as solutions. If a forage harvester or a similar equipment is, the selected additive(s) can be added during harvest at all phases, such as before the intake feed rollers, during intake, at chopping, after chopping, through the blower, after the blower, in the accelerator, in the boom (or spout), and/or after the boom. If acid and enzyme are added, the acid can added near the intake feed rollers, and a microbe and the enzyme can added in
 15 the boom. The selected additive(s) to be added during harvest may be towed behind the harvester on a trailer.

The harvested cellulosic biomass can be stored or ensiled for a period of time to allow for production of ethanol from at least a portion of the fermentable sugar in the cellulosic biomass. Additionally or alternatively to above, selected additive(s) can also be
 20 added prior to storage or ensiling of the cellulosic biomass. Selected additive(s) can be added to the cellulosic biomass in any order. An acid can added to the cellulosic biomass before adding a microbe to prime the material to provide an attractive growth environment for the microbe.

Referring to FIG. 3, an acid 308 can be added to reduce the pH of cellulosic
 25 biomass 302 to a range that facilitates and/or expedites selected indigenous or added microbial growth, which increases production of ethanol. Acid can be added until the pH of the solid biomass is between about 2.5 and about 5.0, preferably in a range of about 3.7 to about 4.3, and more preferably about 4.2. The acid used can include known acids, such as sulfuric acid, formic acid, or phosphoric acid. The following Table 2 provides non-
 30 limiting examples of an acid that can be used individually or in combination.

Table 1

Sulfuric Acid	Formic Acid	Propionic Acid	Malic Acid
Phosphoric Acid	Maleic Acid	Folic Acid	Citric Acid

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After cellulosic biomass 302 has reached the desired pH with the addition of acid, a microbe 306 can be provided to cellulosic biomass 302 in silage pile. A microbe in the additive context refers at least to a living organism added to the solid biomass that is capable of impacting or affecting the prepared biomass material. One exemplary impact or effect from added microbe(s) includes providing fermentation or other metabolism to convert fermentable sugars from various sources, including cellulosic material, into ethanol or other volatile organic compounds. Preferred microbes include *Saccharomyces cerevisiae* strains that can tolerate high ethanol concentrations and are strong competitors in its respective microbial community. The microbes may be mesophiles or thermophiles. Thermophiles are organisms that grow best at temperatures above about 45°C, and are found in all three domains of life: Bacteria, Archaea and Eukarya. If a strain of *Saccharomyces cerevisiae* is used, the strain can come from a commercially available source such as Biosaf from Lesaffre, Ethanol Red from Phibro, and Lallamand activated liquid yeast.

If the microbe is obtained from a commercial source, the microbe can be added according to the recommended rate of the provider, which is typically based on the expected sugar content per wet ton, where water is included in the mass calculation. The term “wet ton” refers at least to the mass unit including water. The recommended amount can be adjusted according to reaction conditions. The microbe added can comprise one strain or multiple strains of a particular microbe. Optionally, the microbes are added at a rate of up to 500 mL per wet ton of solid biomass. In a particular, if commercially available yeast is used, about 300 mL of Lallamand yeast preparation can be added per wet ton of solid biomass. It is understood that one or more additional yeast strains can be added. For example, Ethanol Red can be added at a rate between about 0.001 kg/wet ton to about 0.5 kg/wet ton, particularly about 0.1 kg/wet ton.

Optionally, another yeast strain can be added, e.g., Biosaf, at a rate between about 0.001 kg/wet tone to about 0.5 kg/wet ton, particularly about 0.1 kg/wet ton. It is understood that other amounts of any yeast strain can be added. For example, about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 1.5 times, about 2 times, about 2.5 times, or about 3 times of the provided amounts of microbes can be added.

Optionally, an enzyme can be further added to the cellulosic biomass. The enzyme can be one that assists in the generation of fermentable sugars from plant materials that are more difficult for the microbe to metabolize, such as different cellulosic materials, and/or

to improve the value of an eventual by-product serving as animal feed, such as by making the feed more digestible. The enzyme can also be an antibiotic, such as a lysozyme as discussed further below. The enzyme added can include one type of enzyme or many types of enzymes. The enzyme can come from commercially available enzyme preparations.

5 Non-limiting examples of enzymes that assist in converting certain difficult to metabolize plant materials into fermentable sugars include cellulases, hemicellulases, ferulic acid esterases, and/or proteases. Additional examples also include other enzymes that either provide or assist the provision for the production of fermentable sugars from the feedstock, or increase the value of the eventual feed by-product.

10 Any suitable technique can be used to ensile the cellulosic biomass. For instance, the cellulosic biomass can be stored or ensiled as a free standing pile. Optionally, the pile can be formed in another structure, such as a silage bunker. The storage pile can further optionally include a leachate collection system, which can be used to remove leachate collected from the storage pile. Any suitable leachate collection system known to those skilled in the art can be employed as described. For instance, the leachate collection system can comprise at least one trough along the bottom of the pile, preferably positioned near the middle, of the storage pile or bunker if one is used, where the storage pile can be prepared at a grade designed to direct liquid from the prepared biomass material to the trough and out to a desired collection receptacle or routed to other applications. The leachate collection system can comprise one or more perforated conduits, preferably pipes made of polyvinyl chloride (PVC), that run along the bottom of the pile to allow the liquid collected in the conduits to be directed away from the pile.

Referring to FIG. 3, the cellulosic biomass material containing the selected additive(s) can be stored or ensiled in step 304 for at least about 24 hours and preferably at least about 72 hours (or 3 days) to allow for production of ethanol or other volatile organic compounds. The prepared biomass material is stored for a period of time sufficient to achieve an anaerobiasis environment. Any suitable method can be used to achieve the anaerobiasis environment. For instance, the anaerobiasis environment can be achieved by packing the cellulosic material into a pile, where the packing can range from about 7 lbs/ft³ to about 50 lbs/ft³ per cubic foot of the cellulosic biomass material. The packing can be from about 30 lbs/ft³ to about 50 lbs/ft³, particularly about 44 lbs/ft³. The anaerobiasis environment can be achieved in about 24 hours. Optionally, the anaerobiasis environment can be achieved in more than about 4 hours. The anaerobiasis environment can also be

achieved in up to about 72 hours. The cellulosic biomass material containing the selected additive(s) is stored until it contains no more than about 80 wt% liquid. For instance, the cellulosic biomass material can be stored until it contains between about 2 wt% and about 50 wt% ethanol, and preferably between about 4 wt% and about 10 wt% ethanol.

5 **Pretreatment of the Cellulosic Biomass**

Referring to FIG. 3, preferably, after a sufficient amount of time being stored or ensiled with the selected additives under conditions that allow for ethanol production, ensiled cellulosic biomass 312 comprising ethanol is provided to pretreatment step 310. At least a portion of ethanol may be removed from ensiled cellulosic biomass 312 prior to being provided to pretreatment step 310. Any suitable method may be used to remove at least a portion of ethanol. For instance, a superheated steam dryer (SSD) preferably operated as described in US8946491 may be used to remove at least a portion of the ethanol in the cellulosic biomass. Additionally or alternatively, force can be applied to the cellulosic biomass to remove liquid from the cellulosic biomass, thereby removing some ethanol as at least a portion of the ethanol in the cellulosic biomass is dissolved in the liquid fraction of the cellulosic biomass. The force applied can be achieved via pressing or squeezing of the ensiled cellulosic biomass material. Suitable pressing or squeezing equipment is known to one of ordinary skill, which includes a squeeze press. It is understood that the ensiled cellulosic biomass 312 can be provided to pretreatment step 310 without employing an ethanol removal step. Ensiled cellulosic biomass 312 can contain at least 0.1 wt% ethanol, at least 0.5 wt% ethanol, at least 1 wt% ethanol, at least 1.5 wt% ethanol, or at least 2 wt% ethanol when it is provided to pretreatment step 310. The ethanol amount in ensiled cellulosic biomass 312 may be up to 10 wt%, up to 7.5 wt%, up to 5 wt%, up to 2.5 wt%, or up to 2 wt% when it is provided to pretreatment step 310.

