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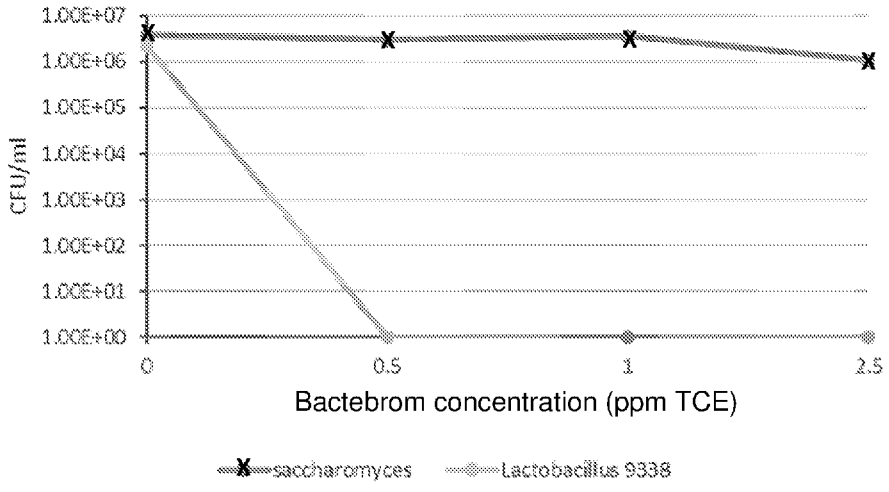


Figure 1

(57) Abstract: The present disclosure provides a method for controlling contaminations of prokaryotes known to reside alongside yeast during fermentation processes, e.g., bacterial contaminations, by using an oxidizing bromine-based biocide. The method enables purifying yeast cultures prone to prokaryotic contamination, while maintaining the viability of the yeast, thereby increasing the yield of ethanol production.

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# METHOD FOR CONTROLLING PROKARYOTIC CONTAMINATION IN YEAST FERMENTATION PROCESSES

## FIELD OF THE INVENTION

The invention provides a method for controlling contaminations of prokaryotes known to reside alongside yeast during fermentation processes, e.g., bacterial contaminations. The method leads to an increase in the yield of end product production, e.g., ethanol, while maintaining the viability of the yeast.

## BACKGROUND OF THE INVENTION

Ethanol-based biofuel (also termed "bioethanol") is considered to be among the sustainable fuels in many countries. It can be used directly as pure ethanol or may be blended with gasoline. Bioethanol is less toxic, readily biodegradable and produces air-borne pollutants to a lesser extent than petroleum fuel.

Conventional production methods of ethanol use a variety of carbohydrate substrates, e.g., sugar (derived, for example, from sugar cane and sugar beet), starch (derived, for example, from corn, wheat, or potatoes) or other polysaccharides. Monosaccharide sugars are converted into ethanol mostly by varieties of the yeast *Saccharomyces cerevisiae* during a fermentation process.

Generally, the fermentation process involves contacting a carbohydrate substrate-rich medium with yeast and fermenting it until the substrate is consumed, or until the yeast is inhibited by the increase in ethanol concentration. In many cases, the fermenting yeast are discarded and fresh *inoculi* are used for each cycle of fermentation. In contrast, distilleries in Brazil have adopted a fermentation process during which the yeast cells are recycled (also referred to herein as the "Brazilian process of ethanol production"). At the end of each fermentation cycle of the Brazilian process for ethanol production, fermented broth is processed to separate the yeast cells in the form of a concentrated cream from the liquid that undergoes further distillation. After being treated with water-diluted sulfuric acid (at a pH of 2.0-2.5, for 1-2 hours), these yeast

cells are returned to large-volume fermentation tanks (250-3000 L) for a new fermentation cycle (Lopes et al. *Ethanol production in Brazil: a bridge between science and industry*. Brazilian Journal of Microbiology: 47S: 64-76. 2016). Overall, the Brazilian process of ethanol production results in a better ethanol yield, since less sugar is consumed for yeast cell multiplication. However, bacterial contaminants are also recycled into the fermentation process and may be troublesome, for example due to substrate competition, thereby reducing the overall ethanol yield during fermentation.

As known in the art, bacteria cause various additional problems during fermentation, generally leading to inhibition of yeast fermentation and reduction of industrial yields. These problems include, for example, production of organic acids and stimulating flocculation of the yeast cells. One of the particular challenges is the control of contamination by lactic and acetic acid bacteria growth, such as bacteria of the genus *Lactobacillus sp* and *Acetobacter sp*., which produce lactic acid or acetic acid instead of ethanol.

In order to reduce the level of bacterial contaminants, the Brazilian process of ethanol production uses dilute sulfuric acid to kill bacteria, as detailed above. Without this acidic treatment step, industrial fermentations are subjected to sugar losses and reduced ethanol yield due to the presence of bacterial contamination.

Currently, there are various methods employed to selectively eliminate bacterial contaminants without harming the yeast during fermentation, among which is by using antibiotics. The most commonly used antibiotics are penicillin, virginiamycin, erythromycin, tylosin, tetracycline and monensin. However, there are several concerns involving the use of antibiotics, for example, the emergence of resistant bacteria due to the use of antibiotics. This concern arises in particular due to the presence of antibiotics residues in dried distillers' grains (DDG), a nutrient-rich co-product of ethanol production derived from starting material such as corn and sold as livestock feed. Various countries do not accept any antibiotic residues in inactive dried yeast for animal feed, thus, it is desirable to avoid the use of antibiotics.

Various other methods are used to overcome the problems relating to bacteria contamination during ethanol production (fermentation). One of the most commonly used methods is the application of a biocide during the steps of the fermentation process. For example, the publication US 2012/0009641 (by Kulkarni *et al.*) suggests a method including a pretreatment in which the sugar cane is washed with water comprising a biocide. Additionally, biocides such as quaternary ammonium compounds, carbamates, glutaraldehyde, and halogenated non-oxidizing organic biocides are among the proposed possible solutions (disclosed in, *inter alia*, US 2011/0027846, and US 2010/0297719). In addition, chlorine dioxide and hop acids derivatives (alpha and beta fraction) are among the new antimicrobials used. Chlorine dioxide ($ClO_2$) is a commonly used oxidative biocide, which has been suggested for the fermentation processes, e.g., in US 9,926,576, however, it suffers from several disadvantages. First, being a strong oxidizing agent, $ClO_2$ lacks selectivity and while eliminating the bacteria it may also harm the yeast and reduce its viability, hence leading to an overall reduction in ethanol yield. Furthermore, use of $ClO_2$ is associated with safety concerns, as production of $Cl_2$ gas may cause an explosion. A further proposed alternative for using antibiotics is a combination of a non-oxidizing biocide, a stabilized oxidizing biocide and an antibacterial peptide (WO 2011/116042).

Furthermore, the publication US 2003/0228373 relates to a composition for inhibiting microbial growth in industrial waters, including triamine and a biocide which is an oxidizing biocide (e.g., a brominated agent), a non-oxidizing biocide or a combination thereof. Additionally, publication WO 2020/240559 discloses methods for microbial control with bromine-based compounds and cis-2-decenoic acid.

## SUMMARY OF THE INVENTION

It has now been unexpectedly shown that it may be possible to control prokaryotic contamination in a yeast culture using an oxidizing bromine-based biocide, which is an on-site oxidation product of an inorganic bromide source that is a solution comprising hydrobromic acid, or a metal bromide salt. Thus, the oxidizing "active" form of the bromine-based biocide is obtained on-site, e.g., by contacting bromine-based compositions (e.g., solution comprising hydrobromic acid, or a metal bromide salt

which may be regarded as "pre-biocides") with an oxidizer, as described in greater detail below. Experimental work conducted in support of this invention in laboratory models examined the effect of the oxidizing bromine-based biocide which is a solution of hydrobromic acid and urea ("Bactebrom™ Solution", manufactured by ICL, https://www.icl-ip.com/product/bactebrom-solution/, also referred to herein as "Bactebrom™"), activated on-site with sodium hypochlorite to provide "activated Bactebrom™" (also referred to herein as the active biocide form) on the viability of various bacterial species (namely, *L. fermentum*, *L. plantarum* and *E. coli*) vis-à-vis the effect thereof on yeast viability (*S. cerevisiae*). Unless the context clearly indicates otherwise, the term "Bactebrom™" as used herein, particularly in reference to its biocidal properties and activity, refers to "activated Bactebrom™" and is used interchangeably with this term.

As detailed above, bacterial contaminants are abundant in alcohol fermentation reactors, affecting yeast performance and alcohol yield. The laboratory models detailed herein seek to mimic the microorganism environment present during alcohol fermentation. Surprisingly, the experiments conducted demonstrate that the activated Bactebrom™ is an effective anti-bacterial agent against the tested bacterial species, as a single biocidal agent. Remarkably, at the tested biocidal dosage of 0.5 and 1 ppm of total chlorine equivalent (herein: "ppm TCE", as elaborated below), there was a negligible effect on the viability of the tested yeast (*S. cerevisiae*) at a variety of pH values. Furthermore, even at a dosage of 2.5 ppm TCE, activated Bactebrom™ had a marginal effect on the yeast tested, while demonstrating an anti-bacterial effect. It is noted that the terms "ethanol" and "alcohol" are used herein interchangeably, both referring to ethyl alcohol.

The effect of activated Bactebrom™ at 2.5 and 5 ppm TCE on yeast viability was further evaluated by optical microscopy analysis, as detailed below, which demonstrated that activated Bactebrom™ did not affect the yeast viability and even had a beneficial effect thereon. It is also worth noting that while the active form of Bactebrom™ selectively affected bacteria without harming the yeast, the non-oxidizing biocide 2,2-dibromo-3-nitrilopropionamide (DBNPA) generally showed negligible effect on both bacterial strains and the yeast.

Therefore, microbial control is achieved using an oxidizing bromine-containing biocide as described herein, leading to selective decontamination of prokaryotic growth during the fermentation process, e.g. bacterial growth, with limited effect on yeast viability, thereby reducing or eliminating the need for the application of antibiotics during fermentation procedures.

In other words, the present invention provides a method for selective bacterial decontamination and/or control during the fermentation process. The method comprises fermenting a fermentable substrate and yeast in the presence of at least one bromine-based oxidizing biocide as defined herein, to produce ethanol and solids content, wherein the oxidizing biocide may be present as the sole biocide and controls growth of bacteria without substantially affecting yeast population, and wherein the method further comprises distilling the fermented substrate to separate at least a portion of the ethanol from the solid content produced during fermentation. The method according to the invention provides a sustainable solution, by reducing or eliminating altogether the use of antibiotics during fermentation.

Therefore, by one of its aspects the present invention provides a method for producing ethanol by fermentation prone to prokaryotic contamination, said method comprising:

a) providing a fermentable substrate;

b) combining said fermentable substrate with yeasts in presence of water to obtain a fermentation broth; and

c) fermenting said fermentation broth to obtain a fermented broth;

wherein the process further comprises contacting the yeasts with at least one oxidizing bromine-based biocide, obtained by on-site oxidation of an inorganic bromide source, wherein the bromide source is a solution comprising hydrobromic acid or a metal bromide salt.

In some embodiments the method according to the invention further comprises

d) separating said fermented broth into spent yeasts and ethanol-containing liquid; and optionally

e) recovering said spent yeast,

wherein the recovering step may comprise contacting said spent yeast with said oxidizing bromine-based biocide and optionally with dilute sulfuric acid to obtain recovered yeast.

In some embodiments the method according to the invention is performed essentially in the absence of antibiotics. Alternatively, some amount of antibiotics may be added as needed, as substantially described herein. In specific embodiments, the antibiotic is added in an amount of below 70% of the amount needed to control the contamination under identical conditions, but without the use of oxidizing bromine-based biocide.

In particular, the method according to the invention is applicable wherein the inorganic bromide source is selected from a solution comprising HBr and/or alkali metal bromide.

The on-site oxidized inorganic bromide source may be selected from the group consisting of HBr; a solution of HBr and urea; and sodium bromide. The inorganic bromide source may be oxidized on-site with any one of hypochlorite, chlorine, hydrogen peroxide, ozone, or electrochemically to produce its active form (i.e., the oxidizing bromine-based biocide). In particular, the method according to the invention is applicable wherein said oxidizing bromine-based biocide is an aqueous solution of hydrobromic acid and urea, which reacts with hypochlorite on-site, to produce the oxidizing active bromine species.

