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(54) **Title:** USE OF SYNERGISTIC FORMULATIONS CONTAINING PEROXIDE AND QUATERNARY AMMONIUM REDUCE GROWTH OF CONTAMINANT MICROORGANISMS IN ETHANOL FERMENTATION

(57) **Abstract:** A method for controlling growth of contaminant microorganisms in a fermentation process using a combination of (a) peroxide compound (PC) and (b) a quaternary ammonium compound (QAC). The method comprises adding PC and QAC to one or more steps of a fermentation process. In this method, the PC and QAC may be added to one or more components of a fermentation broth comprising inoculant, fermentable sugar and process water.



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# USE OF SYNERGISTIC FORMULATIONS CONTAINING PEROXIDE AND QUATERNARY AMMONIUM REDUCE GROWTH OF CONTAMINANT MICROORGANISMS IN ETHANOL FERMENTATION

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## FIELD OF THE INVENTION

The present invention relates to synergistic combinations of a peroxide compound and a quaternary ammonium compound and method of use, particularly in fermentation processes.

## BACKGROUND OF THE INVENTION

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In the last decade, the use of ethanol as a transportation fuel has increased significantly. Ethanol production in the United States rose from approximately 6.4 billion liters in the year 2000 to over 37 billion liters in 2009. The number of ethanol plants increased from 54 in 2000 to 170 in 2009. Similar increases in production and plant construction have occurred in Latin America and Europe. In 2007, the United States Congress enacted the Energy Independence and Security Act (H.R. 6), which set the renewable fuel standard at 136 billion liters of ethanol by the year 2022. If this standard is to be met, the ethanol industry will continue to grow.

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Currently both industrial ethanol (e.g., fuel) and beverage ethanol are produced on large scale from agricultural (natural) feedstocks by fermentation processes in which sugar is converted to ethanol and carbon dioxide by inoculant yeast. Many feedstocks can be used to provide the sugar for fermenting, including potentially, any starch or cellulosic material, which includes nearly all plants, as any starch or cellulose can be a precursor to sugar. Some of the common feedstocks particularly suitable for producing fuel ethanol include corn, milo, sorghum, sugar cane, sugar beets and molasses.

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The feedstocks used for ethanol production are natural products therefore, a wide variety of microorganisms such as bacteria, fungi, and yeasts are likely to be naturally present in the feedstocks. Commercial fermentation process conditions are not completely sterile, hence these "contaminant microorganisms" will be present in the process. In commercial ethanol production, microorganisms of greatest concern are lactic acid-producing bacteria and acetic acid-producing bacteria. Such bacteria enter the process from several sources including raw materials, equipment, process water, air,

and inoculant yeast, among others. Concentrations of such bacteria may increase in the process environment either through introduction with incoming materials (raw materials, water, air, yeast) or naturally proliferate as a result of conditions favorable to bacterial growth. The optimum atmosphere for yeast production is also extremely conducive to the growth of these bacteria. Organic acids produced by the bacteria inhibit the growth of yeasts and thus reduce ethanol production rate. The bacteria may also consume sugars and other nutrients intended for use by the yeast to produce desired products, rendering the entire process less economical.

Many fermentation processes use antibiotics as antimicrobial compositions. Such use has become disfavored due to suspected development of antibiotic-resistant bacteria and accumulation of antibiotic residues in fermentation byproducts. Antibiotic-resistant bacteria are a significant concern in human health.

Byproducts of ethanol production include solids that are collected after distillation of the ethanol product. Such solids include distillers dried grains with solubles (DDGS) and distiller's wet grains with solubles (DWGS). Many countries are considering regulatory actions that would limit or eliminate the use of antibiotics for ethanol production. DDGS and DWGS are sold as animal feed products.

The need for antimicrobial treatments is increasing, not only because of the growth in production volume of ethanol but also the expansion in size of ethanol production facilities. Whereas a plant producing 150-200 million liters per year (MMly) was considered a large facility just a few years ago, 380 MMly (or more) facilities are today's industry standard. In fed-batch processes, the volume of individual fermentation batches has increased significantly. To accommodate this added capacity, the flow rate of feedstock (commonly known as "mash" once it has been prepared for entry into fermentation) into a fermentation system has increased from approximately 2000-3000 liters per minute to 4500-6000 liters per minute in the largest ethanol production facilities.

WO 2006/138271 discloses a method to control growth of a microorganism in an aqueous system or on a substrate comprising contacting the system or substrate with an antimicrobial agent comprising (a) a lactoperoxidase, (b) a peroxide source, (c) a halide or a thiocyanate, wherein the halide is not a chloride, and (d) an ammonium source. The lactoperoxidase is present in the aqueous system in a range of 0.01 to 1000

ppm; the peroxide source provides hydrogen peroxide in a range of 0.01 to 1000 ppm; the halide has a concentration in the aqueous system of 0.1 to 10,000 ppm and the ammonium source has a concentration in the aqueous system of 0 to 10,000 ppm.

WO 00/57730 discloses composition and method using composition as an antimicrobial treatment for food surfaces, which composition comprises (a) a quaternary ammonium compound, (b) an oxidant, (c) a halide source and optionally (d) a food grade source of acidity to lower pH to 9 or less. The halide is present at 0.1 to 30 parts by weight of the ammonium compound, at 0.1 to 40 parts by weight of the oxidant and at 0 to 80 parts by weight of the acidity source. The composition is used to reduce microbial count on food surfaces in a dilute aqueous composition comprising 0.1 to 400 grams per liter of the composition.