As mentioned, ethanol can be present in the cellulosic biomass in pretreatment step 310. Referring to FIG. 3, pretreatment step or process 310 is performed in a manner to achieve a high degree of hydrolysis of the hemicellulose and a low degree of cellulose hydrolysis to a sugar during pretreatment. The pretreatment process is generally intended to deliver a sufficient combination of mechanical and chemical action to disrupt the fiber structure of the cellulosic feedstock and increase the surface area of the feedstock to make it accessible to cellulase enzymes. Pretreatment step 310 produces pretreated material 316 that is provided to enzymatic hydrolysis process or step 314 where cellulase enzymes are

introduced to the pretreated material to hydrolyze cellulose and/or hemicellulose to a sugar (including xylose, glucose, arabinose, mannose, galactose or a combination thereof). Enzymatic hydrolysis step 314 produces hydrolysate 106 that can be fermented in vessel 108 with yeast 110 propagated in vessel 102 as described above to produce a fermentation product, including ethanol.

At least some of the ethanol in pretreatment step 310 can also be in pretreated material 316 that is provided to enzymatic hydrolysis step 314. Accordingly, enzymatic hydrolysis step 314 is conducted in the presence of ethanol, which results in hydrolysate 106 containing exogenous ethanol that comes from ethanol produced during the ensiling step 304. It is understood that one of ordinary skill can employ known methods to adjust the amount of ethanol in each of the pretreatment step 310 and/or enzymatic hydrolysis step 314 to generate hydrolysate 106 with a certain amount of ethanol. The pretreated material 316 can contain at least 0.1 wt% ethanol, or preferably at least 0.5 wt% ethanol, when it is provided to enzymatic hydrolysis step 314. As described herein, the process described can generate a hydrolysate that contains at least 5 g/L exogenous ethanol.

Turning back to pretreatment step 310, preferably the pretreatment process is performed in a manner to achieve a high degree of hydrolysis of the hemicellulose and a low degree of cellulose hydrolysis to glucose during pretreatment. While pretreatment step 310 can comprise any suitable pretreatment method known to one of ordinary skill, it is preferred that pretreat step 310 comprises an acid pretreatment process that uses alpha-hydroxysulfonic acid because the pretreated material from an alpha-hydroxysulfonic acid may be provided to enzymatic hydrolysis without a washing step and/or a liquid/solid separation step, which typically is conducted to remove cellulase inhibitors generated during acid pretreatment using acid(s) other than alpha-hydroxysulfonic acid. In particular, referring to FIG. 3, an alpha-hydroxysulfonic acid 318 is introduced to ensiled cellulosic biomass 312 in pretreatment step 310.

Alpha-hydroxysulfonic acids have been shown to be effective in the pretreatment and hydrolysis of biomass with the additional benefit of being recoverable and recyclable through reversal to the acids primary components (aldehyde, SO₂ and water). Additional information regarding alpha-hydroxysulfonic acids can be found in US20120122152, the disclosure of which is incorporated herein by reference.

The α -hydroxysulfonic acid is effective for treatment of biomass hydrolyzing the biomass to fermentable sugars like pentose such as xylose at lower temperature, (e.g.,

about 100°C for α -hydroxymethane sulfonic acid or α -hydroxymethane sulfonic acid) producing little furfural in the process. A portion of the cellulose has also been shown to hydrolyze under these comparatively mild conditions. Other polysaccharides such as starch are also readily hydrolyzed to component sugars by α -hydroxysulfonic acids.

5 Further, the α -hydroxysulfonic acid is reversible to readily removable and recyclable materials unlike mineral acids such as sulfuric, phosphoric, or hydrochloric acid. The lower temperatures and pressures employed in the biomass treatment leads to lower equipment cost. The ability to recycle fragile pentose sugars from the end of pretreatment to the inlet of pretreatment, without their subsequent conversion to undesirable materials

10 such as furfural, allows lower consistencies in the pretreatment reaction itself, yet still passing a high consistency solids mixture containing high soluble sugars out of pretreatment. Biomass pretreated in this manner has been shown to be highly susceptible to additional saccharification, especially enzyme mediated saccharification.

Using pretreatment at high temperatures and dilute acid, free xylose is readily

15 dehydrated to form a toxic byproduct, furfural. Thus, in elevated temperature dilute acid processes it is desirable to terminate the pretreatment reaction as soon as the majority of the xylan has been hydrolyzed in order to minimize xylose decomposition. Any free sugars recycled into the front end of an elevated temperature pretreatment process would immediately decompose and result in very high levels of furfurals with no real increase of

20 sugars. This would preclude any attempts at recycling pretreatment liquids to build soluble sugar levels. Thus, in higher temperature, once through pretreatments, the amount of acid solution to “dry weight” biomass introduced in pretreatment determines the ultimate concentration of fermentable sugar obtained. This is balanced by the absorptive nature of biomass with mixing, transport and heat transfer becoming increasingly difficult as the

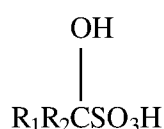
25 relative amount of biomass solids to liquid is increased. The process utilizes low severity conditions (e.g. low temperature) that are possible with pretreatment using higher concentrations of alpha-hydroxysulfonic acids, enabling the recycle and buildup of sugars in the pre-treatment reactor stage. The lower temperature process dramatically reduces the rate of C5 and C6 sugar decomposition to other species such as furfural. Thus, free sugars

30 can be introduced (via recycle) into the front end of a low temperature process and they will pass largely unchanged through pretreatment. This allows buildup of high concentrations of steady state sugars while handling lower consistency in the pretreatment process. The lower temperature has other advantages as if the temperatures are below the

reported lignin melting point, the lignin in the biomass is largely unaltered in texture which results in a non-fouling free flowing pre-treated material. This enables a facile liquid/solid separation at the end of the pretreatment.

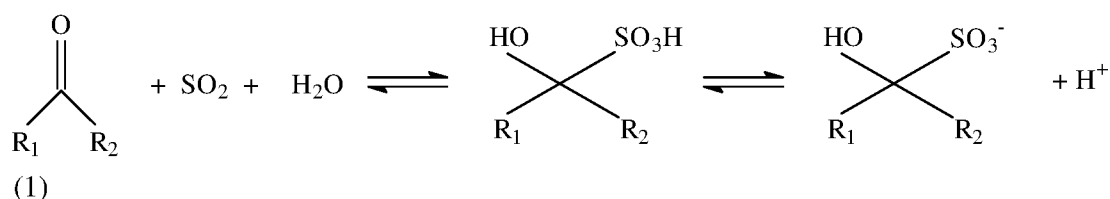
By adding about a molar equivalent amount of a mineral acid (e.g., hydrochloric, sulfuric or phosphoric acid) to a solution of salts of alpha-hydroxysulfonic acids, an equilibrium can be achieved between the protonic and mineral salt versions of the acids. As only the alpha-hydroxysulfonic acid is reversible to volatile components, following Le Chatelier's principle, all of the alpha-hydroxysulfonic acid can be recovered and the salt of the mineral acid is formed.

The α -hydroxysulfonic acids have the general formula



wherein R_1 and R_2 are individually hydrogen or hydrocarbyl with up to about 9 carbon atoms that may or may not contain oxygen can be used in the treatment of the instant invention. The alpha-hydroxysulfonic acid can be a mixture of the aforementioned acids.

The acid can generally be prepared by reacting at least one carbonyl compound or precursor of carbonyl compound (e.g., trioxane and paraformaldehyde) with sulfur dioxide or precursor of sulfur dioxide (e.g., sulfur and oxidant, or sulfur trioxide and reducing agent) and water according to the following general equation 1.



where R_1 and R_2 are individually hydrogen or hydrocarbyl with up to about 9 carbon atoms or a mixture thereof.