The method according to the invention is particularly applicable to inhibition of prokaryotic contamination arising from lactic acid bacteria, for example of the genera (genus) *Lactobacillus* (e.g., *Lactobacillus fermentum* or *Lactobacillus plantarum* or a combination thereof*), E. coli* or any combination thereof.

Preferably, the method according to the invention is applicable wherein said recovered yeast are used for at least one additional fermentation cycle(s).

In some embodiments the method according to the invention is wherein said at least one oxidizing bromine-based biocide is applied or added at a dosage level of 0.2 to 25 ppm TCE. Specifically, depending on the yeasts' strain, said at least one oxidizing

bromine-based biocide is applied at a dosage level of 0.2 to 10 ppm TCE, of 0.2 to 7 ppm TCE, or of 0.2 to 5 ppm TCE, e.g. of 0.5 – 5 ppm TCE.

The present disclosure further provides a method for purifying a yeast culture prone to prokaryotic contamination, said method comprising contacting said yeast culture with at least one oxidizing bromine-based biocide, as herein defined, obtained by on-site oxidation of an inorganic bromide source which is a solution comprising hydrobromic acid, or a metal bromide salt.

The at least one oxidizing bromine-based biocide as herein defined may be added at a continuous or an intermittent mode. In some embodiments, contacting the yeasts with the at least one oxidizing bromine-based biocide as herein defined is performed during fermenting of said fermentation broth.

In some embodiments, the method as herein defined is applicable to the yeast *S. cerevisiae*, preferably selected from *S. cerevisiae* strains PE-2 strain, S288c, baker's yeast, and CEN.PK113-7D strain.

In the above and other embodiments of the method as herein defined, separating the fermented broth comprises centrifuging said fermented broth and collecting at least a portion of said spent yeast. In some embodiments the method according to the present disclosure further comprises separating ethanol from said ethanol-containing liquid. In specific embodiments the separation is performed by distillation.

In various embodiments the method according to the present disclosure is wherein the fermentable substrate is derived from sugar-containing raw materials, preferably sugar beet, sugarcane, molasses, whey, sorghum or fruits, starch-containing feedstocks, preferably grain or root crops, such as corn, wheat, rice or cassava, or any combination thereof.

In specific embodiments the fermentable substrate is derived from sugar-containing raw materials, preferably sugar beet, sugarcane, molasses, whey, sorghum or fruits or any combination thereof. In further specific embodiments the fermentable substrate is

derived from starch-containing feedstocks, preferably grain or root crops, such as corn, wheat, rice or cassava, or any combination thereof.

## BRIEF DESCRIPTION OF THE FIGURES

Embodiments will now be described, by way of non-limiting example only, with reference to the accompanying drawings, in which:

**Figure 1** is a graph showing the effect of Bactebrom™ at the indicated concentration range (as ppm total chlorine equivalents, TCE) on viability of the yeast *Saccharomyces cerevisiae* and the bacterial strain *Lactobacillus fermentum* (9338) at pH 5.5. **Abbreviations**: CFU, cell forming units; ml, milliliter; *Saccharomyces, Saccharomyces cerevisiae; Lactobacillus, Lactobacillus fermentum,* ppm, parts per million.

**Figure 2** is a graph showing the effect of Bactebrom™ at the indicated concentration range (as ppm TCE) on viability of the yeast *Saccharomyces cerevisiae* and the bacterial strain *Lactobacillus fermentum* (9338) at pH 3.5. **Abbreviations**: *Lactobacillus, Lactobacillus fermentum.*

**Figure 3** is a graph showing the effect of Bactebrom™ at the indicated concentration range (as ppm TCE) on viability of the yeast *Saccharomyces cerevisiae* and the bacterial strain *Lactobacillus plantarum* (8014) at pH 5.5.

**Figure 4** is a graph showing the effect of Bactebrom™ at the indicated concentration range (as ppm TCE) on viability of the yeast *Saccharomyces cerevisiae* and the bacterial strain *Lactobacillus plantarum* (8014) at pH 3.5.

**Figure 5** is a graph showing the effect of Bactebrom™ at the indicated concentration range (as ppm TCE) on viability of the yeast *Saccharomyces cerevisiae* and the bacterial strain *E. coli* (8739) at pH 5.5.

**Figure 6** is a graph showing the effect of Bactebrom™ at the indicated concentration range (as ppm TCE) on viability of the yeast *Saccharomyces cerevisiae* and the bacterial strain *E. coli* (8739) at pH 3.5.

**Figure 7** is a graph showing the effect of 2,2-dibromo-3-nitrilopropionamide (DBNPA) at the indicated concentration range on viability of the yeast *Saccharomyces cerevisiae* and the bacterial strain *Lactobacillus fermentum* (9338) at pH 5.5. **Abbreviations**: DBNPA, 2,2-dibromo-3-nitrilopropionamide.

**Figure 8** is a graph showing the effect of DBNPA at the indicated concentration range on viability of the yeast *Saccharomyces cerevisiae* and the bacterial strain *Lactobacillus fermentum* (9338) at pH 3.5.

**Figure 9** is a graph showing the effect of DBNPA at the indicated concentration range on viability of the yeast *Saccharomyces cerevisiae* and the bacterial strain *Lactobacillus plantarum* (8014) at pH 5.5.

**Figure 10** is a graph showing the effect of DBNPA at the indicated concentration range on viability of the yeast *Saccharomyces cerevisiae* and the bacterial strain *Lactobacillus plantarum* (8014) at pH 3.5.

**Figure 11** is a micrograph showing yeast cells stained by Erythrosine, in a Neubauer Chamber. Red-stained cells (indicated by an arrow) are dead cells and unstained cells are viable cells in optical microscope (100X).

**Figure 12** is a schematic representation of the Brazilian process for alcohol production using fermentation.

## DETAILED DESCRIPTION OF THE INVENTION

In one aspect provided herein is a method for controlling a prokaryotic contamination in a yeast-based process for producing ethanol, also referred to herein as a fermentation process. It has been unexpectedly found that by using an oxidizing bromine-based biocide obtained by on-site oxidation of an inorganic bromide source (the bromide source is a solution comprising hydrobromic acid, or a metal bromide salt), it is possible to selectively eradicate prokaryotic growth without substantially affecting yeast viability. Thus, the method of the invention comprises contacting yeast with an oxidizing bromine-based biocide as defined herein.

In other words, by one of its aspects, the present disclosure provides a method for purifying a yeast culture prone to prokaryotic contamination, said method comprising contacting said yeast culture with at least one oxidizing bromine-based biocide, obtained by on-site oxidation of an inorganic bromide source which is a solution comprising hydrobromic acid, or a metal bromide salt.

Furthermore, the present disclosure provides a method for producing ethanol by fermentation prone to prokaryotic contamination, said method comprising:

a) providing a fermentable substrate;

b) combining said fermentable substrate with yeasts in the presence of water to obtain a fermentation broth; and

c) fermenting said fermentation broth to obtain a fermented broth;

wherein the process further comprises contacting the yeasts with at least one oxidizing bromine-based biocide, obtained by on-site oxidation of an inorganic bromide source which is a solution comprising hydrobromic acid, or a metal bromide salt.

As may be appreciated by those skilled in the art, by the term "purifying" it is meant decontaminating or disinfecting at least partially, from prokaryotic contamination, such that the yeast culture or any medium comprising thereof (e.g., the fermentation broth) is substantially purified, namely at least 60% free, preferably at least 75% free, and more preferably at least 90% free from prokaryotic contamination.

The contacting step may be carried out by adding an oxidizing bromine-based biocide into a liquid containing yeast or a liquid containing a yeast culture contaminated with a prokaryotic growth. This step may be performed at any time during the course of the process (e.g., for producing ethanol) and at any stage thereof, by way of example, during the stage in which yeast are recovered (recycled) after a fermentation step is completed (such yeast are also referred to herein as "spent yeast") or during the pre-treatment step of the fermentable substrate, in order to decontaminate the substrate before the fermentation step commences. The contacting step may alternatively or additionally be performed during fermenting of the fermentation broth. The contacting step of adding an oxidizing bromine-based biocide into a liquid containing yeast is generally carried out with a dosage level of at least 0.2 ppm of total chlorine equivalent (TCE), with the upper limit dictated by the process needs, as generally described below, but may be up to, e.g. 25 ppm of total chlorine equivalent. Generally, as it has been demonstrated in the examples below, dosage levels of the bromine-based biocide of between 0.5 and 5.0 ppm TCE are efficient in selectively eradicating prokaryotic contamination, when performed in an organic-free aqueous medium. One should understand that in the presence of reducing sugars and other constituents of fermentation/fermented broth, the required dosage of the bromine-based biocides may be larger, which may correspond to significantly higher TCE concentrations in organic-

free medium. Thus, the dosage levels in organic-free medium may serve as a standardized starting point to determine the required dosage levels of the bromine-based biocide in the ethanol-production process. The process may thus also comprise pre-determining the maximum tolerable amount of the bromine-based biocides for the specific strain of the yeast that is being used in the process (i.e. the dosage level that does not kill the yeast just yet), and based on the experimental results demonstrated herein, to select a dosage level that is lower than that maximum tolerable amount for the process, expecting that it will be sufficient to eradicate any reasonable prokaryotic contamination.

Thus, specifically, depending on the yeast's strain, said at least one oxidizing bromine-based biocide as defined herein may be used at a dosage level of 0.2 to 25 ppm TCE, e.g. of 0.2 to 10 ppm TCE, 0.2 to 7 ppm TCE, 0.2 to 5 ppm TCE, e.g. of 0.5 to 5 ppm TCE. The actual concentration of the oxidizing bromine-based biocide required for the process may be determined via monitoring, continuously or intermittently, the TCE values, and adjusting the dosage level (e.g., the rate of introduction) of the oxidizing biocide accordingly during the process, or by relying on pre-determined values, e.g. as described above. The required amounts will also be dependent on the specific composition of the medium wherein the oxidizing bromine-based biocide is added, particularly by the organic content thereof.

The fermentation process is usually carried out in an aqueous medium. Thus, all the components of the fermentation process, as described in greater detail below, may be added into, or contained in, an aqueous medium, for example distilled water.

Oxidizing bromine-based biocides (for example HBr, HBr and urea, alkali metal bromides) provide active bromine species in the media into which they are delivered, for example water, through bromide oxidation that converts the $Br^{(-)}$ into said active bromine species, e.g. elemental bromine species and/or $Br^{+}$ species (e.g., hypobromous acid / hypobromite). The oxidation is usually achieved with the aid of a chemical oxidant, e.g. sodium hypochlorite, chlorine, hydrogen peroxide or ozone or electrochemically. The dosage of the oxidizing bromine-based biocides described herein is usually expressed as parts per million of total chlorine equivalent (ppm TCE),

i.e. the total oxidizing species present in the solution expressed as titrimetric equivalents of free chlorine.

The total chlorine equivalent concentration may be determined, e.g., by an iodometric titration. The titration may be performed directly, e.g. using titrating burettes, or using an automated titrimeter, e.g., titroprocessor Titrino 848 plus. The oxidative species may be reacted with excess iodide solution to form iodine which is then titrated directly with a calibrated solution of thiosulfate, or with an excess of calibrated solution of thiosulfate, the remainder whereof is then quantitatively titrated with iodine. Additionally, total chlorine equivalent may be determined by the DPD (Diethyl-p-PhenyleneDiamine) reagent method using a spectrophotometer, e.g., Merck SQ-300.

The inorganic bromide sources, namely solutions comprising metal bromide salts, and/or hydrobromic acid (HBr), including a solution of HBr and urea, provide active bromine species into the medium to which they are delivered (e.g., water) upon oxidation, e.g., by an oxidant (such as by chemical oxidation using hypochlorite, chlorine gas, hydrogen peroxide or ozone and/or by electrochemical oxidation, namely, anodically-generated bromine). Commercially available products include activated sodium bromide (consisting of an aqueous solution of sodium bromide and sodium hypochlorite prepared on-site and delivered immediately to the system to be treated); a solution of hydrobromic acid which reacts with sodium hypochlorite on-site; a solution of HBr and urea (e.g., Bactebrom™ solution, composed of hydrobromic acid and urea, from ICL-IP), reacting on-site with sodium hypochlorite. Such composition may be prepared, for example, as described in WO 2012/038954, incorporated herein by reference.