US Patent 5,320,805 discloses a method of using a chemical composition as a cleanser, sanitizer, disinfectant, sporicide, and fungicide, said composition comprising from about 10 to about 90% by weight of an alkaline water soluble salt having hydrogen peroxide of crystallization and from about a fraction of a percent to about 30% by weight of a positively charged phase transfer agent selected from the group consisting of a phosphonium salt, a sulfonium salt, and a quaternary ammonium, said composition forming both a water soluble and lipid soluble phase transfer ion pair.

WO 2009/0108365 discloses a method of producing a fermentation-based product comprising fermenting a sugar containing medium with yeast, in the presence of an organic biocide and a quaternary ammonium compound, in amounts sufficient to reduce or control a bacterial population in the medium.

Although some methods are known, there remains demand for improved methods for addressing contaminant microorganisms in the fermentation industry, both for carbohydrate-containing feedstocks and in fermentation processes. An improved method should preferably be antibiotic-free and not result in residues that accumulate in fermentation coproducts or give rise to antibiotic-resistant bacteria. The method should be efficacious at a wide variety of pH ranges and conditions encountered in the fermentation industry. The method should also use treatment that has a reasonable shelf life, prior to use. There is also a need to improve economics of today's larger volume fermentation processes. The present invention meets these needs.

### **SUMMARY OF THE INVENTION**

The present invention provides a method for controlling growth of contaminant microorganisms in a fermentation process using a combination of (a) peroxide  
5 compound (PC) and (b) a quaternary ammonium compound (QAC). The method comprises adding PC and QAC to one or more steps of a fermentation process, wherein the fermentation process wherein the fermentation process comprises (i) providing an inoculant, a fermentable sugar and process water; (ii) introducing separately, or in any combination, the inoculant, fermentable sugar and process water into a fermentation  
10 vessel to provide a fermentation broth; (iii) contacting the inoculant with the fermentable sugar in the fermentation vessel at a temperature at which the inoculant converts the fermentable sugar to ethanol. Optionally nutrients are added to one or more of the inoculant, fermentable sugar and process water. Nutrients may also be added directly to the fermentation vessel. The PC and QAC may be added to the  
15 inoculant, fermentable sugar or process water prior to introducing each of these to the fermentation vessel. Alternatively the PC and QAC may be added directly to the fermentation vessel.

The peroxide compound and quaternary ammonium compound may be added separately to the process. When adding the components separately, the components  
20 may be added simultaneously or sequentially in any order. That is, components may be added at the same time (simultaneously) or one component may be added prior to adding the other component (sequentially). Alternatively, the method may comprise contacting the components (a) and (b) prior to adding to the fermentation process.

The present invention provides a method for controlling the growth of  
25 contaminant microorganisms in the reactants (defined hereinbelow) in the fermentation broth, and in the products of a fermentation process. The method also controls growth of contaminant microorganisms on surfaces of components of a fermentation system. The method consists of, consists essentially of, or comprises the step of adding a peroxide compound and quaternary ammonium compound to a reactant or the  
30 fermentation broth, or to a surface or into a vessel of the fermentation system.

The present invention also provides a combination of components that can be used in cleaning-in-place (CIP) applications to treat surfaces of equipment used in fermentation processes. By "CIP" is meant herein that surfaces can be cleaned without the need to disassemble the equipment.

5           The method includes adding a combination of components which comprise PC and QAC in amounts effective to control the growth of contaminant microorganisms without detrimental effect on the inoculant used in the fermentation process. The effective amount varies in accordance with how the combination of components is used, but can be determined by one skilled in the art in view of the disclosures herein. PC is  
10 typically added in an amount to provide a concentration of PC in the fermentation broth of 10 ppm to 1000 ppm, based on the total weight of the fermentation broth. QAC is also typically added in an amount to provide a concentration of QAC in the fermentation broth of 10 to 1000 ppm based on the total weight of the fermentation broth.

15           The method of the present invention further provides a method to control growth of at least one contaminant microorganism in a carbohydrate feedstock wherein the step of providing a fermentable sugar comprises providing a carbohydrate feedstock and contacting the feedstock with a peroxide compound and quaternary ammonium compound, wherein the carbohydrate content of the feedstock is at least 1% and  
20 preferably 1 to 70%, by weight, based on the total feedstock weight, and wherein PC and QAC are added in effective amounts. Typically PC is added in an amount of 10 ppm to 1000 ppm of PC, based on total weight of the feedstock, and QC is typically added in an amount of 50 ppm to 500 ppm of the peroxide compound, based on the total weight of the feedstock.