Illustrative examples of carbonyl compounds useful to prepare the alpha-hydroxysulfonic acids used in this invention are found where

$\text{R}_1=\text{R}_2=\text{H}$ (formaldehyde)

$\text{R}_1=\text{H}$, $\text{R}_2=\text{CH}_3$ (acetaldehyde)

$\text{R}_1=\text{H}$, $\text{R}_2=\text{CH}_2\text{CH}_3$ (propionaldehyde)

$\text{R}_1=\text{H}$, $\text{R}_2=\text{CH}_2\text{CH}_2\text{CH}_3$ (n-butyraldehyde) $\text{R}_1=\text{H}$, $\text{R}_2=\text{CH}(\text{CH}_3)_2$ (i-butyraldehyde)

$\text{R}_1=\text{H}$, $\text{R}_2=\text{CH}_2\text{OH}$ (glycolaldehyde)

$R_1=H, R_2=CHOHCH_2OH$ (glyceraldehyde)

$R_1=H, R_2=C(=O)H$ (glyoxal)

$R_1=H, R_2=$  (furfural)

$R_1=H, R_2=$

$R_1=H, R_2=$  (salicylaldehyde)

$R_1=H, R_2=$

$R_1=H, R_2=$  (benzaldehyde)

$R_1=R_2=CH_3$ (acetone)

$R_1=CH_2OH, R_2=CH_3$ (acetol)

$R_1=CH_3, R_2=CH_2CH_3$ (methyl ethyl ketone)

$R_1=CH_3, R_2=CHC(CH_3)_2$ (mesityl oxide)

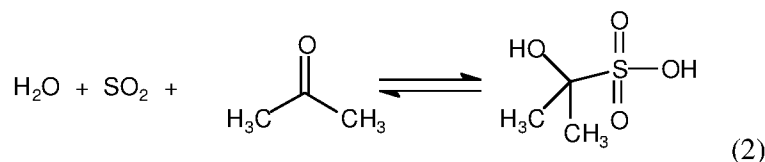
$R_1=CH_3, R_2=CH_2CH(CH_3)_2$ (methyl i-butyl ketone)

$R_1, R_2=(CH_2)_5$ (cyclohexanone) or

$R_1=CH_3, R_2=CH_2Cl$ (chloroacetone)

- 15 The carbonyl compounds and its precursors can be a mixture of compounds described above. For example, the mixture can be a carbonyl compound or a precursor such as, for example, trioxane which is known to thermally revert to formaldehyde at elevated temperatures, methyldehyde which is known to thermally revert to acetaldehyde at elevated temperatures, or an alcohol that may be converted to the aldehyde by
- 20 dehydrogenation of the alcohol to an aldehyde by any known methods. An example of such a conversion to aldehyde from alcohol is described below. An example of a source of carbonyl compounds maybe a mixture of hydroxyacetaldehyde and other aldehydes and ketones produced from fast pyrolysis oil such as described in "Fast Pyrolysis and Bio-oil Upgrading, Biomass-to-Diesel Workshop", Pacific Northwest National Laboratory,
- 25 Richland, Washington, September 5-6, 2006. The carbonyl compounds and its precursors can also be a mixture of ketones and/or aldehydes with or without alcohols that may be converted to ketones and/or aldehydes, preferably in the range of 1 to 7 carbon atoms.

30 The preparation of α -hydroxysulfonic acids by the combination of an organic carbonyl compounds, SO_2 and water is a general reaction and is illustrated in equation 2 for acetone.



The α -hydroxysulfonic acids appear to be as strong as, if not stronger than, HCl since an aqueous solution of the adduct has been reported to react with NaCl freeing the weaker acid, HCl (see U.S. Patent 3549319).

5 The reaction in equation 1 is a true equilibrium, which results in facile reversibility of the acid. That is, when heated, the equilibrium shifts towards the starting carbonyl, sulfur dioxide, and water (component form). If the volatile components (e.g. sulfur dioxide) are allowed to depart the reaction mixture via vaporization or other methods, the acid reaction completely reverses and the solution becomes effectively neutral. Thus, by
 10 increasing the temperature and/or lowering the pressure, the sulfur dioxide can be driven off and the reaction completely reverses due to Le Châtelier's principle, the fate of the carbonyl compound is dependent upon the nature of the material employed. If the carbonyl is also volatile (e.g. acetaldehyde), this material is also easily removed in the vapor phase. Carbonyl compounds such as benzaldehyde, which are sparingly soluble in water, can form
 15 a second organic phase and be separated by mechanical means. Thus, the carbonyl can be removed by conventional means, e.g., continued application of heat and/or vacuum, steam and nitrogen stripping, solvent washing, centrifugation, etc. Therefore, the formation of these acids is reversible in that as the temperature is raised, the sulfur dioxide and/or aldehyde and/or ketone can be flashed from the mixture and condensed or absorbed
 20 elsewhere in order to be recycled. These reversible acids, which are approximately as strong as strong mineral acids, are effective in biomass treatment reactions.

These treatment reactions produce very few of the undesired byproducts, such as furfurals, produced by other conventional mineral acids at higher temperatures. Additionally, since the acids are effectively removed from the reaction mixture following
 25 treatment, neutralization with base to complicate downstream processing is substantially avoided. The ability to reverse and recycle these acids also allows the use of higher concentrations than would otherwise be economically or environmentally practical. As a direct result, the temperature employed in biomass treatment can be reduced to diminish the formation of byproducts such as furfural or hydroxymethylfurfural.

It has been found that the position of the equilibrium given in equation 1 at any given temperature and pressure is highly influenced by the nature of the carbonyl compound employed, steric and electronic effects having a strong influence on the thermal stability of the acid. More steric bulk around the carbonyl tending to favor a lower thermal stability of the acid form. Thus, one can tune the strength of the acid and the temperature of facile decomposition by the selection of the appropriate carbonyl compound.

Various factors affect the conversion of the biomass feedstock in the hydrolysis reaction with α -hydroxysulfonic acids. The carbonyl compound or incipient carbonyl compound (such as trioxane) with sulfur dioxide and water should be added to in an amount and under conditions effective to form alpha-hydroxysulfonic acids. The temperature and pressure of the hydrolysis reaction should be in the range to form alpha-hydroxysulfonic acids and to hydrolyze biomass into fermentable sugars. The amount of carbonyl compound or its precursor and sulfur dioxide should be to produce alpha-hydroxysulfonic acids in the range from about 1 wt.%, preferably from about 5 wt.%, to about 55 wt.%, preferably to about 40 wt.%, more preferably to about 20 wt.%, based on the total solution. For the reaction, excess sulfur dioxide is not necessary, but any excess sulfur dioxide may be used to drive the equilibrium in eq. 1 to favor the acid form at elevated temperatures. The contacting conditions of the hydrolysis reaction may be conducted at temperatures preferably at least from about 50 °C depending on the alpha-hydroxysulfonic acid used, although such temperature may be as low as room temperature depending on the acid and the pressure used. The contacting condition of the hydrolysis reaction may range preferably up to and including about 150 °C depending on the alpha-hydroxysulfonic acid used. In a more preferred condition the temperature is at least from about 80°C, most preferably at least about 100°C or about 125 °C. In a more preferred condition the temperature range up to and including about 90°C to about 120 °C. The reaction is preferably conducted at as low a pressure as possible, given the requirement of containing the excess sulfur dioxide. The reaction may also be conducted at a pressure as low as about 0.1 bara, preferably from about 3 bara, to about pressure of as high as up to 11 bara. The temperature and pressure to be optimally utilized will depend on the particular alpha-hydroxysulfonic acid chosen and optimized based on economic considerations of metallurgy and containment vessels as practiced by those skilled in the art.

The acetaldehyde starting material to produce the alpha-hydroxysulfonic acids can be provided by converting ethanol, produced from the fermentation of the treated biomass of the invention process, to acetaldehyde by dehydrogenation or oxidation. Such processes are described in US20130196400 which disclosure is herein incorporated by reference in
5 its entirety.

A plurality of reactor vessels may be used to carry out pretreatment step 310. These vessels may have any design capable of carrying out a pretreatment reaction. Suitable reactor vessel designs can include, but are not limited to, batch, trickle bed, co-current, counter-current, stirred tank, down flow, or fluidized bed reactors. Staging of
10 reactors can be employed to arrive the most economical solution. The remaining biomass feedstock solids may then be optionally separated from the liquid stream to allow more severe processing of the recalcitrant solids or pass directly within the liquid stream to further processing that may include enzymatic hydrolysis, fermentation, extraction, distillation and/or hydrogenation. A series of reactor vessels may be used with an
15 increasing temperature profile so that a desired sugar fraction is extracted in each vessel. The outlet of each vessel can then be cooled prior to combining the streams, or the streams can be individually fed to the next reaction for conversion.