In specific embodiments, the inorganic bromide source is selected from the group consisting of: HBr, a solution of HBr and urea and sodium bromide. In the above and other embodiments, the inorganic bromide source is oxidized on-site with any one of hypochlorite, chlorine, hydrogen peroxide, ozone, and/or electrochemically, to produce said oxidizing bromine-based biocide.

Oxidizing bromine-based biocides as defined herein suitable for use in the present

invention are available in the marketplace in different forms, i.e., solids (such as powders and compacted forms, e.g., granules and tablets) and liquids (e.g., aqueous concentrates or other flowable formulations that can be easily supplied to the fermentation system to be treated).

In general, an aqueous solution of the bromide source and an aqueous solution of the oxidant are held in separate tanks. With the aid of dosing pumps, two separate streams are supplied from the tanks and mixed just prior to use, such that the combined stream enters the intended site of use shortly after it has been formed (also referred herein "*on-site*" or "*in situ*"). The oxidizing bromine-based biocide as defined herein may be supplied in continuous mode or at intermittent mode (dosing) at pre-determined time intervals, depending on the requirements of the process. For example, to produce the active Bactebrom™, a first stream, consisting of HBr and urea, and a second stream, comprising sodium hypochlorite, are mixed shortly before use.

In specific embodiments, the present disclosure provides a method for producing ethanol by fermentation prone to prokaryotic contamination, said method comprising:

a) providing a fermentable substrate;

b) combining said fermentable substrate with yeasts in the presence of water to obtain a fermentation broth; and

c) fermenting said fermentation broth to obtain a fermented broth; and optionally

d) separating said fermented broth into spent yeasts and ethanol-containing liquid; and

e) recovering said spent yeast,

wherein the process further comprises contacting the yeast recovered with at least one oxidizing bromine-based biocide, obtained by on-site oxidation of an inorganic bromide source which is a solution comprising hydrobromic acid, or a metal bromide salt.

The recovering step of the spent yeast may therefore comprise contacting the spent yeast (recovered) with at least one oxidizing bromine-based biocide as herein defined and optionally further comprises contacting the spent yeast with dilute sulfuric acid,

before, after or concurrently with contacting the spent yeast with the oxidizing bromine-based biocide, as further detailed below. The recovered yeast may be used for at least one additional fermentation cycle(s).

The contacting step (namely the step involving bringing yeasts and the oxidizing bromine-based biocide into physical connection) may be conducted at any suitable pH value, yet it may be especially advantageous to select the pH value according to the requirements of the process. For example, between 2 and 6, e.g., between 2 and 4 for a contacting step performed during the recovery step of the yeast, or between 4 and 6 for a contacting step performed during the fermenting step. As demonstrated in the Examples section below, selective eradication of bacteria over yeasts occurs both at pH 3.5 and 5.5, and without being bound by a particular theory it is believed that this selectivity is not affected by the pH of the medium.

As discussed herein, the contacting step with the oxidizing bromine-based biocides as defined herein may be performed as a stand-alone decontamination step, e.g., as a method for purifying yeast culture from bacterial decontamination or as a step during yeast-based fermentation, in particular during a yeast recovering step, and optionally may be associated with an acidification step as known for the Brazilian process of ethanol production (e.g., contacting the spent yeast with water-diluted sulfuric acid ($H_2SO_4$), at a pH of 2.0-2.5, for 1-2 hours). The contacting step with the oxidizing bromine-based biocides may be performed before or after the acidification step as defined above or concurrently therewith.

Without being bound by a particular theory, it is believed that contacting potentially contaminated yeast culture by at least one oxidizing bromine-based biocide according to the invention during the recovery step of the yeast, may be sufficient to essentially eradicate prokaryotic contamination, thereby selectively inhibiting bacterial growth without significantly affecting the viability of the yeast, if performed at a dosage level of the oxidizing bromine-based biocide of between about 0.2 and about 25 ppm TCE, e.g. between 2 to 10 ppm TCE, between 0.2 to 7 ppm TCE or between 0.2 to 5 ppm TCE, e.g. between 0.5 to 5 ppm TCE; and/or at pH value of 2 to 4. In the above and other embodiments, the contacting time between the yeast and the oxidizing bromine-

based biocide of the present disclosure is of e.g. between 30 and 120 minutes, or between 45 and 75 minutes. The specific contacting time may be dependent on the nature of the prokaryotic contamination, on the particular strain(s) of the yeasts, on the dosage level of the bromine-based biocide, and on many other factors. Ideally, the contacting time, as well as the effective concentration / dosage level for each particular process, may be predetermined in a separate experiment prior to the mass production process.

Generally, the fermentable substrate (namely the substance or material being fermented) is a mixture of nutritious sugars and/or oligo- and polysaccharides, consumed by the microorganisms utilized in the fermenting step. The raw materials for the process are pre-processed, mechanically, chemically, enzymatically, and/or by pre-fermentation with another microorganism, to make these nutrients accessible to the ethanol-fermenting microorganisms, as described below. Generally, there are three major steps in ethanol production by fermentation: (1) obtaining the fermentation broth, namely the solution that contains fermentable substrate, from raw materials, (2) converting sugars and/or other nutrients in the fermentable broth to ethanol by fermentation, thereby obtaining the fermented broth, and (3) separating ethanol and/or purifying it, by further processes, including for example distillation.

The starting material for ethanol production, also referred to herein as “fermentable substrate”, “substrate”, "fermentable mash", and the like, suitable for the present invention, may be derived, for example, from sugar-containing raw materials (for example, sugar beet, sugarcane, molasses, whey, sorghum and fruits) or starch-containing feedstocks (for example grain or root crops, such as corn, wheat, rice, or cassava, etc.). Preferably, the process may be used for producing so-called “first generation bioethanol”, namely ethanol directly related to a biomass that is more than often edible. In particular, the fermentable substrate is sugarcane juice, molasses or mixtures thereof.

In specific embodiments, the fermentable substrate is derived from sugar-containing raw materials, preferably sugar beet, sugarcane, molasses, whey, sorghum or fruits, starch-containing feedstocks, preferably grain or root crops, such as corn, wheat, rice

or cassava, or any combination thereof.

The fermentable substrate is therefore derived from the various suitable feedstocks, e.g., as referred to above, which have been (optionally) subjected to pretreatment processes suitable to the particular feedstock used, as known in the art. Methods used for pretreatment include physical pretreatment (e.g., mechanical milling to grind the substrate and facilitate downstream mashing), chemical pretreatment (e.g., acid hydrolysis, alkaline hydrolysis, ozonolysis, enzymatic hydrolysis), physicochemical pretreatment (e.g. ammonia fiber explosion or stream fiber explosion) and biological pretreatment (e.g., using different fungal species) procedures. Overall, pretreatment procedures as defined herein include any process necessary for converting the raw material feedstocks into fermentable sugars available for bioethanol production by yeast fermentation.

Various methods of pre-treating feedstocks for ethanol fermentation and numerous other aspects of the process have been reviewed, e.g., in Mohd-Azhar *et al.*, *Biochemistry and Biophysics Reports 10 (2017) 52–61* incorporated herein by reference.

For example, currently in Brazil, the fermentable substrate comprises molasses (which is the residue or by-product of sugar production) diluted with water, or a mix of sugar cane juice and molasses, and currently in the USA the fermentable substrate comprises a liquor of glucose resulting from the process of breakdown of starch extracted from corn.

The different starting materials vary in the extent to which they should be pre-processed (also termed herein "pre-treated") prior to use thereof for fermentation by yeast, as well known in the art. Thus, the process may comprise pre-treating the starting material to obtain a fermentable substrate or mash. For example, when sugar cane is used as the source material for the ethanol production process, the cane may be first washed. Preferably, the washing of the cane is carried out in alkaline water with pH value of about 11, e.g. between 10 and 12. The washed cane is then milled to obtain a raw juice and solid residue. The cane solids can be used as a regular burning fuel (e.g.,

for heating of the liquids in the further steps of the process). The raw juice may then be pre-heated, e.g., to a temperature between 60 and 80°C, and then clarified, e.g., by addition of calcium oxide (quicklime), and/or passing sulfur dioxide through it. The clarified raw juice may then be phosphated to precipitate the residues of calcium, and heated in a decanting assembly to separate the inorganic component, e.g. at about 105 °C for about 2 hours. The decanted liquid may then be filtered off to remove the inorganic solids, which may be used as a fertilizer, whereas the liquid (called at this stage "clarified broth") may be used for sugar manufacturing. The residues of the sugar manufacturing (molasses), or the clarified broth, may be diluted with water of clarified sugarcane juice, as described above, to produce wort, used as a fermentable mash.

By way of a further example, when the starch-containing feedstock corn is used as source (raw) material, the pre-treatment thereof is a multistep process, as known in the art and as briefly described below. The first step involves milling of the corn, by dry or wet milling. When wet milling is employed, corn kernels are broken down into starch, fiber, corn germ, and protein by heating in a sulfurous acid solution for prolonged time intervals, e.g. 1-3 days, such as about 2 days. The starch is then separated and may be used as the starting material for producing ethanol (namely the fermentable substrate as herein defined), as well as corn syrup, or food-grade starch. The first step of corn milling by a dry milling process is grinding the corn by using a suitable mill, e.g. hammer-mill or roller mill. Once the corn is broken down, it is mixed with heated water to form a mash or slurry. The slurry comprises at this stage, among other constituents, corn particles and cornstarch granules. Next, the corn slurry undergoes gelatinization and liquefaction (also termed "cooking") under conditions of temperature and acidity as detailed below, during which water interacts with the starch granules in the corn when the temperature is above 60°C and forms a viscous suspension. The liquefaction step is partial hydrolysis that lowers the viscosity, essentially breaking up long starch chains into shorter chains. In order to accomplish liquefaction, the reaction conditions are usually maintained at a pH in the range of 5.9 – 6.2, and ammonia and sulfuric acid are added to the tank to maintain the pH value. The enzyme alfa-amylase may also be added to the mash before jet cooking (for 2-7 minutes at 105-120°C) to improve the flowability of the mash. At this stage, shorter dextrin is produced but glucose is not yet formed. When alfa-amylase is utilized, there

are several processes known in the art, involving enzymatic and heat hydrolysis in various order. The first process consists of adding alfa-amylase and incubating the material at 85-95°C. The second process consists of placing the mash in the jet cooker at 105-120°C for 2-7 minutes, then flowing thereof to a flash tank at 90°C and adding alfa-amylase three hours later. The third process consists of adding the alfa-amylase, heating in the jet cooker at 150°C, followed by flow to the flash tank at 90°C and adding more alfa-amylase. As known in the art, the enzyme alfa-amylase acts on the internal glycosidic bonds to yield dextrin and maltose, for liquefaction of the material. The next step of corn pre-treatment is saccharification, namely the process by which further hydrolysis to glucose monomers occurs, using the enzyme glucoamylase, which cleaves glycosidic bonds at dextrin ends to form glucose. The optimal reaction conditions required are a pH of 4.5 and a temperature of 55-65°C.

As known in the art, while pretreatment of raw feedstock material may be separated from the fermentation process, the fermentation process may commence along-side the hydrolysis of the raw material into fermentable substrate, provided that the pretreatment conditions are suitable for fermentation.

In general, fermentation starts by the addition of a substrate (fermentable substrate), which may be composed, by way of a specific example, of sugar cane juice and/or molasses at any proportion, to an inoculum of a yeast culture contained in a bioreactor (fermenter vessel) in the presence of water. The substrate usually contains reducing sugars (e.g., sucrose, glucose, fructose, and their mixtures), usually present at concentrations of about 150-250 g/L. Fermenting may be performed as generally known in the art, e.g., in a batch mode (e.g., by a fed batch process), in a continuous mode, or in any combinations of these two.

The fermentable substrate is contacted with the yeast culture and water under desired conditions of, for example, inoculum (yeast culture) concentration, sugar concentration, temperature, oxygenation, pH, incubation time and mixing (agitation rate). The mixture of yeast, water and fermentable substrate (also referred to herein as "fermentation broth") is then incubated for a certain time, to effect fermenting of the fermentable substrate. The typical temperature may be between 20°C and 37°C,

preferably between 32°C and 34°C. The temperature may be controlled as generally known in the art, e.g. by using a double-jacketed vat and circulating a heat-exchange liquid, e.g. water, through the jacket. The temperature of the heat-exchange liquid may be controlled by temperature-controlling unit, e.g. by a heating unit and/or cooling unit. The temperature of the heat-exchange liquid may be controlled by a temperature measured in a fermenting vat, e.g. by an indwelling thermocouple. The optimum pH range for *S. cerevisiae* is 4.0-5.0.