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### **DETAILED DESCRIPTION OF THE INVENTION**

The present invention provides methods for controlling the growth of a contaminant microorganism comprising, consisting essentially of, or consisting of, adding a combination of components comprising peroxide compound (PC) and  
30 quaternary ammonium compound (QAC) to one or more steps of a fermentation process. Synergistic combinations of PC and QAC are added to the process in amounts

effective to control growth of contaminant microorganisms without inhibiting the ability of inoculant yeast to convert fermentable sugars into ethanol and carbon dioxide. PC and QAC are typically added to provide a concentration of PC in the fermentation broth of 10 ppm to 1000 ppm on a weight/weight basis, based on the total weight of the fermentation broth and concentration of QAC in the fermentation broth of 10 to 1000 ppm on a weight/weight basis, based on the total weight of the fermentation broth.

The present invention further provides in the step of providing a fermentable sugar, a method to control growth of at least one contaminant microorganism in a carbohydrate feedstock comprising, consisting essentially of, or consisting of, providing a carbohydrate feedstock and adding a peroxide compound and a quaternary ammonium compound to the feedstock.

### Definitions

The following terms have the definitions as provided below for use herein.

Aqueous medium means the medium is substantially water, such as for example greater than 80% water, preferably greater than 90% water, more preferably greater than 95% water. The aqueous medium can be greater than 99% water. Process water is an example of an aqueous medium.

Aqueous medium means the medium is substantially water, such as for example greater than 80% water, preferably greater than 90% water, more preferably greater than 95% water. The aqueous medium can be greater than 99% water. Process water is an example of an aqueous medium.

Carbohydrate feedstock means a feed used in preparation of or directly as a fermentable sugar. That is, a carbohydrate feedstock is a fermentable sugar or a composition comprising a fermentable sugar or a composition that can be converted to a fermentable sugar.

The carbohydrate feedstock may comprise up to 100% by weight of carbohydrates. Generally the carbohydrate feedstock comprises between 1% and 70% carbohydrate based on the total weight of the feedstock, preferably between 2 and 40%, as a solution or suspension in an aqueous medium. The amount and composition of the carbohydrates in the feedstock can vary depending on the intended end use. For example, corn steep liquor, which is a carbohydrate solution obtained from a wet mill

process, may comprise 16.5% carbohydrates. In a wet mill process, corn is soaked or steeped and then separated into various components. The corn steep liquor is the aqueous liquid obtained after the corn has been soaked for an extended period, during which readily fermentable soluble components are extracted from the corn solids into the steep water. The starch component from the wet mill process may comprise up to 40% by weight carbohydrates.

The carbohydrate feedstock may comprise other components generally functioning as adjuncts to the solutions and/or suspensions. For example, the carbohydrate feedstock may comprise enzymes, surfactants, dispersants, antifoaming compositions, minerals, trace elements, and combinations of two or more thereof. These components and other components that act as adjuncts are well-known to those skilled in the art.

Control as applied to growth of a microorganism herein means to reduce and/or prevent the growth of a targeted undesirable contaminant microorganism. Controlling the growth of a microorganism as used herein also includes to maintain the microorganism population at a desired level, to reduce the population of the microorganism to a desired level (partially reduce or even reduce to undetectable limits, e.g., zero population), and/or to inhibit growth of the microorganism.

An effective amount refers herein to an amount of each of the PC and QAC that, in combination, when added to a fermentation process, is effective to control the growth of contaminant microorganisms without detrimental effect on the inoculant used in the fermenta A fermentable sugar is a reactant and common nutrient for inoculants used in ethanol fermentation processes. The fermentable sugar is a sugar (e.g. glucose) or starch that is converted by action of yeast to ethanol and carbon dioxide. Equation (1) illustrates the process for glucose ( $C_6H_{12}O_6$ ).



A fermentable sugar as used herein is a carbohydrate that is derived from essentially any plant source comprising sugar, starch and/or cellulose, generally provided in the form of a solution or suspension of the sugar, starch and/or cellulose in water. Starch and/or cellulose can be converted by processes known in the art, e.g., using enzymes, to a fermentable sugar for use in the method of this invention. The



fermentable sugar can be derived from sources of starch or one or more cellulosic material such as corn, sorghum, wheat, wood chips, milo, barley, millet, sugar cane, sugar beets, molasses, whey, potatoes, wheat straw, corn stover, switch grass, algae, seaweed, and/or other biological sources. The fermentable sugar may also be derived from fruit juice (e.g., grapes, apples). The fermentable sugar may alternatively be derived from non-traditional feedstocks such as wood waste, bagasse, paper waste, and municipal solid waste. Processes are known to those skilled in the art to convert these sources to fermentable sugar. For reference, see, J. Y. Zhu, et al. *Bioresource Technology* (2010) vol. 101 (13), pp. 4992-5002; and A. L. Demain, *J. Ind. Microbiol. Biotechnol.* (2009) vol. 36(3), pp. 319-332.

Conveniently, the fermentable sugar is derived from corn, using either the dry grind or wet mill process. In a dry grind process, corn is ground into meal and processed without separation into its constituent components comprising essentially fiber, starch, oil, and protein. In a wet mill process, corn is soaked or steeped in water and then mechanically separated into its constituent components. The corn starch component from the wet mill process or meal (corn flour) from the dry grind process is mixed with water and enzymes and cooked to solubilize the starch.

Corn starch is a polysaccharide, that is, a polymer made of individual units of glucose. The corn starch is converted to smaller (shorter) polysaccharides, i.e., dextrins, by enzymes ( $\alpha$ -amylase). The smaller polysaccharides are converted to glucose (monosaccharide) using the enzyme glucoamylase, thus forming the fermentable sugar.