Suitable reactor designs can include, but are not limited to, a backmixed reactor (e.g., a stirred tank, a bubble column, and/or a jet mixed reactor) may be employed if the
20 viscosity and characteristics of the partially digested bio-based feedstock and liquid reaction media is sufficient to operate in a regime where bio-based feedstock solids are suspended in an excess liquid phase (as opposed to a stacked pile digester). It is also conceivable that a trickle bed reactor could be employed with the biomass present as the stationary phase and a solution of alpha-hydroxysulfonic acid passing over the material.

The reactions described below can be carried out in any system of suitable design, including systems comprising continuous-flow (such as CSTR and plug flow reactors), batch, semi-batch or multi-system vessels and reactors and packed-bed flow-through reactors. For reasons strictly of economic viability, it is preferable that the invention is practiced using a continuous-flow system at steady-state equilibrium. In one advantage of
25 the process in contrast with the dilute acids pretreatment reactions where residual acid is left in the reaction mixture (< 1% wt. sulfuric acid), the lower temperatures employed using these acids (5 to 20% wt.) results in substantially lower pressures in the reactor
30

resulting in potentially less expensive processing systems such as plastic lined reactors, duplex stainless reactors, for example, such as 2205 type reactors.

Enzymatic Hydrolysis

Referring to FIG. 3, as mentioned above, pretreatment step 310 generates pretreated material 312, which is provided to enzymatic hydrolysis step 314, preferably without a washing step and/or a liquid/solid separation step. Enzymatic hydrolysis step 314 may be conducted according to any suitable manner known to one of ordinary skill.

For instance, in enzymatic hydrolysis step 314, one or more cellulase enzymes 318 can be provided to pretreated material 312. Cellulase enzymes 318 can be any type of suitable cellulase enzymes suitable for enzymatic hydrolysis and effective at the pH and other conditions utilized, regardless of their source. Among the most widely studied, characterized and commercially produced cellulases are those obtained from fungi of the genera *Aspergillus*, *Humicola*, *Chrysosporium*, *Melanocarpus*, *Myceliophthora*, *Sporotrichum* and *Trichoderma*, and from the bacteria of the genera *Bacillus* and *Thermobifida*. Cellulase produced by the filamentous fungi *Trichoderma longibrachiatum* comprises at least two cellobiohydrolase enzymes termed CBHI and CBHII and at least four EG enzymes. As well, EGI, EGII, EGIII, EGV and EGVI cellulases have been isolated from *Humicola insolens* (see Lynd et al., 2002, Microbiology and Molecular Biology Reviews, 66(3):506-577 for a review of cellulase enzyme systems and Coutinho and Henrissat, 1999, "Carbohydrate-active enzymes: an integrated database approach." In Recent Advances in Carbohydrate Bioengineering, Gilbert, Davies, Henrissat and Svensson eds., The Royal Society of Chemistry, Cambridge, pp. 3-12, each of which are incorporated herein by reference).

In addition to CBH, EG and beta-glucosidase, there are several accessory enzymes that aid in the enzymatic digestion of cellulose (see co-owned WO 2009/026722 (Scott), which is incorporated herein by reference and Harris et al., 2010, Biochemistry, 49:3305-3316). These include EGIV, also known as glycoside hydrolase 61, swollenin, expansin, lucinen and cellulose-induced protein (Cip). Glucose can be enzymatically converted to the dimers gentiobiose, sophorose, laminaribiose and others by beta-glucosidase via transglycosylation reactions.

An appropriate cellulase dosage can be about 1.0 to about 40.0 Filter Paper Units (FPU or IU) per gram of cellulose, or any amount therebetween. The FPU is a standard measurement familiar to those skilled in the art and is defined and measured according to

Ghose (Pure and Appl. Chem., 1987, 59:257-268; which is incorporated herein by reference). A preferred cellulase dosage is about 10 to 20 FPU per gram cellulose.

The conversion of cellobiose to glucose is carried out by the enzyme β -glucosidase. By the term " β -glucosidase", it is meant any enzyme that hydrolyzes the glucose dimer, cellobiose, to glucose. The activity of the β -glucosidase enzyme is defined by its activity
5 by the Enzyme Commission as EC#3.2.1.21. The β -glucosidase enzyme may come from various sources; however, in all cases, the β -glucosidase enzyme can hydrolyze cellobiose to glucose. The β -glucosidase enzyme may be a Family 1 or Family 3 glycoside hydrolase, although other family members may be used in the practice of this invention. The preferred
10 β -glucosidase enzyme for use in this invention is the Bgl1 protein from *Trichoderma reesei*. It is also contemplated that the β -glucosidase enzyme may be modified to include a cellulose binding domain, thereby allowing this enzyme to bind to cellulose.

Enzymatic hydrolysis step 314 can be conducted at a pH between about 4.0 and 6.0 as this is within the optimal pH range of most cellulases. This includes ranges
15 therebetween having numerical limits of 4.0, 4.25, 4.5, 4.75, 5.0, 5.25, 5.5, 5.75 or 6.0. When the pH of the pretreated cellulosic feedstock is acidic, its pH will typically be increased with alkali to about pH 4.0 to about 6.0 prior to enzymatic hydrolysis, or more typically between about 4.5 and about 5.5. However, cellulases with pH optima at more acidic and more alkaline pH values are known.

The alkali can be added to pretreated biomass material 312 after it is cooled, before cooling, or at points both before and after cooling. The point of alkali addition can coincide with addition of cellulase enzymes 318, or the addition point can be upstream or downstream of the location of the enzyme addition. If the enzyme is added upstream of the alkali addition point, the contact time of the enzyme at the lower pH of the pretreated
20 feedstock would typically be minimized to avoid enzyme inactivation. Without being limiting, it is preferred that alkali is added prior to enzyme addition or simultaneously therewith.

Enzymatic hydrolysis step 314 can be carried out at a temperature that is preferably adjusted so that it is within the optimum range for the activity of the cellulase enzymes. Generally, a temperature of about 45° C. to about 70° C., or about 45° C. to about 65° C.,
30 or any temperature therebetween, is suitable for most cellulase enzymes. For example, the temperature of the reactor content of reactor 102 may be adjusted to about 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64 or 65° C. However, the

temperature of the content of reactor 102 may be higher for thermophilic cellulase enzymes.

Enzymatic hydrolysis step 314 can be carried out for a time period in a range of 2 and 36 hours, between 4 and 24 hours or between 6 and 12 hours.

5 It is understood that enzymatic hydrolysis step 314 may be carried out in any number of suitable reactor vessels that may or may not be connected to one another. The reactor vessel(s) may be operated in batch and/or continuous mode. One of ordinary skill would know how to select the type, number, and arrangement of reactor vessel(s) to carry out enzymatic hydrolysis.

10 Referring to FIG. 3, enzymatic hydrolysis step 314 generates hydrolysate 106 that comprises ethanol, particularly at least 10 g/L, which had been generated during ensiling process 304 prior to pretreatment step 310 and enzymatic hydrolysis step 314. Referring to FIGS. 1 – 3, hydrolysate 106 generated as shown in FIG. 3 can be fermented as shown in FIGS. 1 – 2 where a portion of hydrolysate 106 is used as part of 104 propagation medium
15 and/or optional pre-propagation growth medium 104A.

Accordingly, the present disclosure provides a method for propagating a fermentation microorganism for fermentation comprising:

(a) providing a hydrolysate for fermentation, wherein the hydrolysate comprises at least 10 g/L ethanol and wherein the hydrolysate is generated according to a method
20 comprising:

- ensiling a cellulosic biomass material for at least 24 hours to generate ethanol in the cellulosic biomass material;

- pretreating the ensiled cellulosic biomass material with an alpha-hydroxysulfonic acid to produce a pretreated material comprising ethanol; and

- 25 - introducing one or more cellulases to the pretreated material to produce the hydrolysate;

- (b) providing a propagation reactor containing:

- a propagation medium comprising: the hydrolysate in an amount of at least 25% by volume and glucose in an amount of greater than 14 g/L; and

- 30
 - a first cell mass of a fermentation microorganism; and

- (c) propagating the first cell mass in the propagation medium for a time period to form a propagated culture comprising a second cell mass of the microorganism that is greater than the first cell mass.