The time necessary for the process, e.g., for full consumption of the starting material (i.e., until less than about 0.1% of fermentable sugars remain present) or other pre-determined end-point, is usually dictated by the consumption rate of the sugars and/or by the production of alcohol; either or both may be monitored continuously or at discrete time points, for process control purposes.

The fermenting step may comprise gradually feeding the fermentable substrate to be combined with the yeast and water (namely, by fed batch mode). This gradual addition may be particularly advantageous, in order to reduce production of toxic co-products, thereby causing less stress to the yeast, and consequently increasing the yield of ethanol production. Alternatively, the fermentable substrate may be combined substantially completely with the yeast and water to initiate the fermenting step. When gradual addition is employed, it can be carried out within about 4-6 hours, whereas fermentation is completed within 6-10 hours after the initiation of feeding, attaining 7-10% (v/v) ethanol in the fermented broth (i.e. the mixture obtained from the fermenting step). During feeding and fermentation, the temperature inside the fermenter vessel is maintained at between 27°C and 37°C, preferably between 32°C and 34°C. Ethanol may be thus advantageously produced at an efficiency of at least 80%, preferably at least 90%, meaning that 90% of the sugar contained in the substrate has been converted into ethanol.

Yeast suitable for industrial ethanol production by fermentation are usually *S. cerevisiae* strains, however, other genera known in the art may also be used, e.g., *Pichia Stipidis* (for example, strain NRRL-Y-7124), or *Kluyveromyces fagilis* (for example, strain Kf1). The present invention is thus applicable to any yeast suitable for industrial

ethanol production by fermentation, in particular at least one *S. cerevisiae* strain. Some non-limiting examples of *S. cerevisiae* strains are PE-2 strain, S288c, baker's yeast, and CEN.PK113-7D. The yeast suitable for the process may be native, or genetically modified, e.g., to consume pentoses or to express genes encoding lytic enzymes effective against bacteria. Additionally, or alternatively, the yeast may be a mixture of several genera and/or strains, to maximize the utilization of the nutrients and conversion to ethanol. Some additional yeast microorganisms, suitable for fermenting the fermentable substrate into ethanol are described, e.g. in Mohd Azhar 2017 (*vide supra*). As known in the art, yeast cells reproduce by budding. Therefore, the yeast cells (interchangeably referred to herein as yeast culture) of the present invention are a yeast culture containing a heterogeneous collection of yeast cells at various stages of life cycle and may occasionally also include dead yeast cells. Yeast utilized in the process according to the invention may be free yeast or immobilized yeast.

After the fermentation step is completed, according to the acceptable Brazilian process for ethanol production, the yeasts are separated from the fermented broth and treated with an acid to remove contaminants, and thereby recycled or recovered, whereas according to other (non-Brazilian) processes the yeasts are processed into products generally non-related to further fermentation.

The inoculum for fermentation may be prepared from fresh yeast cells or, as in the Brazilian process for ethanol production (for example, as schematically presented in Figure 12), yeast cells or yeast culture collected and recovered at the end of a fermentation cycle (e.g., the previous cycle) are used again after being treated (recovered) for example as detailed herein.

According to the present invention, the contacting of yeasts with the oxidizing bromine-based biocides as defined herein may also be performed after the step of fermentation of sugars into ethanol is completed.

Depending on the starting material, the fermented broth may contain coarse solids, e.g., remainders from the mashing process, as well as dispersed yeast mass. The fermented broth is first subjected to separating the liquid fraction from the solids fraction. When

the substrate contains insoluble solids and/or forms insoluble fermentation products, these can be separated from the fermented broth by decantation, filtration or any other method known in the art. Yeast may be separated from the fermented broth (which does not contain any appreciable amount of other insoluble products), e.g., by centrifuging the fermented broth at a suitable g-force. The fermented broth may contain between 8% and 16% of yeast, preferably between 10% and 14%; after centrifugation the obtained yeast cream (also termed herein "spent yeast") may contain between 40% and 80% of yeast. The liquid stream separated as supernatant during the centrifuging step (also termed "fermented wine", i.e., liquid without the yeast cells) may be further processed to extract ethanol, e.g., distilled for ethanol recovery in a distillation unit.

The yeast cream may then be subjected to a yeast recovering step, by contacting the spent yeast with at least one oxidizing bromine-based biocide as detailed above.

In other words, in some embodiments the method for producing ethanol by fermentation prone to prokaryotic contamination comprises:

a) providing a fermentable substrate;

b) combining said fermentable substrate with yeasts in the presence of water to obtain a fermentation broth;

c) fermenting said fermentation broth to obtain a fermented broth;

d) separating said fermented broth into spent yeasts and ethanol-containing liquid; and

e) recovering said spent yeast,

wherein the process further comprises contacting the recovered yeasts with at least one oxidizing bromine-based biocide, obtained by on-site oxidation of an inorganic bromide source which is a solution comprising hydrobromic acid, or a metal bromide salt (e.g., a solution of HBr and urea oxidized on site with hypochlorite) and optionally with dilute sulfuric acid to obtain recovered yeast.

Generally, the amount of yeasts undergoing recovery (recycling) is dictated by the needs of the process, with excess yeast produced in the process being optionally removed for further processing or discarded. Briefly, the yeasts may be contacted consecutively with the oxidizing bromine-based biocide(s) as defined herein, and the

acid to ensure decontamination. Alternatively, the yeasts may be first contacted with acid, to reduce the primary prokaryotic bioburden, and then with the oxidizing bromine-based biocide(s) as defined herein. The pH of the medium may be adjusted to any suitable value according to the requirements of the particular yeast strain. Alternatively, the yeasts may be recovered using only the oxidizing bromine-based biocide(s), obviating the need to expose the fermenting organisms to acid.

In other words, the yeast may be contacted with oxidizing bromine-based biocide solution as described herein, optionally concurrently with or preceded or succeeded by the acid treatment. Yeast acid treatment may be performed in a designated yeast treatment vessel, for example by diluting the spent yeast with water (e.g., in a ratio 1:1) and reducing the pH to 1.8-2.5, e.g., with sulfuric acid (98%), and incubating for about 1 hour, at a temperature between 32 and 34°C.

Furthermore, the yeast may be contacted with the oxidizing bromine-based biocide of the present invention in combination with natural products having antibacterial properties, such as, for example Hop compounds (extracted from *Humulus Lupulus*), *Propolis* and *Chitosan* as well as with bacteriophages, which naturally antagonize bacteria.

The recovered yeast suspension may be at least partially transferred for a further (e.g., consecutive) fermenting step with fresh substrate, e.g., to an empty fermentation vat (reactor). Even using the conventional Brazilian process for ethanol production, it may be possible to perform two fermentation cycles per day. It is readily evident that increasing the efficiency of yeast recycling will improve the overall process outcomes.

The present invention is directed to provide microbial control over prokaryotic contamination of yeast, namely, bacterial species the presence of which is associated with yeast-based ethanol fermentation, sometimes referred to as "*natural contaminants*" of yeast. By a non-limiting example, the methods according to the invention are suitable for the control of lactic acid bacteria (LAB), for example of the genera (genus) *Lactobacillus* (e.g., *Lactobacillus fermentum, Lactobacillus plantarum, L. vini, L. paracasei, L. delbrueckii, L. buchneri and L. brevis*), *Leuconostoc* (e.g., *Leuconostoc mesenteroides, L.citrovorum, L.dextranicum*),

*Oenococcus*, *Pediococcus* and *Weissella*, for the control of acetic acid bacteria (AAB), for example of the genera *Acetobacter, Gluconobacter* and *Xanthomonas,* for the control of *E. coli* or any combination thereof.

In specific embodiments, the lactic acid bacteria on which the present disclosure applies are of the genus *Lactobacillus*. In further specific embodiments, the lactic acid bacteria of the genus *Lactobacillus* are *Lactobacillus fermentum*, *Lactobacillus plantarum* or any combination thereof.

The fermented broth according to the present invention contains a liquid fraction and solid sediment, which may be separated. Separating the liquid fraction of the fermented broth from the solid fraction results *inter alia* in a solid fraction containing a minimal amount of bromine-based oxidizing biocide as described herein. The solid fraction according to the present invention may be further converted into a distillers dried grains (DDG) product, containing a minimal amount of bromine-based oxidizing biocide and being essentially free of antibiotics.

Preferably, the method according to the present invention obviates the use of antibiotics altogether, therefore in some particularly preferred embodiments the process is essentially antibiotic-free (namely performed in the absence of antibiotics). That is, the oxidizing bromine-based biocide as described herein is usually sufficient to maintain the yeast culture free from contamination. Nevertheless, it is envisaged that some contaminants may be particularly hard to control even at the highest applied dosage levels of the oxidizing bromine-based biocide. In such cases the process may comprise providing an antibiotic during the process, e.g. to the fermentable substrate, to the fermentable broth, and/or to the yeast. In other words, the oxidizing bromine-based biocide as described herein may be applied in combination with antibiotics that is, before, after, or concurrently with antibiotics. Any antibiotic agent may be used in the framework of the present invention, preferably an antibiotic agent that eliminates bacterial contaminants without harming the yeast during fermentation. By way of example, the antibiotic agent may be monensin, penicillin, virginiamycin, erythromycin, tylosin and tetracycline, to name but a few. The antibiotic agent may be applied at a dosage of about 0.2 to about 5 ppm. The antibiotic agent may preferably be applied during the yeast recycling step. It is noted that when the antibiotic is used,

it is used in a concentration that is lower that the concentration needed to control the contamination under identical conditions, but without the use of oxidizing bromine-based biocide. For example, when antibiotic is used, it may be used in an amount of below 70% of the amount needed to control the contamination under identical conditions, but without the use of oxidizing bromine-based biocide, preferably, below 50%, more preferably below 30%.

## EXAMPLES

### Materials

Culture media, reagents and solutions used are as detailed in Table 1 below.

**Table 1 List of materials**

| Product | Description | Manufacturer |
|---|---|---|
| Sabouraud Dextrose Agar | Culture medium containing dextrose (40 gr/l), peptic digest of animal tissue (5 gr/l), pancreatic digest of casein (5 gr/l) and agar (15 gr/l) | Neogen |
| Nutrient Agar (NA) | Culture medium containing beef extract (3 gr/l), enzymatic digest of gelatin (5 gr/l) and agar (15 gr/l) | Neogen |
| MRS agar | Culture medium containing beef extract (10 gr/l), proteose peptone no 3 (10 gr/l), yeast extract (5 gr/l), dextrose (20 gr/l), polysorbate 80 (1 gr/l), ammonium citrate (2 gr/ l), sodium acetate (5 gr/ l), magnesium sulfate (0.1 gr/l), manganese sulfate (0.05 gr/l), dipotassium phosphate (2 gr/l) and Agar (15 gr/l) | Difco |
| Universal Neutralizer | A solution containing Tween 80 (30 gr/l, Sigma), saponin (30 gr/l, Sigma), lecithin (3 gr/l, Across), sodium thiosulfate (7.84 g/l, Merck), NaCl (8.5 gr/l, Merck), histidine (1 gr/l, Merck) and tryptone (1 gr/l, Neogen) | Prepared by the inventors |
| Citric acid buffer solution, pH=3.5 | A solution containing $Na_2HPO_4$ (4 gr/l, Merck) and citric acid (4.66 gr/l, Merck) | Prepared by the inventors |

| Product | Description | Manufacturer |
|---|---|---|
| Citric acid buffer solution, pH=5.5 | A solution containing $Na_2HPO_4$ (7.89 gr/l, Merck), citric acid (4.66 gr/l, Merck) | Prepared by the inventors |
| Sodium thiosulfate Neutralizer | Sodium thiosulfate (7.84 g/l, Merck) | Merck |
| Phosphate buffer solution | 1.25 ml of a $KH_2PO_4$ stock solution (containing 34 gr/l $KH_2PO_4$ (Merck) in 1 liter of water) | Prepared by the inventors |
| Glass beads | Used for preparing the test cultures | |
| Bactebrom™ | Urea-HBr aq. Solution. 35% as HBr | ICL |
| DBNPA | 2,2-dibromo-3-nitrilopropionamide | ICL |
| natural sugar cane juice | natural sugar cane juice derived from sugarcane by milling | Brazilian producer |
| papain | Pure papain | Dinamica |
| Erythrosine | Erythrosine | Dinamica |

*Microorganisms*

The tests were conducted using the following three (3) bacterial and one yeast species, all obtained from the American Type Culture Collection (ATCC):

*Escherichia coli*, also referred to as *E. coli* or *E. coli* 8739 (ATCC 8739);

*Lactobacillus fermentum*, also referred to as *L. fermentum* or "*Lactobacillus* 9338" (ATCC 9338);

*Lactobacillus plantarum*, also referred to as *L. plantarum* or *Lactobacillus plantarum* 8014 (ATCC 8014); and

*Saccharomyces cerevisiae*, also referred to as *S. cerevisiae* or "saccharomyces" (ATCC 9763).