As an alternative to corn, the fermentable sugar can be derived from molasses. Molasses can be obtained from a variety of sources including sugar cane or sugar beets, for example, as a byproduct of the process to manufacture crystalline sugar. Molasses is typically obtained as a syrup, to which other ingredients may be added in preparation for fermentation. These other ingredients include sugarcane juice, beet juice, water, and vitamins or other nutrients. Whether one or more of the other ingredients are added and the amount added will vary in a molasses-derived fermentable sugar.

As an alternative to corn, the fermentable sugar can be derived from sugar cane juice. Sugar cane juice is juice extracted with water from sugar cane. The extraction of juice from sugarcane is also accomplished by physical crushing, diffusion in water, or

other methods generally well known to those skilled in the art. See, for example, H. V. Amorim, et al, in “The Alcohol Textbook” (2009), Nottingham University Press , Nottingham, pp. 39-46; and EPA Food and Agricultural Industries Handbook, chapters 9.10.1.1 (sugar cane) and 0.10.1.2 (sugar beets), available at  
5 <http://www.epa.gov/ttnchie1/ap42/ch09/> (accessed December 18, 2011). Sugar cane juice can be used without further processing and added directly to a fermentation vessel as the fermentable sugar.

For purposes herein, steps used to produce carbohydrate feedstocks and fermentable sugars are steps of the fermentation process . That is, the fermentation  
10 process comprises steps to produce carbohydrate feedstocks, and steps to convert starch and/or cellulose to a fermentable sugar.

In the fermentation process, carbohydrates, including sugars and starches, is typically present in the fermentation broth at a concentration of about 5 to about 40% (weight/volume), preferably in the range of about 10 to 35% (weight/volume), based on  
15 the total volume of the fermentation broth.

Fermentation broth as used herein means the contents of a fermentation vessel during the fermentation process after all reactants have been added and typically comprises fermentable sugar, inoculant, optional nutrients and process water.

Fermentation process as used herein means a process comprising contacting an  
20 inoculant, such as yeast, with a fermentable sugar, and optional nutrients, in process water to produce ethanol. A fermentation process can be batch or continuous. In addition, for purposes herein, fermentation also includes steps for preparing or pretreating one or more of the reactants, such as processes to prepare the fermentable sugar from natural sources, and yeast propagation.

25 The contacting step is performed at a temperature at which the inoculant converts sugar to ethanol and carbon dioxide.

When ethanol is produced via a dry grind process using corn as a feedstock, the fermentation process may comprise one or more of the steps of slurring of the corn, liquefaction, saccharification, preparing inoculant yeast (propagation), fermenting the  
30 mash(actual step of action of inoculant on sugar to produce ethanol), separating and recovering the resultant ethanol, and optionally recycling the spent inoculant yeast.

The fermentation process may comprise one or more of the steps of preparing inoculant yeast, preparing the juice by dilution, pasteurization, or other such process, fermenting, separating and recovering the ethanol, and optionally recycling the yeast. It will be understood by those skilled in the art that these process steps may vary, depending on feedstock availability and plant design and plant location.

Fermentation system as used herein comprises components (equipment), such as vessels and pipes in which and through which one or more of the reactants and products of a fermentation process resides or passes. Such equipment have surfaces on which bacteria and other contaminant (undesirable) microorganisms may be present.

As part of a fermentation system is a fermentation vessel – the vessel in which the fermentation step of reacting a fermentable sugar with inoculant to produce ethanol occurs.

When ethanol is produced via a dry grind process, the fermentation system may comprise one or more vessels such as a slurry tank, liquefaction tank, saccharification tank, yeast propagation tank, and fermentation vessel. Such vessels and their use are known to those skilled in the art. See, for example, R. J. Bothast and M. A. Schlicher, Applied Microbiology and Biotechnology ( 2005), vol. 67(1), pp. 19-25. When ethanol is produced via a wet mill process, the fermentation system may comprise one or more vessels such as a steep tank, a separation tank, yeast propagation tank, and fermentation vessel. It will be understood by those skilled in the art that these process steps may vary, depending on feedstock availability and plant design and plant location.

An inoculant is any microorganism added purposely to a process in order to convert a feedstock (input) to a desired product (output). For purposes herein, an inoculant is a microorganism which is capable of converting a fermentable sugar to ethanol. Yeasts are common inoculants used in ethanol fermentation. Yeasts are microorganisms capable of living and growing in either aerobic (with oxygen) or anaerobic (lacking oxygen) environments. An inoculant may also be selected from the group consisting of fungi, and algae that are capable of converting a fermentable sugar to ethanol. For example, ethanol can be produced from cellulose using ethanol-producing bacteria as the inoculant. When used in accordance with the method of this invention, the inoculant bacteria is not adversely affected by the stabilized

chlorine dioxide and peroxide compound as are lactic acid- and acetic acid-producing bacteria.

The following discussion is directed to a process in which the inoculant is yeast.