The present disclosure also provide a method for generating a hydrolysate comprising:

- (a) pretreating an ensiled cellulosic biomass material comprising at least 2 wt% of ethanol,
- 5 wherein the ensiled cellulosic biomass material is generated by adding at least one of a microbe and an acid to cellulosic biomass to produce a prepared cellulosic biomass material and storing the prepared cellulosic biomass material for at least 24 hours to generate the ensiled cellulosic biomass material, and
- 10 wherein the pretreating comprises contacting the ensiled cellulosic biomass material with a solution containing an acid thereby hydrolyzing the ensiled cellulosic biomass material to produce a pretreated product comprising ethanol and at least one fermentable sugar;
- wherein the ensiled cellulosic biomass material comprises ethanol when it is contacted with the acid solution; and
- 15 contacting the pretreated product with one or more cellulases to produce the hydrolysate, wherein the pretreated product comprises ethanol when it is contacted with the one or more cellulases.

While the invention is susceptible to various modifications and alternative forms, specific embodiments thereof are shown by way of examples herein described in detail. It

20 should be understood, that the detailed description thereto are not intended to limit the invention to the particular form disclosed, but on the contrary, the intention is to cover all modifications, equivalents and alternatives falling within the spirit and scope of the present invention as defined by the appended claims. The present invention will be illustrated by the following illustrative embodiment, which is provided for illustration only and is not to

25 be construed as limiting the claimed invention in any way.

The person skilled in the art will readily understand that, while the invention is illustrated making reference to one or more a specific combinations of features and measures, many of those features and measures are functionally independent from other features and measures such that they can be equally or similarly applied independently in

30 other embodiments or combinations.

ILLUSTRATIVE EMBODIMENTS

The following Examples 1 – 2 demonstrate the different propagation conditions and subsequent results of fermentation of hydrolysate using the yeast propagated under those conditions.

Sweet sorghum was harvested, and *S. cerevisiae* and sulfuric acid were added to the harvested material as described above to generate prepared sweet sorghum. The prepared sweet sorghum was ensiled as described for 43 days to generate ensiled sweet sorghum. The ensiled sweet sorghum was contacted with a solution containing about 5 wt% alpha-hydroxyethane sulfonic acid under pretreatment temperature of about 120 °C for a time period of about 1 hour to generate pretreated biomass material. The pH of the pretreated biomass was adjusted to 5.3 using 28-30% NH₄OH. The pretreated biomass was not subject to a washing and/or liquid/solid separation step. Enzymatic hydrolysis of the pretreated biomass was then conducted in a 5-L reactor for 96 hours at 53 °C by adding commercially available cellulase enzymes, particularly CTec3, to generate a hydrolysate. Table 2 below provides compositions of four Hydrolysates, which are labeled as Hydrolysates A – D, which were produced according to embodiments described herein. As can be seen, the process generates hydrolysates having at least 5 g/L exogenous ethanol, particularly here, an average of about 14 g/L exogenous ethanol.

Table 2

	Glu	Xylo	Lacti	Aceti	Ethan
Hydrol	93.	61.8	5.2	13.4	14.9
Hydrol	94.	57.4	3.7	13.2	13.8
Hydrol	98.	59.1	4.7	12.6	14.0
Hydrol	96.	58.7	3.8	13.0	13.0
Averag	95.	59.2	4.4	13.0	13.7

Hydrolysates A – D have about 26% total solids as measured by the AACC Method 44-01.01 Calculation of Percent Moisture method.

Examples 1 – 2 uses a *S. cerevisiae* yeast strain, which was a xylose and glucose fermenting yeast and commercially available. This yeast strain was propagated in Examples 1 – 2 to produce an amount to inoculate fermentation of Hydrolysate D.

Example 1 is a control experiment where the propagation medium is 100% YPD20. The propagation medium for Example 2 is 50% YPD20 and 50% Hydrolysate D. YPD is known to one of ordinary skill as a common medium in which to grow yeast that

includes yeast extract, peptone, and dextrose and the numeral “20” designates the amount of glucose in the medium, which was 20 g/L. The pre-propagation of yeast cultures in Examples 1 – 2 included re-suspending about five *S. cerevisiae* colonies from an agar plate in fresh YPD to form a homogenous mixture, which was used to inoculate the pre-propagation medium, which may or may not include the hydrolysate. The pre-propagation culture was then grown for about 12 hours – 19 hours at 30 degrees C and 200 rpm in a baffled, vented flask with volume ratio of medium:flask of 1:5. The optical density of the respective pre-propagation culture was then measured and used to calculate the volume required to initiate a propagation culture at an initial optical density at OD₆₀₀ = 0.05. The pre-propagation medium had was done with 75% YPD20 and 25% Hydrolysate D.

The propagation culture(s) for Examples 1 – 2 were grown at 30 degrees C and 200 rpm for 12 – 18 hours to generate sufficient biomass for inoculation of the hydrolysate fermentation. It is understood that the concentrations of glucose, xylose, lactic acid, acetic acid, and ethanol in the hydrolysate-containing propagation medium can be calculated by multiplying the percent of hydrolysate used.

The respective propagated culture was used for fermentation of Hydrolysate 4. The portion of the respective Hydrolysate used for the pre-propagation medium and propagation medium was centrifuged then filtered through glass fiber filters to remove large particulate matter followed by filter sterilization via a 0.45 µm Nalgene filter.

Fermentation was performed in 125 mL baffled, one-way vented shake flasks using a cell pitch of 1 g (DW)/L at 32°C and initial pH of 5.8 adjusted using 8 – 10 N KOH. One mL samples were taken from the flasks for High Performance Liquid Chromatography (HPLC) analysis using an HPX 87H Biorad column at 65°C with 0.005% sulphuric acid mobile phase with a flow rate of 0.6 mL/min. Samples were diluted 10 fold in 10 mM sulphuric acid and run at 20µL injections. Yield calculations were performed on the consumed sugar assuming a theoretical conversion of glucose and xylose to ethanol of 0.51 g/g.

In Example 1, the control example, there was no ethanol in the initial propagation medium and the glucose amount was about 20 g/L. In Example 2, the ethanol concentration in the initial propagation medium prior to propagation was about 6 g/L and the glucose amount was about 60 g/L. As shown in FIG. 4, about 8 g/L of ethanol was produced during propagation in Example 1 and about 30 g/L of ethanol was produced during propagation in Example 2. FIG. 5 shows the ethanol yield from fermentation in

Examples 1 and 2 measured at 24 hours, 48 hour, and 72 hour, which shows an improved ethanol yield in Example 2 as compared to control Example 1. In particular, the 24-hour ethanol yield in Example 1 was 9 g/L as compared to 56 g/L in Example 2. The 48-hour ethanol yield in Example 1 was 41 g/L as compared to 62 g/L in Example 2. The 72-hour ethanol yield in Example 1 was 59 g/L as compared to 63 g/L in Example 2.

The ethanol yield in Example 2 as compared to the control Example 1 demonstrates that propagating a fermentation organism, e.g., yeast, according to embodiments described herein results in improved fermentation product yield over the control. It is expected that the difference between the yeast propagated according to methods described herein and the control would be greater for hydrolysates that contain ethanol amounts of greater than about 13 – 14 g/L ethanol. For instance, the difference in yields between Examples 1 – 2 at 72-hour fermentation was 4 g/L. It is expected that this difference can increase to greater than 5 g/L or greater than 10 g/L if the hydrolysate contains about 20 g/L endogenous ethanol where propagating the yeast as described herein provides better fermentation performance as the endogenous ethanol inhibitor level goes up. For instance, this difference can increase to greater than 15 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L, 40g/L, 45 g/L, or 50 g/L as the endogenous ethanol amount increases to 25 g/L, 30 g/L 35 g/L, 40 g/L, or greater.