*Yeast for optical microscopy*

Yeast for optical microscopy were based on commercial fresh bread yeast (*Saccharomyces cerevisiae*, Itaiquara® fresh bread yeast, stored at about 4°C until use) and on industrial *Saccharomyces cerevisiae* consisting of yeast cream collected at a distillery in São Paulo state (season 2020/21), just after the centrifuge step and before acid treatment. The yeast cream was stored at 4 °C until use.

*Biocide preparation by activation of HBr/urea solution*

Biocide was prepared by activation of HBr/urea solution using sodium hypochlorite (also referred to herein as activated Bactebrom™) as follows.

Stock solution 1: 8.94 gr of Bactebrom™ from ICL-IP (HBr : urea solution) diluted with 241.06 gr of distilled water.

Stock solution 2: NaOCl ~1% prepared by 23.58 gr of NaOCl 10.6% w/w diluted with 226.42 gr of distilled-water.

Stock solution 2 (250.00 gr of NaOCl 1.0%) was added gradually while stirring to the above diluted Bactebrom™ solution (stock solution 1), to obtain the active biocide (orange solution) - total weight 500.00 gr. Expected biocide concentration as determined by iodometric titration using Titroprocessor: Titrino 848 plus.: ~ 0.5% as $Cl_2$ (~5000 ppm as $Cl_2$ or TCE). The desired biocide dosage level in each experiment was obtained by dilution with distilled water.

**Methods**

*Preparation of microbial suspensions*

*Saccharomyces cerevisiae*, *E.coli* and the two *Lactobacillus* bacteria were sub-cultured from stock culture by streaking them onto culture plates containing Sabouraud Dextrose, NA and MRS agar, respectively. The plates were incubated for 18-24 hours at 30 ± 2 °C for *E. coli* and *Saccharomyces cerevisiae* and at 37 ± 2°C for the two Lactobacillus. Then, a second subculture was prepared from the first subculture, in the same manner, and the cultures were incubated as detailed above, namely, 18-24 hours at 30 ± 2°C for *E. coli* and *Saccharomyces cerevisiae* and at 37 ± 2°C for *Lactobacillus*.

In order to prepare a microbial test suspension, sterile phosphate buffer solution (10 ml) was placed in a 100 ml flask with 5 gr of glass beads. A loopfull of cells from the over-night grown culture was transferred into the phosphate buffer. The flask was shaken for 3 minutes, using vortex. The suspension was aspirated from the glass beads and transferred to another tube. The number of cells in the suspension was adjusted, by

means of a calibration curve, to $1.0\times10^6$-$1.0\times10^7$ CFU/ml (colony forming units per ml), using phosphate buffer solution.

*Efficacy test procedure*

The efficacy (the anti-microbial activity) of activated Bactebrom™ (at concentrations of between 0.5 to 2.5 ppm as $Cl_2$) and the efficacy of 2,2-dibromo-3-nitrilopropionamide (DBNPA, at concentrations of between 0.5 to 5 ppm) were tested against the yeast *Saccharomyces cerevisiae*, against *E. coli* and against two lacto-bacteria commonly found in fermentative process: *Lactobacillus fermentum* and *Lactobacillus plantarum* at pH=5.5 and pH=3.5 (each having specified strain number and source as detailed above) according to a modification of the European standard EN 1040: 2005: "Chemical disinfectants and antiseptics – Quantitative suspension test for the evaluation of basic bactericidal activity of chemical disinfectants and antiseptics – Tests method and requirements (phase 1)", as generally described below.

Tests were performed as follows. Phosphate buffer solution (100 ± 2 ml) was transferred into sterile flasks. Duplicate flasks were prepared for each tested biocide concentration. In addition, duplicate controls with no biocides were prepared. Each test vessel was brought to 30 ±2°C and inoculated with pure culture to achieve microbial count of at least $10^6$ CFU/ml at time zero, after which the biocide stock solution was added to the duplicate flasks in a manner such that the volume of biocide stock added does not exceed 1% of the total volume of the flask. Flasks were placed in a shaker to provide mixing, under a constant temperature of 30 ±2°C during the contact periods.

Following a time period of 1 hour ± 5 minutes of contact time, 1 ml of the tested mixture was pipetted into a tube containing 9.0 ml neutralizer (Sodium thiosulfate neutralizer for conditions of pH=5.5 and universal neutralizer for conditions of pH=3.5, both neutralizers prepared as described above). After a time period of 5 min ± 10 sec, a sample of 1 ml was taken (in duplicate) and after a serial dilution, inoculated by the pour plate method into a Petri dish containing NA agar for *E.coli*, Sabouraud agar for *S. cerevisiae* and MRS agar for the two lactobacillus (*L. fermentum* and *L. plantarum*). The plates were incubated at 37 ±2°C for 48 hours for both lactobacillus and at 30 ±2°C

for 48 hours for *E.coli* and S. *cerevisiae*. After incubation, visible growth was observed and the colonies forming units (CFU) were counted and recorded.

*Preparation of yeast samples for optical microscopy*

Commercial fresh bread yeast were mashed in natural sugar cane juice (4 gr yeast in 500 mL of juice) resulting in a yeast cells concentration of approximately $10^8$ CFU/mL. Industrial *Saccharomyces cerevisiae* (namely yeast cream obtained from a distillery as detailed above) consisted of a concentrate of yeast cells (culture) and bacterial contamination (naturally present) obtained from the sugar & ethanol industry. Samples were thawed 24 hours before the test, at environmental temperature (namely room temperature of about 25°C) and then added to fresh sugar cane juice (100 mL of juice) and left overnight at room temperature.

*Yeast viability analysis by optical microscopy*

Yeast viability was evaluated according to the standard method applied by the Brazilian ethanol distillers (Ceccato-Antonini, S.R. Microbiologia da fermentação alcoólica – A importância do monitoramento microbiológico em destilarias. Tecnologia Sucroalcoleira – Coleção UAB-UFSCar 105p, 2010), as detailed below. Fresh sugar cane juice (250 ml) was added to the yeast cream (bread yeast or industrial yeast collected as detailed above) while mixing. In each assay, 50 mL of the resulting yeast mixture were treated with different concentrations of Bactebrom™ at 0 ppm (control), 2.5 and 5 ppm (as $Cl_2$ or TCE) for a time period of 1 hour, by adding activated Bactebrom™ at the desired dosage level. Samples were collected at the initial and final time points of treatment, and papain (at 5 mg) was added to each one of the samples. Next, (after 5 minutes), samples were diluted 10 or 100-fold and stained by Erythrosine (0.02% solution prepared in phosphate buffer). The numbers of viable (transparent cells) and dead yeast cells (colored by red) were counted by optical microscopy in a Neubauer Chamber Improved (depth: 0.1 mm, 25 squares with 16 reticles and 400 chambers, HBG), using a 40x lens. Viability was calculated as the percentage of viable cells out of the total number of yeast cells (both viable and dead cells).

**EXAMPLES 1-6**

***The anti-bacterial effect of Bactebrom™***

The effect of activated Bactebrom™ was tested on the three different bacterial strains, *L. fermentum*, *L. plantarum* and *E. coli* and was compared to the effect thereof on the yeast *Saccharomyces cerevisiae*, as detailed above and summarized in Table 2 below (relating to Examples 1-6). The results are graphically shown in Figure 1 through Figure 6 (corresponding to Examples 1-6).

**Table 2 Bacterial strains and pH conditions in Examples 1-6**

| Example No. | Bacterial strain | pH | Figure No. |
|---|---|---|---|
| 1 | *L. fermentum* | 5.5 | 1 |
| 2 | *L. fermentum* | 3.5 | 2 |
| 3 | *L. plantarum* | 5.5 | 3 |
| 4 | *L. plantarum* | 3.5 | 4 |
| 5 | *E. coli* | 5.5 | 5 |
| 6 | *E. coli* | 3.5 | 6 |

Figures 1-6 show that activated Bactebrom™ is an effective anti-bacterial agent against all tested bacteria, at all of the tested concentrations thereof and under both tested pH conditions, after a contact time of 1 hour with the bacteria. Remarkably, at the biocidal concentrations of 0.5 and 1 ppm, there was either none or a negligible effect on yeast viability. Furthermore, even at a concentration of 2.5 ppm, Bactebrom™ had a marginal effect on the yeast tested, while demonstrating a biocidal effect.

**EXAMPLES 7-10 - comparative**

***The anti-bacterial effect of DBNPA***

Next, the effect of DBNPA was tested on the two different bacterial strains, *L. fermentum* and *L. plantarum* and was compared to the effect thereof on the yeast *Saccharomyces cerevisiae*, as detailed above and summarized in Table 3 below (relating to Examples 7-10). The results are graphically shown in Figure 7 through Figure 10, corresponding to Examples 7-10.

**Table 3 Bacterial strains and pH conditions in Examples 7-10**

| Example No. | Bacterial strain | pH | Figure No. |
|---|---|---|---|
| 7 | *L. fermentum* | 5.5 | 7 |
| 8 | *L. fermentum* | 3.5 | 8 |
| 9 | *L. plantarum* | 5.5 | 9 |
| 10 | *L. plantarum* | 3.5 | 10 |

As shown in Figures 7-10, when DBNPA was at concentrations comparable to the biocidal concentrations of activated Bactebrom™, there was either none or negligible effect on both bacterial strains and the yeast, after a contact time of 1 hour, under both pH conditions tested. Notwithstanding the above, a minor biocidal effect was shown against *Lactobacillus fermentum* at pH 3.5.

**EXAMPLE 11**

*Yeast viability tests by optical microscopy*

The effect of activated Bactebrom™ at 2.5 and 5 ppm TCE on yeast viability was also evaluated by optical microscopy analysis, as detailed above.

Briefly, yeast viability in the presence of Bactebrom™ was evaluated by applying activated Bactebrom™ at dosage levels of 0, 2.5 and 5 ppm TCE to bread yeast or industrial yeast samples prepared as detailed above. After an incubation period of one hour, papain (5 mg) was added and after 5 minutes of further incubation, the samples were diluted, stained by Erythrosine and the numbers of viable and dead yeast cells were counted using optical microscopy as described above. Viability was calculated as the percentage of viable cells out of the total number of yeast cells (i.e., viable and dead yeast cells). The results are summarized in Table 4 below.

**Table 4**: Viability of yeast (% of viable cells/total yeast) after no treatment (Control), 2.5 and 5 ppm (as $Cl_2$) of activated Bactebrom™.

| Exp. No. | 1 | | 2 | | 3 | | 4 | | 5 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Yeast | Bread Yeast | | Bread Yeast | | Bread Yeast | | Ind. Yeast | | Ind. Yeast | |
| | Yeast Viability (%) | | Yeast Viability (%) | | Yeast Viability (%) | | Yeast Viability (%) | | Yeast Viability (%) | |
| Treat. | T0 | Tf (1h) | T0 | Tf (1h) | T0 | Tf (1h) | T0 | Tf (1h) | T0 | Tf (1h) |
| Control | ND | 79,3 | 65,1 | 38,8 | 94 | 94,7 | 83 | 85,6 | 87,5 | 81,3 |
| 2.5 ppm Bacteb. | ND | 98,6 | ND | 42,1 | ND | 100,0 | ND | 87,3 | ND | 89,7 |
| 5 ppm Bacteb. | ND | 89 | ND | 41,3 | ND | 94,5 | ND | 85,9 | ND | 87,0 |

**Abbreviations**: T0, measurement of cell viability obtained before incubation with Bactebrom; Tf(1h), measurement of cell viability after 1 hour of incubation with Bactebrom; ND, not determined; Bacteb, Bactebrom; Ind. Yeast, industrial yeast.