For purposes herein, an inoculant yeast is a yeast which has been deliberately  
5 selected for a particular conversion in a reaction system. For example, an inoculant yeast may be selected for production of additional yeast in yeast propagation (such as for use as baker's yeast); an inoculant yeast may be selected for metabolism of a particular nutrient for production of enzymes. In this specific application, an inoculant yeast is selected for fermentation of a fermentable sugar to produce ethanol of desired  
10 quality and in desired quantity. Inoculant yeasts are generally selected because of their ability to completely ferment available sugars, tolerance to high levels of osmotic stress, elevated temperatures, and high concentrations of ethanol. Yeast are commonly used in ethanol fermentation. Yeast are microorganisms capable of living and growing in either aerobic (with oxygen) or anaerobic (lacking oxygen) environments. Suitable  
15 inoculant yeasts for use in the process of this invention include, but are not limited to *Saccharomyces cerevisiae* (*S. cerevisiae*), *Saccharomyces uvarum* (*S. uvarum*), *Schizosaccharomyces pombe* (*S. pombe*), and *Kluyveromyces* sp.

The inoculant yeast may be introduced into the fermentation vessel as a yeast inoculum, which is an activated form of the inoculant yeast. That is, prior to  
20 introducing inoculant yeast into a fermentation vessel, a yeast inoculum is produced by contacting a yeast starter culture and a nutrient composition in a propagation tank to propagate (increase the quantity of) the yeast. The nutrient composition comprises one or more fermentable sugar, enzyme, additional nutrients, and water to grow or activate the yeast. The propagation tank is a separate vessel from the fermentation vessel and is  
25 operated at suitable conditions of temperature (e.g., 68-104°F, 20-40°C). Each inoculant will have preferred temperature for propagation. For example, a temperature of 30-32°C (86-90°F) may be particularly suitable for propagating *S. cerevisiae*. While it is recognized that yeast propagation can occur in the fermentation vessel during the fermentation process, activation of yeast in a propagation tank provides a highly active  
30 inoculant yeast. Thus, highly active yeast is introduced to the fermentation vessel. Such yeast propagation techniques are known to those skilled in the art. See, for

example, E. Bellissimi, et al., *Process Biochemistry* (2005), vol. 40(6), pp. 2205-2213; and M. Knauf, et al., *Sugar Industry* (2006), vol. 131, pp. 753-758.

For continuous fermentation processes, there is often no separate yeast propagation tank. Yeast may or may not be recycled in a continuous process. When no recycle is available, yeasts grow and produce ethanol continuously in a stage called a primary fermentation. When recycle is available, such as is common when molasses or sugarcane juice is used as a feedstock for the fermentable sugar, yeasts are recycled by separation from the other fermentation components, (usually using centrifugation) and typically treated with acid in a separate tank (generally called a yeast recycle tank) to condition the yeast cells for a new fermentation cycle. Alternatively, the yeast may be separated from the fermentation broth, then dried and sold as a co-product. These steps are well known to those skilled in the art. See, for example, E. A. N. Fernandes, et al., *Journal of Radioanalytical and Nuclear Chemistry* (1998) vol. 234 (1-2), pp. 113-119; and L. C. Basso, et al., *FEMS Yeast Res.* (2008) vol. , pp. 1155-1163.

Relative to bacteria, yeasts may have moderate to slow fermentation rates. To compensate for their metabolic rate, large amounts of yeast may be required in large scale industrial ethanol production. Inoculant yeast is generally added to the fermentation process in an amount typically about  $1 \times 10^5$  to  $1 \times 10^7$  cells per gram of fermentation broth. It will be recognized by those skilled in the art that this amount may vary depending on the method of fermentation employed.

Mash is used herein to refer to a composition comprising a fermentable sugar. Mash includes any mixture of mixed grain or other fermentable carbohydrates in water used in the production of ethanol at any stage from mixing of a fermentable sugar with water to prior to any cooking and saccharification through to completion of fermentation, as defined in Jacques, K.A., Lyons, T.P., Kelsall, D.R., "The Alcohol Textbook", 2003, 423-424, Nottingham University Press, UK.

Microorganisms in the context of this invention are in two categories, desirable and contaminant (undesirable) microorganisms. A desirable microorganism has the capability of consuming nutrients to convert a fermentable sugar to ethanol. Desirable microorganisms include inoculants such as the yeast, *Saccharomyces cerevisiae*, which is used in the fermentation of glucose into ethanol and carbon dioxide. Other desirable microorganisms are used in other biorefinery processes. Desirable microorganisms are

not typically present in carbohydrate feedstocks. When desirable microorganisms are present in a carbohydrate feedstock, the number of viable cells present is typically too low to compete favorably with the other microorganisms commonly also present in the feedstock.

5           A contaminant microorganism is a microorganism that competes with the inoculant for and consumes the fermentable sugar and nutrients. Contaminant microorganisms produce undesirable products and/or produce alcohol at a much lower rate than would be produced by the inoculant.

10           Contaminant microorganisms include bacteria, fungi, wild or contaminant yeasts, and other microorganisms capable of metabolizing components of a carbohydrate feedstock to sustain the viability of the microorganism. Contaminant microorganisms contaminate carbohydrate feedstocks, consume the feedstock as a food source in support of their growth, multiply, and thus deplete the feedstock.

15           Contaminant microorganisms such as contaminant yeasts are often found in both industrial and beverage ethanol production, and can cause severe episodes of contamination, resulting in reduced ethanol productivity in a fermentation process. These unwanted microorganisms may be introduced into the process through the fermentable sugar (feedstock), process water, air, operators, and numerous other sources.