Example 3 – Ensiling Process

The following examples demonstrate the ethanol production capabilities of the ensiling process described herein. In this example, various samples of fresh chopped sorghum are mixed with a variety of added components as listed in Table 3 and are stored in silage tubes for 258 days. The amount of ethanol produced in each experiment is shown in the bottom row of the table. The addition rates of selected additives are shown in Table 4.

ble 3

Experiments	WITHOUT ACID	WITHOUT ACID	WITHOUT ACID	WITHOUT ACID	WITHOUT ACID	WITH ACID	WITH ACID	WITH ACID
Plot #	1	2	3	4	5	6	7	8
Seed Mass	20 tonnes	20 tonnes	20 tonnes	20 tonnes	20 tonnes	20 tonnes	20 tonnes	20 tonnes
Seed Content	68%	68%	68%	68%	68%	68%	68%	68%
Harvest Method	Silage Tube	Silage Tube	Silage Tube	Silage Tube	Silage Tube	Silage Tube	Silage Tube	Silage Tube
Harvest	Ethanol Red	Ethanol Red	Ethanol Red	Ethanol Red	Ethanol Red	Ethanol Red	Ethanol Red	Ethanol Red
Harvest			BioSaf	BioSaf	BioSaf			BioSaf
Inhibitor	Lactrol	Lactrol	Lactrol	Lactrol	Lactrol	Lactrol	Lactrol	Lactrol
Enzyme		Cellulase CE-2		Cellulase CE-2	Cellulase CE-2		Cellulase CE-2	Cellulase CE-2
Glucose		Amylase		Amylase	Amylase		Amylase	Amylase
Enzyme Accessories					Liquicell 2500			
Yield (initial dry weight)	36	48	35	35	46	36	45	36

Table 4

ADDITIVE Rates		
LACTROL	1.6	g/wet tonne
Ethanol Red	0.11	kg/wet tonne
BioSaf	0.11	kg/wet tonne
Cellulase CE-2	0.22	kg/wet tonne
Amylase	0.11	kg/wet tonne
Liquicell	0.11	kg/wet tonne
93% Concentrated Sulfuric Acid	0.42	L/wet tonne

The experiments of Example 3 demonstrated the principle of ethanol production in silage piles and the duration of that storage. Further, they demonstrated effects of certain additive. The bottom row of Table 3 describes the result in terms of ethanol production in the respect experiments of Example 3. All experiments in the example produced a significant amount of ethanol, demonstrating the ethanol production capabilities of the ensiling process described herein. In general, the experiments with acid showed superior stability to those without acid. Nevertheless, experiments without acid still yielded ethanol production, indicating that an acid additive is optional.

Example 4 – Ensiling Process

In Example 4, three additional experiments are shown in Table 5. The addition rates of selected additives are shown in Table 6.

Table 5

2011 Experiments	WITH ACID	WITH ACID	WITH ACID
Experiment #	1	2	3
Estimated Mass	450 tonnes	450 tonnes	100 tonnes
Moisture Content	76%	76%	69%
Storage Method	Bunker	Bunker	Silage Tube
Yeast	Lallemand Liquid Yeast	Lallemand Liquid Yeast	Lallemand Liquid Yeast
Bacterial Inhibitor	Lactrol	Lactrol	Lactrol
Cellulose to Glucose	Novozymes Cellic CTec2	Novozymes Cellic CTec2	Novozymes Cellic CTec2
Chop Size	3 mm	13 mm	13 mm
Result (gallons Ethanol/initial dry ton)	50	51	48
Days in Storage	330	330	315

Table 6

ADDITIVE	Rates
LACTROL	3.2 g/wet ton
Lallemand Stabilized Liquid Yeast	18 fl oz/wet ton
Novozymes Cellic CTec2	20 fl oz/wet ton
9.3% Concentrated Sulfuric Acid	3.8 L/wet ton

The experiments of Example 4 also demonstrated the effects of certain additives, as well as the effects of scale. Experiments 1 and 2 of Example 4 were conducted in the same
5 bunker demonstrating that this fermentation technology is stable and efficient at commercial scale.

Therefore, the present invention is well adapted to attain the ends and advantages mentioned as well as those that are inherent therein. The particular embodiments disclosed above are illustrative only, as the present invention may be modified and practiced in different
10 but equivalent manners apparent to those skilled in the art having the benefit of the teachings herein. Furthermore, no limitations are intended to the details of construction or design herein shown, other than as described in the claims below. It is therefore evident that the particular illustrative embodiments disclosed above may be altered, combined, or modified and all such variations are considered within the scope and spirit of the present invention. The invention
15 illustratively disclosed herein suitably may be practiced in the absence of any element that is not specifically disclosed herein and/or any optional element disclosed herein. While compositions and methods are described in terms of “comprising,” “containing,” or “including” various components or steps, the compositions and methods may also “consist essentially of” or “consist of” the various components and steps. All numbers and ranges
20 disclosed above may vary by some amount. Whenever a numerical range with a lower limit and an upper limit is disclosed, any number and any included range falling within the range is specifically disclosed. In particular, every range of values (of the form, “from about a to about b,” or, equivalently, “from approximately a to b,” or, equivalently, “from approximately a-b”) disclosed herein is to be understood to set forth every number and range encompassed within
25 the broader range of values. Also, the terms in the claims have their plain, ordinary meaning unless otherwise explicitly and clearly defined by the patentee. Moreover, the indefinite