As evident from Table 4, exposure of yeast to the activated Bactebrom™ biocide resulted in either no reduction in yeast viability or with an improvement of the viability of the yeast, as shown in the presence of activated Bactebrom™ at 2.5 ppm TCE. Interestingly, in all assays performed (5 experiments in total) the viability after treatment with activated Bactebrom™ at 2.5 ppm TCE was higher as compared to the control measurement, demonstrating that activated Bactebrom™ did not affect the yeast viability and even had a beneficial effect.

The improved viability can be contributed to the fact that the biocide has eliminated specifically and only the harmful bacteria that can cause a reduction in yeast viability, leading, among others, to a reduction in the yield of the fermentation process.

Overall, the results obtained show that activated Bactebrom™ applied in determined concentrations can selectively act on bacteria and eliminate bacterial contaminations without damaging yeast growth. The beneficial effect shown for activated

Bactebrom™ can improve the viability of yeast and consequently improve ethanol yield in yeast-based fermentation processes. Furthermore, since Bactebrom™ is a non-antibiotic biocide, use thereof will avoid formation of tolerant and resistant bacteria in the fermentation process, and no residues of antibiotics in the dry yeast recovered from the fermentation process.

**CLAIMS:**

**1.** A method for producing ethanol by fermentation prone to prokaryotic contamination, said method comprising:

a) providing a fermentable substrate;

b) combining said fermentable substrate with yeasts in the presence of water to obtain a fermentation broth; and

c) fermenting said fermentation broth to obtain a fermented broth;

wherein the process further comprises contacting the yeasts with at least one oxidizing bromine-based biocide, obtained by on-site oxidation of an inorganic bromide source which is a solution comprising hydrobromic acid, or a metal bromide salt.

**2.** The method according to claim 1, further comprising

d) separating said fermented broth into spent yeasts and ethanol-containing liquid; and optionally

e) recovering said spent yeast.

**3.** The method according to claim 2, wherein the recovering step comprises contacting said spent yeast with said oxidizing bromine-based biocide and optionally with dilute sulfuric acid to obtain recovered yeast.

**4.** The method according to claim 2 or claim 3, wherein said recovered yeast are used for at least one additional fermentation cycle(s).

**5**. A method for purifying a yeast culture prone to prokaryotic contamination, said method comprising contacting said yeast culture with at least one oxidizing bromine-based biocide, obtained by on-site oxidation of an inorganic bromide source which is a solution comprising hydrobromic acid, or a metal bromide salt.

**6.** The method according to any one of the preceding claims, wherein the inorganic bromide source is a solution comprising HBr, or an alkali metal bromide.

**7.** The method according to any one of the preceding claims, wherein the inorganic bromide source is selected from the group consisting of: HBr, a solution of HBr and urea, and sodium bromide.

**8.** The method according to any one of the preceding claims, wherein the inorganic bromide source is oxidized on-site with any one of hypochlorite, chlorine, hydrogen peroxide, ozone, and/or electrochemically, to produce said oxidizing bromine-based biocide.

**9.** The method according to any one of the preceding claims, wherein said oxidizing bromine-based biocide is an aqueous solution of hydrobromic acid and urea, which reacts with hypochlorite on-site to produce the oxidizing bromine-based biocide.

**10.** The method according to any one of the preceding claims, wherein said at least one oxidizing bromine-based biocide is added at a dosage level of 0.2 to 25 ppm TCE.

**11.** The method according to any one of the preceding claims, wherein the at least one oxidizing bromine-based biocide is added at a continuous or an intermittent mode.

**12.** The method according to any one of the preceding claims, wherein said prokaryotic contaminants comprise lactic acid bacteria, *E. coli, or* any combination thereof.

**13.** The method according to claim 12, wherein said lactic acid bacteria are of the genus *Lactobacillus.*

**14.** The method according to claim 13, wherein said lactic acid bacteria of the genus *Lactobacillus* are *Lactobacillus fermentum, Lactobacillus plantarum* or a combination thereof.

**15.** The method according to any one of the preceding claims, wherein said yeast is *S. cerevisiae*, preferably selected from *S. cerevisiae* strains PE-2 strain, S288c, baker's yeast, and CEN.PK113-7D strain.

**16.** The method according to any one of the preceding claims, performed in absence of antibiotics.

**17.** The method according to any one of claims 1 to 15, further comprising adding an antibiotic in an amount of below 70% of the amount needed to control the contamination under identical conditions, but without the use of oxidizing bromine-based biocide.

**18.** The method according to any one of claims 1 to 4 and 6 to 17, wherein said contacting the yeasts with at least one oxidizing bromine-based biocide is performed during fermenting of said fermentation broth.

**19.** The method according to any one of claims 2 to 4 and 6 to 18, wherein separating said fermented broth comprises centrifuging said fermented broth and collecting at least a portion of said spent yeast.

**20.** The method according to any one of claims 1 to 4 and 6 to 19, further comprising separating ethanol from said ethanol-containing liquid.

**21.** The method according to claim 20, wherein said separation is performed by distillation.

**22.** The method according to any one of claims 1 to 4 and 6 to 21, wherein said fermentable substrate is derived from sugar-containing raw materials, preferably sugar beet, sugarcane, molasses, whey, sorghum or fruits, starch-containing feedstocks, preferably grain or root crops, such as corn, wheat, rice or cassava, or any combination thereof.