20           Contaminant microorganisms, such as bacteria, including lactic acid bacteria (species of *Lactobacillus*) produce products such as acetic and lactic acids from glucose feedstocks, that not only consume the feedstock and thus prevent feedstock conversion to desired products, but also adversely affect desirable microorganisms in a biorefining process. For example, acetic and lactic acids adversely affect the rate at which  
25   *Saccharomyces cerevisiae* converts glucose to ethanol. Other contaminant bacteria include species of *Pediococcus*, *Enterococcus*, *Acetobacter*, *Gluconobacter*, and *Clostridium*.

30           A nutrient means an element which supports growth and/or survival of the inoculant yeast or other inoculant microorganism, for example a nutrient may be a source of carbon, nitrogen, oxygen, sulfur, phosphorus, magnesium, and a variety of trace elements such as metals. A carbon source can be an alcohol such as methanol, or

a hydrocarbon such as octane. A carbon source may also be the fermentable sugar. A nitrogen source can be urea, ammonia, an ammonium salt or a nitrate. A salt such as magnesium sulfate can provide magnesium and sulfur. Phosphorus can be supplied as a sodium or potassium salt, while oxygen can be supplied by introducing air into the process. Other forms of gaseous oxygen can be used, such as pure oxygen or oxygen/nitrogen mixtures. Other elements can be added directly or may be naturally present in process components.

Peroxide compounds, as used herein, refers to compounds that contain at least one oxygen-oxygen single bond. Preferred peroxides are selected from the group consisting of hydroperoxides, organic peroxides, inorganic peroxides and peroxygen-releasing compounds.

Hydroperoxides are compounds of the general formula  $R-O-O-H$  wherein  $R$  is a hydrogen or straight, branched and/or cyclic alkyl radical having 1 to 20 carbons atoms and can be optionally interrupted by one or more oxygen and/or carbonyl groups. Examples of hydroperoxides include, but are not limited to, hydrogen peroxide, acetic peroxide, tert-butyl hydroperoxide, cumene hydroperoxide, and diethyl ether hydroperoxide.

Organic peroxides are compounds that have the general formula  $R^1-O-O-R^2$ , where  $R^1$  and  $R^2$  are independently straight, branched, and/or cyclic alkyl radical having 1 to 20 carbons atoms and can be optionally interrupted by one or more oxygen and/or carbonyl groups. Examples of organic peroxides include, but are not limited to, benzoyl peroxide and di-tert-butyl peroxide.

Inorganic peroxides are compounds that contain an  $O_2^{2-}$  anion and have the general formula of  $M_2O_2$  or  $M^1O_2$  wherein  $M$  is an alkaline metal and  $M^1$  is an alkaline earth metals. Examples of inorganic peroxides include, but not limited to, sodium peroxide, calcium peroxide, and barium peroxide.

Peroxygen-releasing compounds are also useful in the present invention. More particularly a nitrogen-free peroxygen-releasing compound is defined herein as any nitrogen-free material capable of releasing hydrogen peroxide or other oxygen-containing radicals upon addition to an aqueous system. Examples of peroxygen-releasing compounds include alkali metal, alkaline earth metal and

transition metal compounds of percarbonates, perborates and peroxides, and suitable mixtures thereof. Preferred peroxygen-releasing compounds include, but are not limited to, sodium percarbonate, sodium perborate, sodium peroxide, calcium peroxide, magnesium peroxide and zinc peroxide.

5            Preferably, the peroxide used in the present invention is hydrogen peroxide, herein referred to as "HP". HP is commercially available in concentrations of 33 to 37 % by weight in water. Commercially available HP may also contain additives. Additives include stabilizers, such as phosphoric or other mineral acids, and inhibitors, such as pyrophosphate salts and stannate compounds.

10            Preserve and preservation means treating a carbohydrate feedstock to prevent reaction or consumption of carbohydrate by contaminant microorganisms such as bacteria. Preservation provides a stable carbohydrate feedstock that does not undergo substantial change, such as would result from reaction or consumption of the carbohydrates due to microbial metabolism, over a period of time of at least one month.

15            One measure of change is the microbial population of the preserved feedstock. When properly preserved, the carbohydrate feedstock does not undergo an increase in the microbial population in the feedstock of more than 1 log<sub>10</sub> CFU/ml or 1 log<sub>10</sub> CFU/g. Typically microbial population is expressed as log<sub>10</sub> CFU/ml for liquid feedstocks and as log<sub>10</sub> CFU/g for solid/semi-solid feedstocks. The expression log<sub>10</sub> CFU/g can also

20            be used for liquid feedstocks.

A second measure of change with respect to preservation is based on the concentrations of particular acids in the feedstock. Acids are known products resulting from reaction and/or consumption of carbohydrates by contaminant bacteria. For example, certain bacteria known to contaminate carbohydrate feedstocks produce lactic

25            acid and acetic acid upon consumption of the carbohydrate. Increasing concentrations of lactic acid and acetic acid in a carbohydrate feedstock can be used to indicate whether the feedstock is properly preserved. When properly preserved, concentration of lactic acid in the carbohydrate feedstock is less than 0.60% (weight/volume) and concentration of acetic acid in the carbohydrate feedstock is less than 0.30 %

30            (weight/volume).