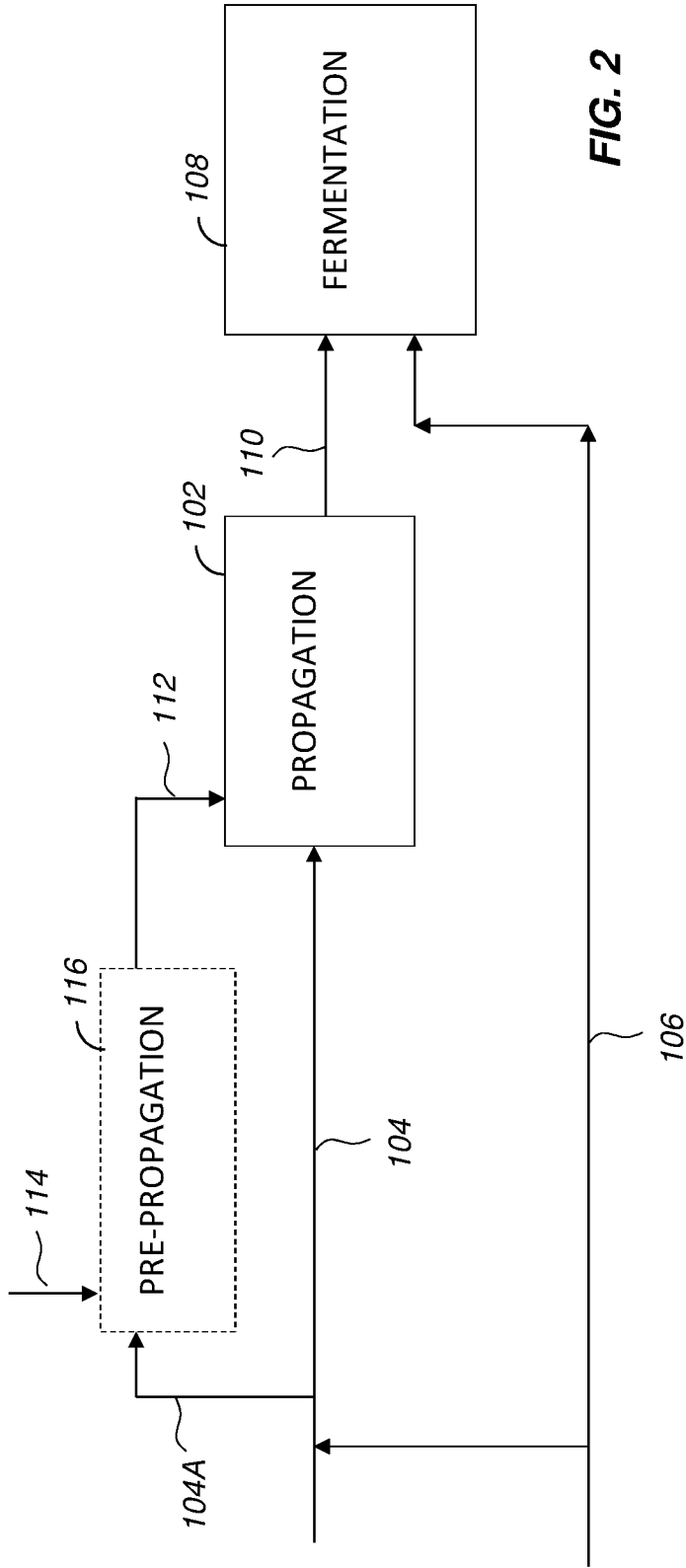
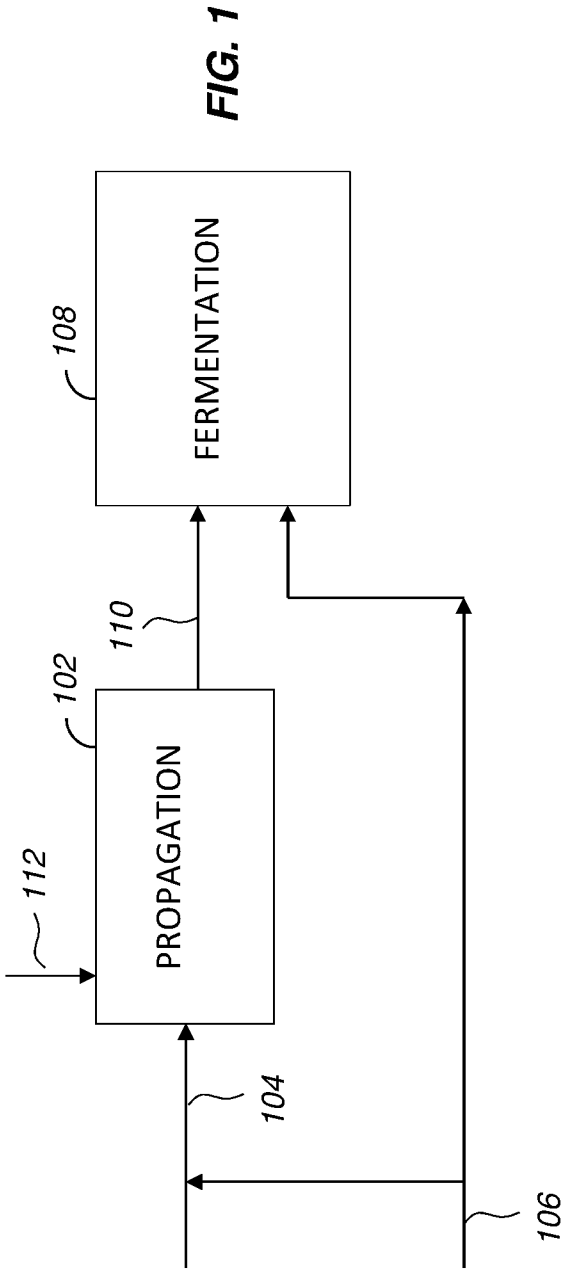
articles “a” or “an,” as used in the claims, are defined herein to mean one or more than one of the element that it introduces. If there is any conflict in the usages of a word or term in this specification and one or more patent or other documents that may be incorporated herein by reference, the definitions that are consistent with this specification should be adopted.

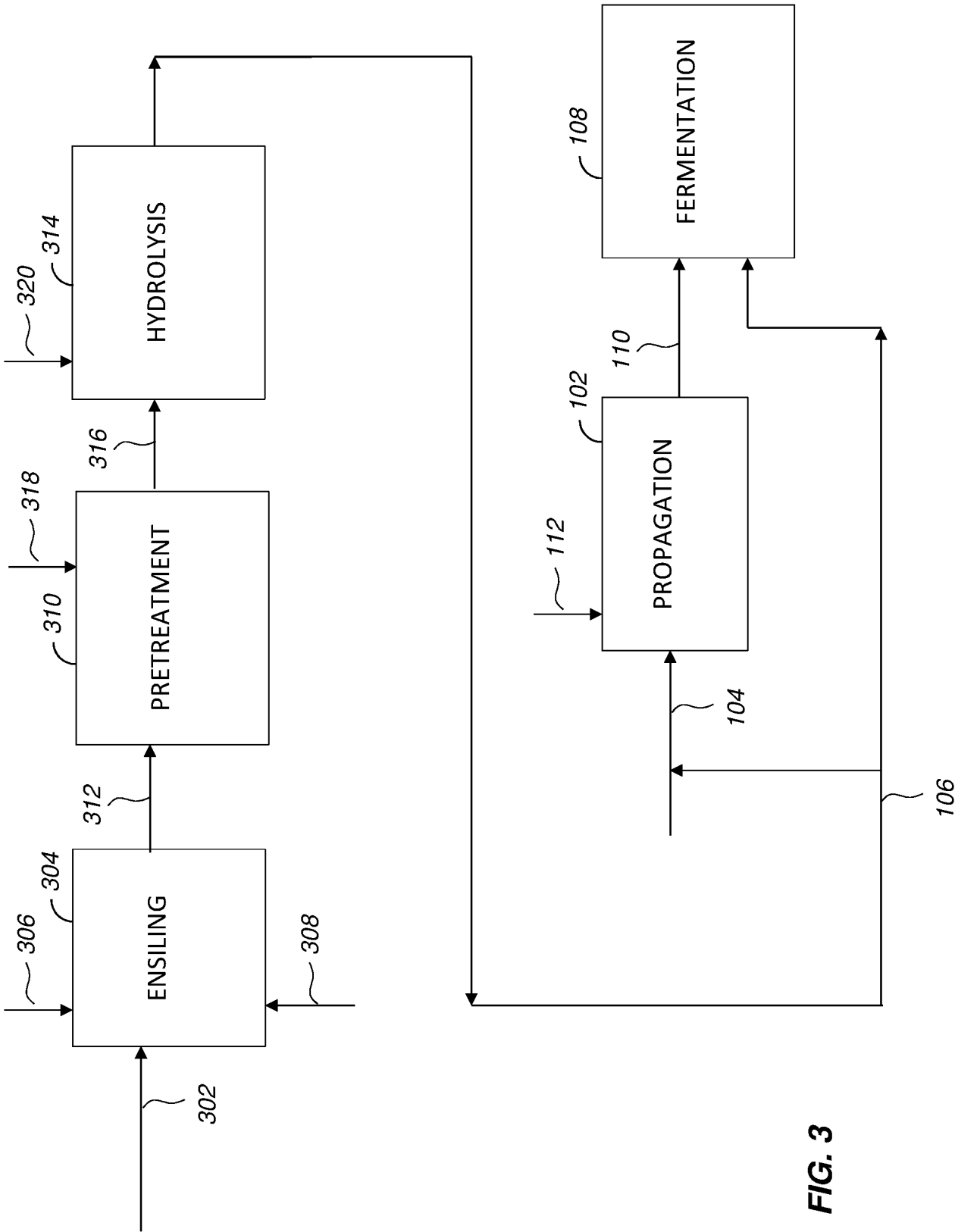
CLAIMS

1. A method of propagating a fermentation microorganism comprising:
 - (a) providing a hydrolysate for fermentation, wherein the hydrolysate comprises exogenous ethanol in an amount of at least 10 g/L;
 - 5 (b) providing a propagation reactor containing:
 - a propagation medium comprising: the hydrolysate in an amount of at least 25% by volume and glucose in an amount of greater than 14 g/L; and
 - a first cell mass of a fermentation microorganism; and
 - (c) propagating the first cell mass in the propagation medium for a time period to form
- 10 a propagated culture comprising a second cell mass of the fermentation microorganism that is greater than the first cell mass.
2. The method of any preceding claim wherein at least 15 g/L of endogenous ethanol is produced by the fermentation microorganism during propagation.
3. The method of any preceding claim wherein the hydrolysate contains at least 20% total
- 15 solids (TS).
4. The method of any preceding claim wherein the fermentation organism comprises a yeast.
5. The method of any preceding claim wherein the hydrolysate comprises at least 14 g/L exogenous ethanol.
- 20 6. The method of any preceding claim wherein the hydrolysate comprises at least 20 g/L exogenous ethanol.
7. The method of any preceding claim wherein the propagation medium comprises at least 50% hydrolysate by volume.
8. The method of any preceding claim wherein the propagation medium comprises at
- 25 least 75% hydrolysate by volume.
9. The method of any preceding claim further comprising:
 - providing the second cell mass of microorganism to the hydrolysate in a fermentation reactor; and

growing the microorganism in the fermentation reactor under anaerobic conditions to produce a fermentation product.