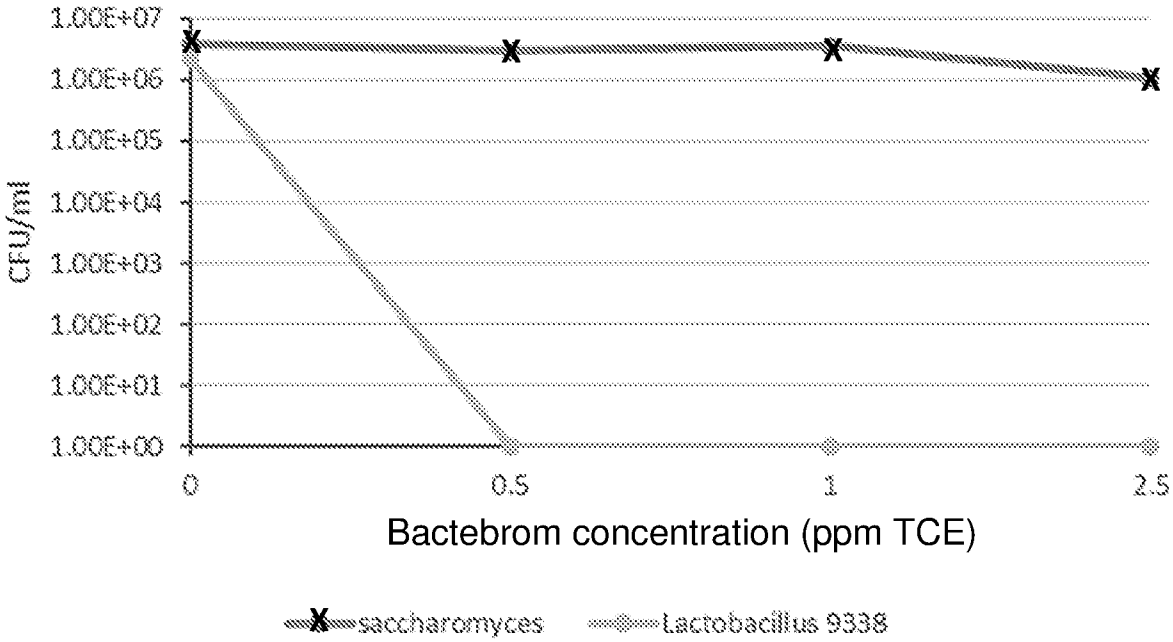



Figure 1

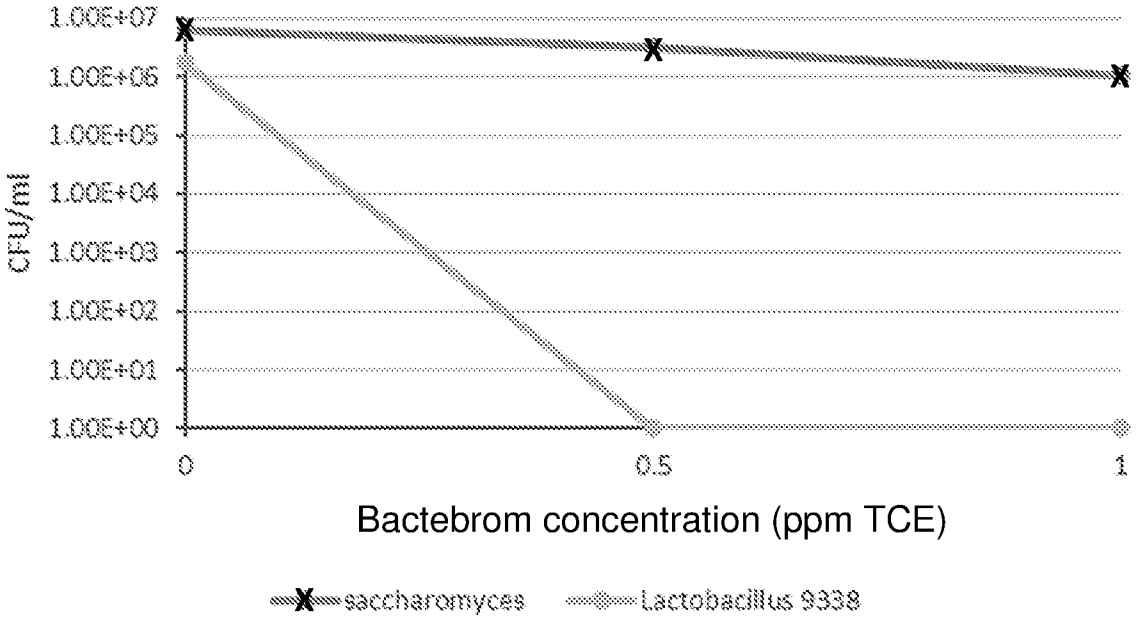



Figure 2

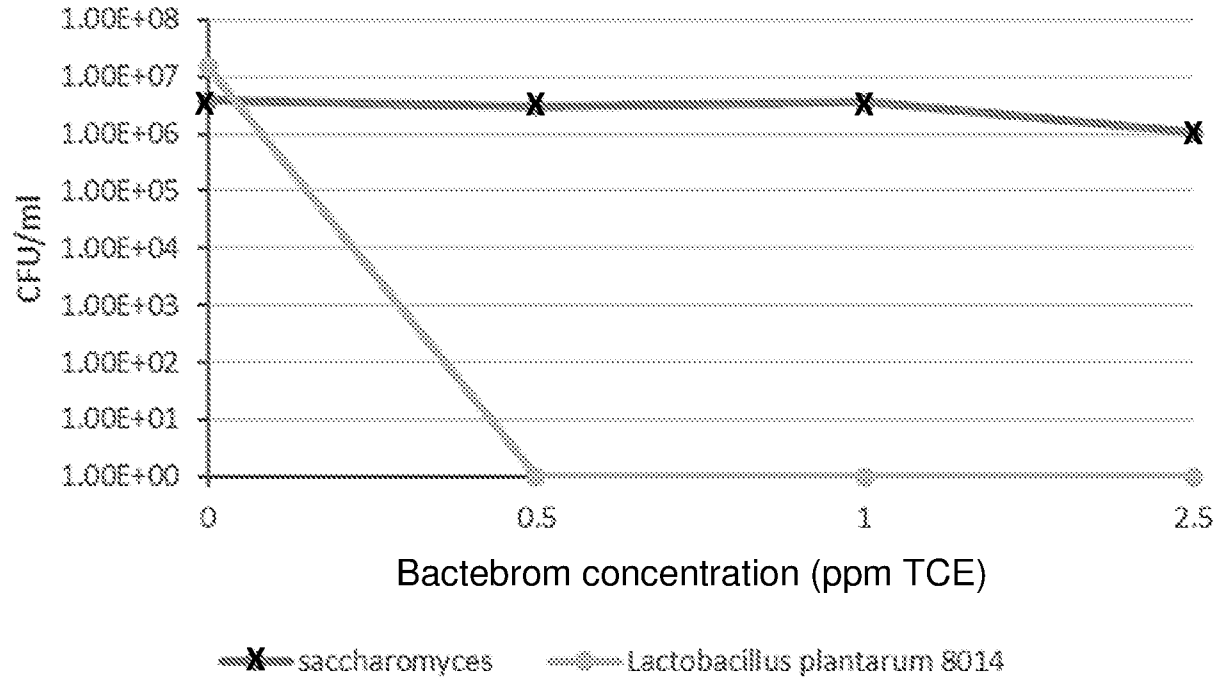



Figure 3

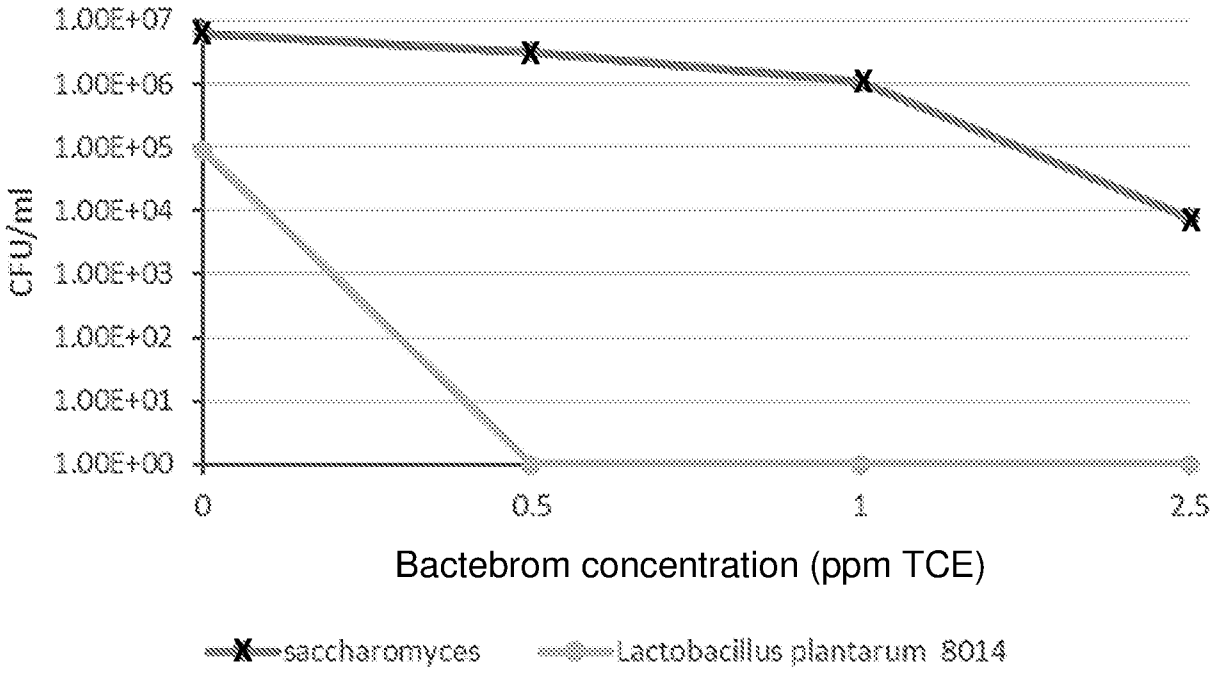



Figure 4

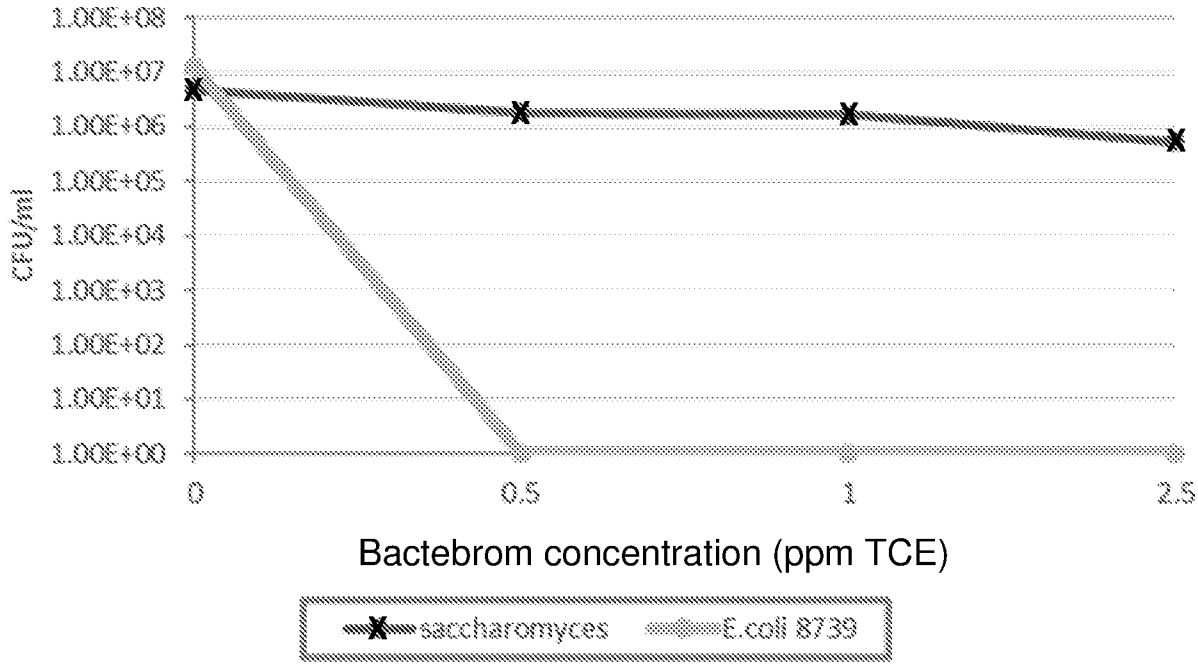



Figure 5

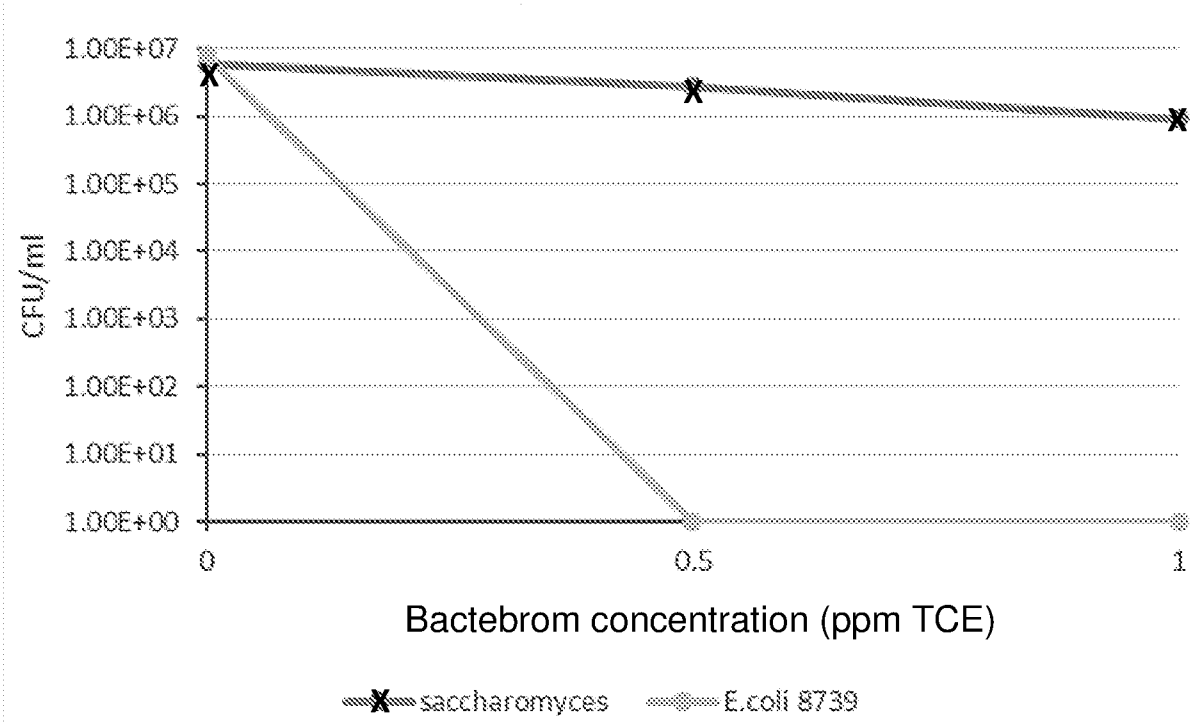



Figure 6

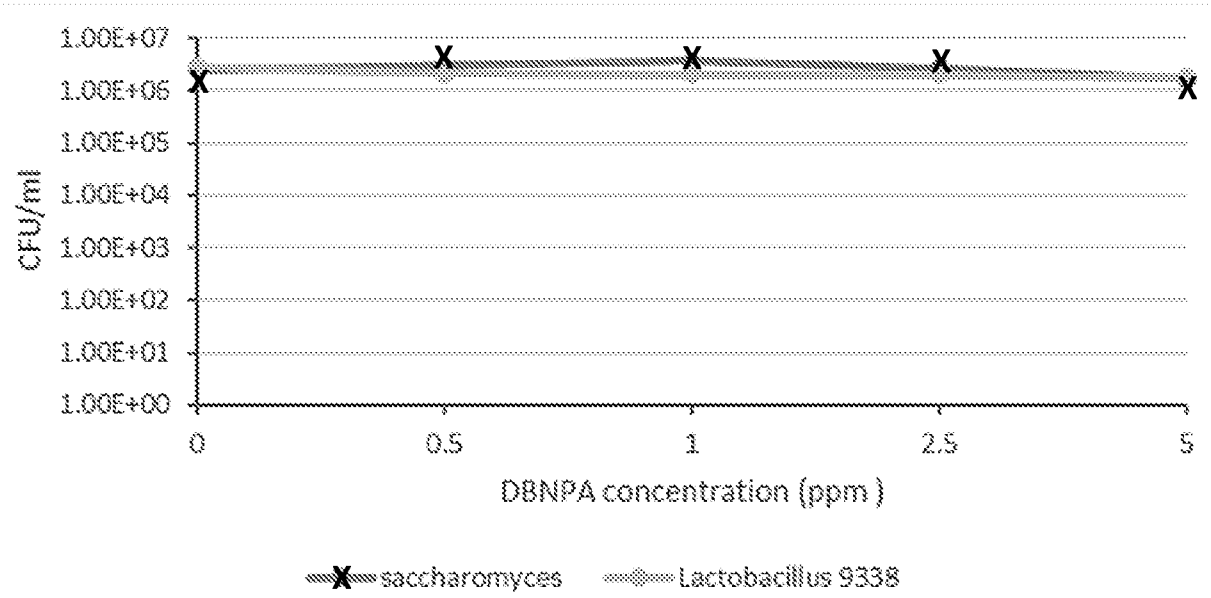



Figure 7

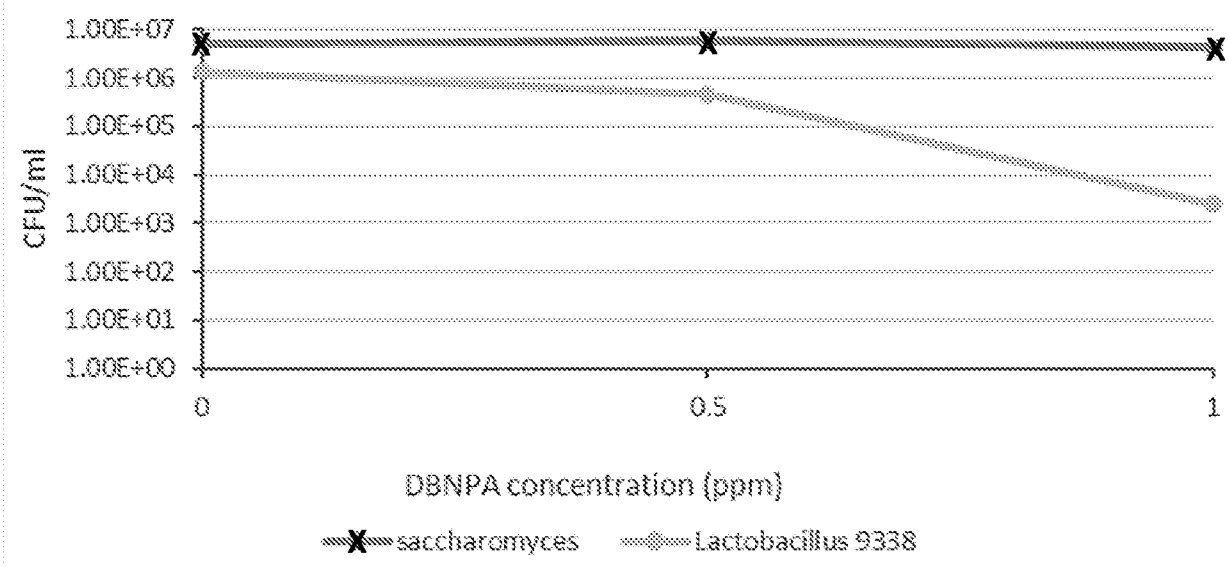



Figure 8

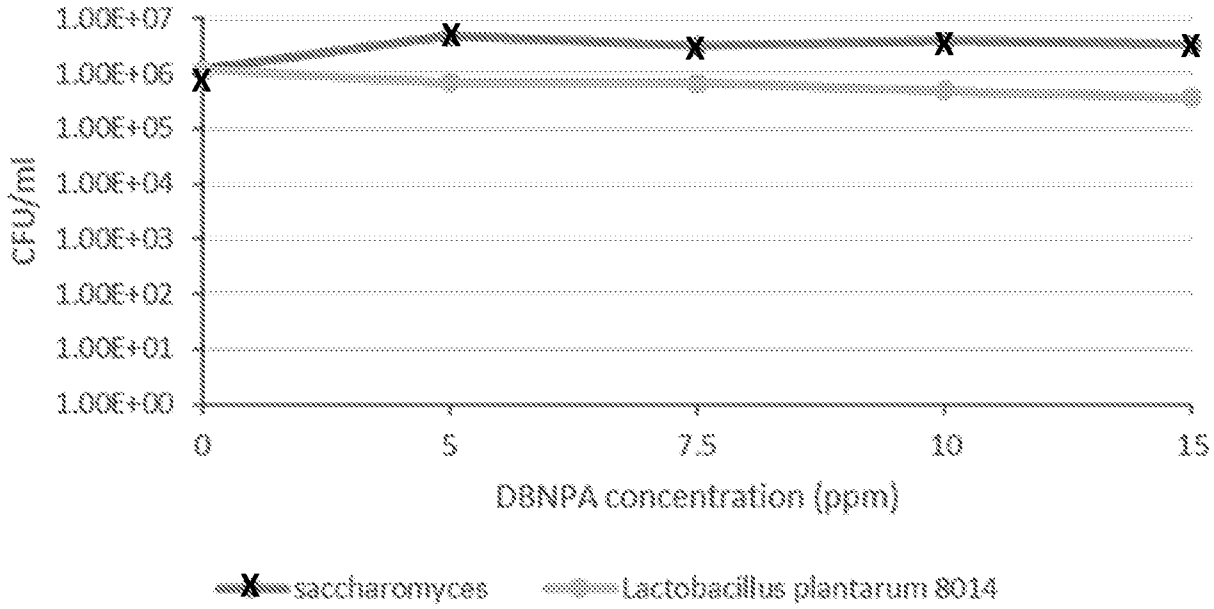



Figure 9

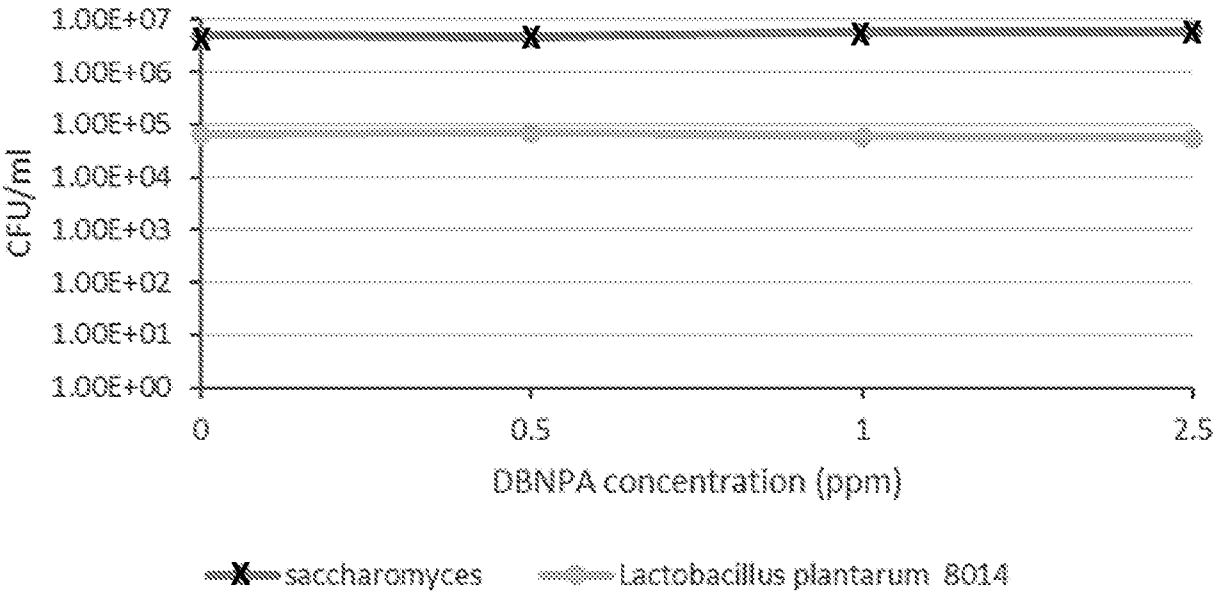



Figure 10

Figure 11

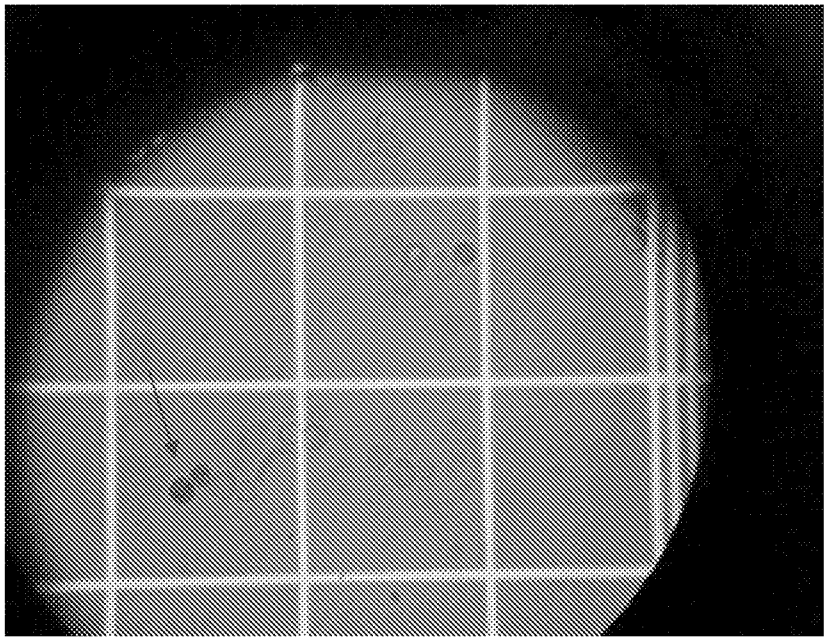
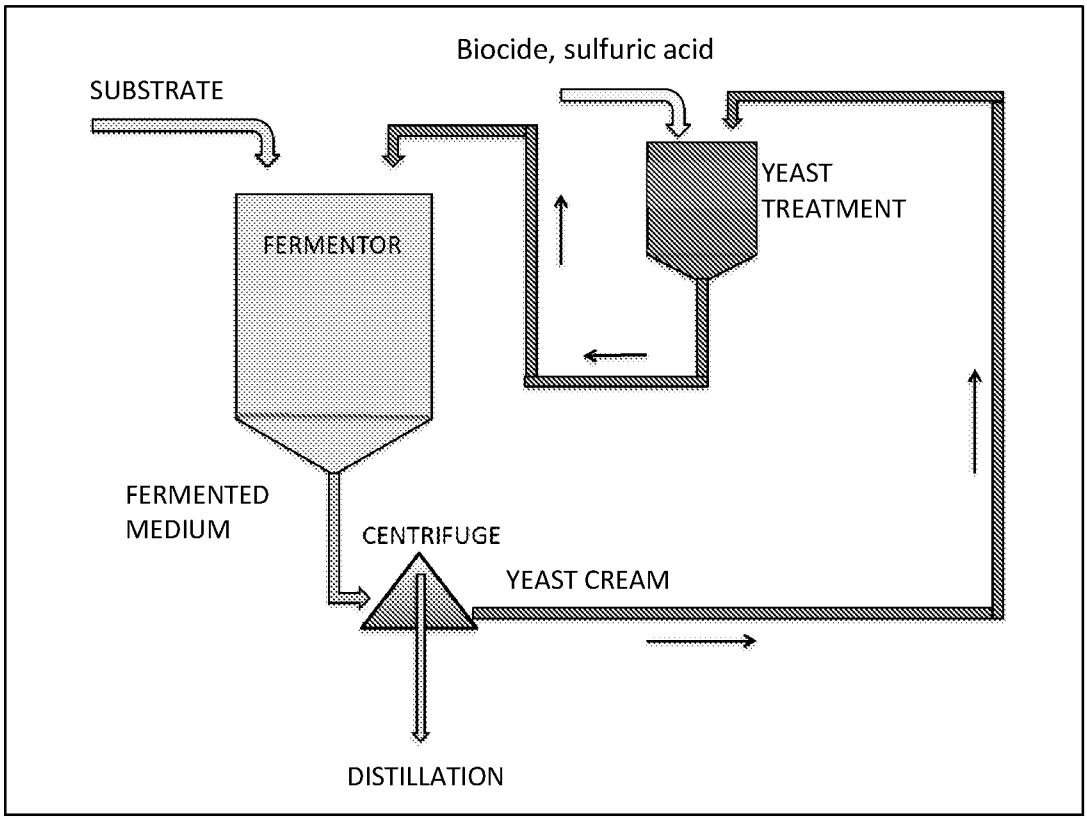


FIGURE 12



# INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2022/051175

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P7/06 A01N25/30 A01N37/06
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P A01N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | WO 2020/240559 A1 (BROMINE COMPOUNDS LTD [IL]; UNIV NEW YORK STATE RES FOUND [US]) 3 December 2020 (2020-12-03) cited in the application page 2, paragraph 3 page 5, paragraph 2 ----- | 1-22 |