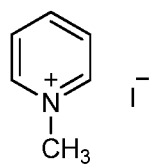
It will be further appreciated by those skilled in the art that other measures of change may be used. For example, detection of the presence of undesired compounds



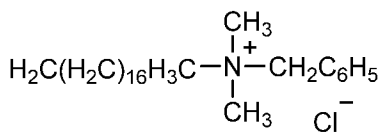
may indicate change, such as a product from metabolism of the carbohydrate feedstock. Detection methods may include spectrophotometry, chromatography, and other methods known to those skilled in the art. Still other measures may include physical changes to the carbohydrate feedstock such as specific gravity or viscosity.

- 5            Quaternary ammonium compounds (QAC) have the general formula  $R_4N^+X^-$  and are a type of cationic organic nitrogen compound, where X is any halogen. R is selected from the group consisting of saturated and unsaturated alkyl, aryl, alkylaryl, phenyl, allyl, and alkylphenyl groups which may be connected together in any combination. QAC are salts having quaternary ammonium cations and halogen anions, wherein the cations remain permanently charged independent of the pH of the solution. R may either be equivalent or correspond to two to four distinctly different moieties. R may be any type of hydrocarbon: saturated, unsaturated, aromatic, aliphatic, branched chain, or normal chain with 1-24 carbons. R may also be an alkylamidoalkylene, hydroxyalkylene, or alkoxyalkylene group having 1-8 carbon atoms, wherein the hydrocarbon or alkylene chain may be interrupted by one or more heteroatoms selected from the group consisting of nitrogen, sulfur, and phosphorus. R may also contain additional functionality and heteroatoms. The nitrogen atom, covalently bonded to four organic groups, bears a positive charge that is balanced by the anion "X". Heterocyclics, in which the nitrogen is bonded to two carbon atoms by single bonds and to one carbon by a double bond, are also considered quaternary ammonium compounds. Examples of QAC include (1) methylpyridinium iodide, (2) benzyldimethyloctadecylammonium chloride, and (3) di(hydrogenated tallow)alkyldimethylammonium chloride, where the alkyl groups, R, can be the same or different and have 14 to 18 carbon atoms, which are illustrated below.

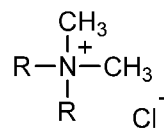
25



(1)



(2)



(3)

Quaternary ammonium compounds also include sources of protonizable nitrogen. Various sources of quaternary ammonium compounds or protonizable

nitrogen can be used in this invention, including cholines, lecithins, betaines, and amine oxides.

Quaternary ammonium compounds useful in the present invention may be selected from the group consisting of alkyl dimethyl benzylammonium chloride;  
5 dioctyl-dimethylammonium chloride; didecyl dimethyl ammonium chloride; diallyl-dimethylammonium chloride; di-n-decyl-dimethylammonium chloride; cetylpyridinium chloride; benzethonium chloride; and mixtures thereof.

Reactant means inoculant, fermentable sugar, optional nutrient as well as any other optional components in a fermentation broth. For purposes herein, reactant also  
10 includes process water.

#### Method for Growth Control

The method of this invention for controlling growth of one or more contaminant microorganisms comprises, consists essentially of or consists of adding a combination  
15 of the components (a) peroxide compound (PC) and (b) quaternary ammonium compound (QAC), to one or more steps of a fermentation process.

The components PC and QAC may be added separately to the fermentation process. When added separately, the PC and QAC may be added simultaneously (at the same time) or sequentially (at different times) in any order to one or more different  
20 process steps.

The components PC and QAC may be added separately or in combination to any of the reactants such as the inoculant, fermentable sugar or process water prior to introducing the reactant and component into the fermentation vessel.

Alternatively, the method may comprise contacting components PC and QAC  
25 of the composition prior to adding to the fermentation process. Preferably components PC and QAC are contacted prior to adding and are thus added as a single composition to the fermentation process.

The fermentation process may be batch or continuous. In a batch fermentation, preferably the components PC and QAC are added as a single composition (contacted  
30 prior to adding) or separately to the fermentation vessel prior to adding a fermentable

sugar, more preferably prior to adding any of the reactants. In a continuous fermentation process, components PC and QAC may be added as a single composition in a yeast propagation step or in the contacting step to the fermentation vessel. If more than one fermentation vessel is used in series, preferably the components are added to  
5 the first or primary fermentation vessel in the series.

Preferably PC and QAC are added to the process prior to the production of significant amount of ethanol, or prior to the production of significant amounts of organic acid associated with growth of contaminant bacteria. By “significant amounts of ethanol”, it is meant that the amount of ethanol in the fermentation broth is not more  
10 than 10% by weight, based on the total volume of the fermentation broth. By “significant amounts of organic acids”, it is meant that the amount of organic acids in the fermentation broth is not more than 0.3% by weight, based on the total volume of the fermentation broth. Preferably PC and QAC are added early in the fermentation process, such as during the propagation of inoculant yeast, before or just after inoculant  
15 yeast has been introduced into the fermentation vessel.