10. The method of any preceding claim further comprising a pre-propagation step, wherein the first cell mass provided to the propagation reactor has been grown in a pre-propagation reactor in a pre-propagation medium comprising hydrolysate in an amount of at least 25% by volume and glucose in an amount of greater than 14 g/L.





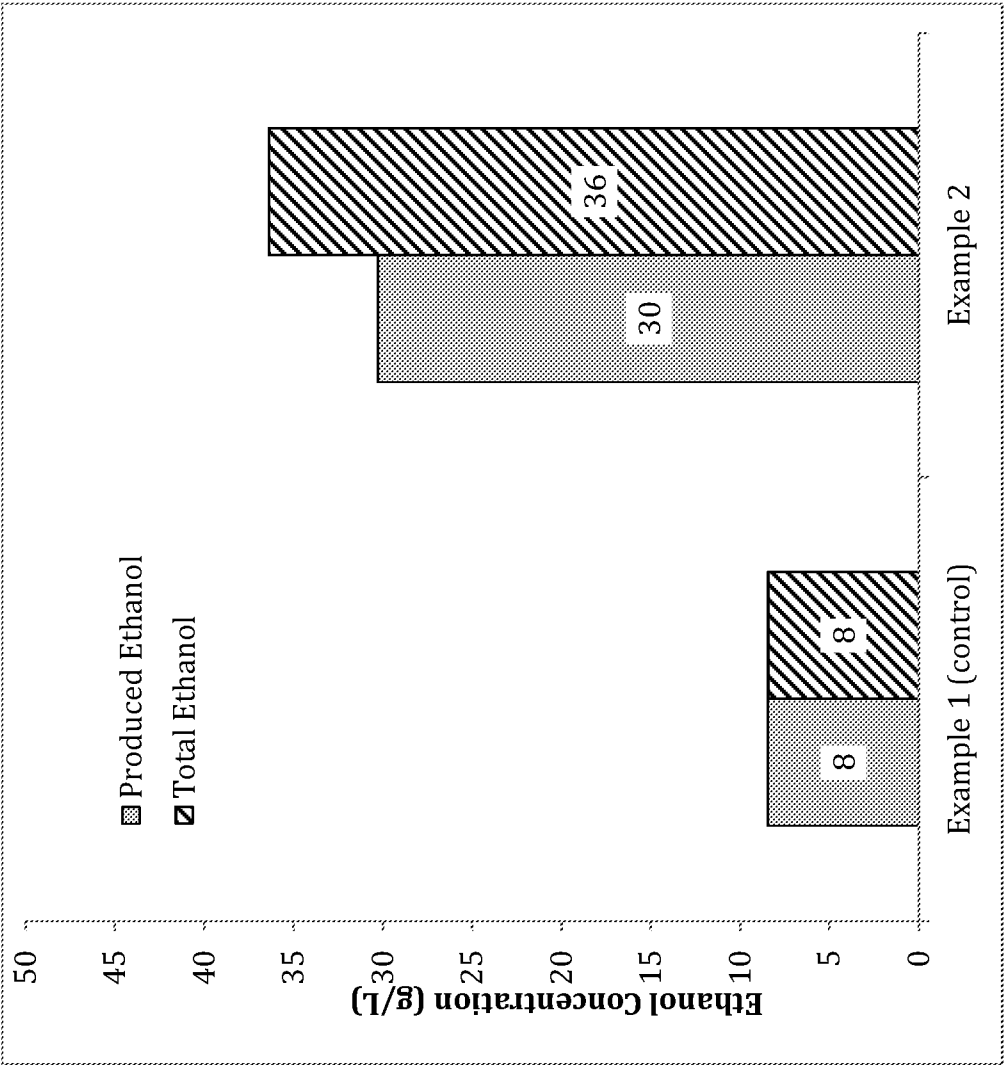


FIG. 4

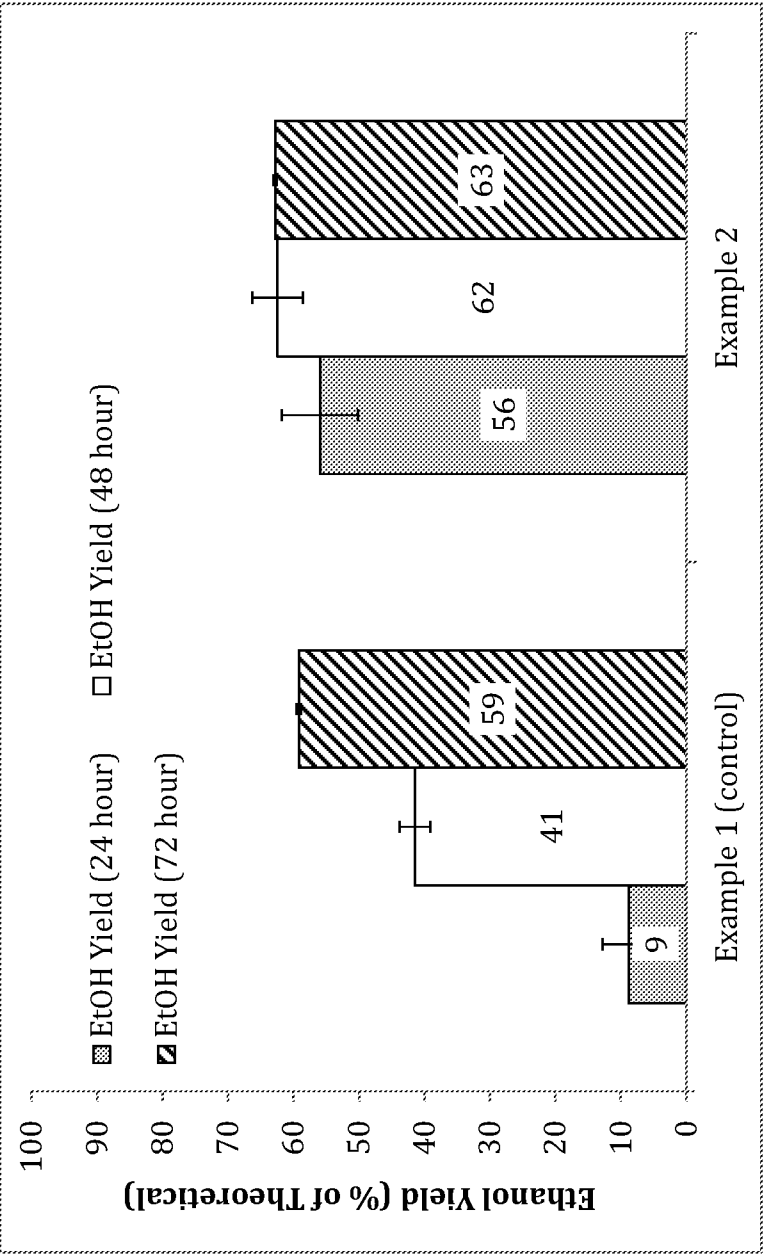


FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/66480

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12P 7/06, 7/08, 7/10; C12N 1/22, 1/32 (2017.01)

CPC - C12P 7/06, 7/08, 7/10; C12N 1/22, 1/32; Y02E 50/17

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)

See Search History document

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

See Search History document

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,242,454 A (MULLER, WC et al.) 30 December 1980; column 3 lines 16-18, column 4 lines 2-5, 8-12	1, 2/1, 3/1-2
Y	US 2011/0014670 A1 (CAIMI, PG et al.) 20 January 2011; paragraphs [0037], [0065]	1, 2/1, 3/1-2
Y	US 2004/0063184 A1 (GRICHKO, V) 1 April 2004; paragraphs [0023], [0141]	1, 2/1, 3/1-2

☐ 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

09 February 2017 (09.02.2017)

Date of mailing of the international search report

17 FEB 2017

Name and mailing address of the ISA/

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/66480

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-10
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.