| A | US 9 149 041 B2 (ANTEBI SHLOMO [IL]; ZOLKOV CHEN [IL] ET AL.) 6 October 2015 (2015-10-06) column 1, paragraph 4 column 4, paragraph 5 - column 6, paragraph 1 column 9, paragraph 2 ----- | 1-22 |

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IL2022/051175

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 2020240559 A1 | 03-12-2020 | EP 3975717 A1 | 06-04-2022 |
| | | JP 2022534915 A | 04-08-2022 |
| | | KR 20220015432 A | 08-02-2022 |
| | | US 2022240503 A1 | 04-08-2022 |
| | | WO 2020240559 A1 | 03-12-2020 |
| US 9149041 B2 | 06-10-2015 | AU 2010258226 A1 | 02-02-2012 |
| | | BR PI1012941 A2 | 15-09-2015 |
| | | CA 2763586 A1 | 16-12-2010 |
| | | EP 2448414 A2 | 09-05-2012 |
| | | ES 2722199 T3 | 08-08-2019 |
| | | JP 5784595 B2 | 24-09-2015 |
| | | JP 6127093 B2 | 10-05-2017 |
| | | JP 2012529496 A | 22-11-2012 |
| | | JP 2016028023 A | 25-02-2016 |
| | | MX 337704 B | 15-03-2016 |
| | | US 2012121728 A1 | 17-05-2012 |
| | | US 2015359224 A1 | 17-12-2015 |
| | | WO 2010143183 A2 | 16-12-2010 |