In another embodiment, PC and QAC are added to the fermentation process prior to the introduction of inoculant into the fermentation vessel. In this embodiment, PC and QAC may be added to the fermentable sugar or process water, or both, prior to introducing either of these reactants to the fermentation vessel and the combined  
20 PC/QAC/fermentable sugar and/or PC/QAC/process water is introduced to the fermentation vessel prior to introducing the inoculant to the fermentation vessel. For example PC and QAC may be added to one or more steps of providing the fermentable sugar, such as to one or more steps of a wet mill or dry grind process.

When the fermentation process includes a sugarcane- or (sugar beet)  
25 molasses-based fermentable sugar, it is typical to have fermentation vessels in series, having a first, second or even third or more fermentation vessel in a series of vessels where the fermentation broth is successively transferred into each fermentation vessel as part of the fermentation cycle. In this embodiment, preferably the PC and QAC are preferably added to the first fermentation vessel.

30 Alternatively, in a fermentation process where sugarcane or sugar beet or molasses is used as the fermentable sugar, PC and QAC are added to the fermentable sugar prior to contacting the fermentable sugar with inoculant. PC and QAC may be

added during the storage of the fermentable sugar. Such step of adding PC and QAC during storage is contemplated herein as a step of the process for providing the fermentable sugar prior to introducing the fermentable sugar into the fermentation vessel. Preferably, PC and QAC are added to the fermentable sugar just prior to  
5 introducing of the fermentable sugar into the fermentation vessel, for example, after process water and nutrients have been added to the fermentation vessel.

PC and QAC may also be added to an empty fermentation vessel, prior to adding the reactants.

In another alternative, for a batch process in which sugarcane or molasses is the fermentable sugar, and in which the inoculant is recycled at the completion of each  
10 fermentation cycle, PC and QAC may be added to a yeast treatment stage (yeast propagation) prior to the beginning of a subsequent batch in a fermentation process. That is, the PC and QAC are added separately, simultaneously, or as a combined composition, into the yeast recycle stream, which is commonly referred to as yeast  
15 cream.

In one embodiment, PC and QAC are added to the process water. Such step of adding PC and QAC to process water is contemplated herein as a step of the process for providing the process water, prior to introducing process water into the fermentation vessel. Process water includes any water that is introduced into the fermentation  
20 process from either external sources or recycled from other parts of the fermentation process.

In a molasses-based fermentation process, process water includes water used to dilute incoming molasses prior to fermentation, water added as part of the yeast preparation process, or water recycled from preceding fermentation steps, including  
25 yeast recycle stream.

In a dry grind fermentation process, process water includes water added into the steep tank, slurry tank, yeast propagation, or fermentation vessels. The process water may also be a recycle stream in a dry grind batch fermentation process, wherein the recycle stream is a stream produced after fermentation is complete, and is commonly  
30 referred to as backset or process condensate. These stages of the dry grind fermentation process are well known to those skilled in the art. See, for example, R. J.

Bothast and M. A. Schlicher, Applied Microbiology and Biotechnology as cited hereinabove.

The fermentation process may further comprise or alternatively comprise following the contacting step, adding PC and QAC to the product of the contacting step which product comprises ethanol, separating ethanol from the product. Alternatively, the fermentation process may further comprise, following the contacting step, separating the ethanol from the remaining product, wherein the remaining product is fed, for example, into a beer-well, or a thin stillage tank and adding the SCD and PC to the beer-well or thin stillage tank.

Peroxide compound (PC) and quaternary ammonium compound are added to the fermentation process in an amount to provide effective amounts so that the combination is effective to control growth of one or more contaminant microorganism. Typically PC is added in an amount to provide a concentration of 10 ppm to 1000 ppm, by weight, based on the total weight of the fermentation broth. Preferably, PC is added to the fermentation process to provide a concentration of PC of 50ppm to 200ppm by weight, based on the total weight of the fermentation broth. More preferably, PC is added to the fermentation process to provide a concentration of PC of 50 ppm to 100 ppm by weight, based on the total weight of the fermentation broth. Typically QAC is added in an amount to provide a concentration of QAC in the fermentation broth of 10 to 1000 ppm, by weight, based on the total weight of the fermentation broth. Preferably, QAC is added to provide a concentration of QAC of 50 to 500 ppm, based on the total weight of the fermentation broth. More preferably, QAC is added to provide a concentration of QAC of 100 to 200 ppm, based on the total weight of the fermentation broth.

#### Preservation method

As an embodiment of the present invention for controlling growth of contaminant microorganisms in a fermentation process, PC and QAC may be added as a step in providing the fermentable sugar. More particularly, as defined herein, the fermentation process comprises process steps to produce and store carbohydrate feedstock wherein the carbohydrate feedstock comprises a fermentable sugar or is used to prepare a fermentable sugar. Thus, the step of providing a fermentable sugar may comprise contacting a carbohydrate feedstock with PC and QAC. Thus, the method of

this invention is also useful to prevent degradation in storage of a carbohydrate feedstock.

The carbohydrate content of the feedstock is at least 1% and preferably 1 to 70%, by weight, based on the total feedstock weight. The peroxide compound and quaternary ammonium compound are added in effective amounts. Typically the peroxide compound is added in an amount of 10 ppm to 1000 ppm, by weight of peroxide compound, based on total weight of the feedstock, and the quaternary ammonium compound is typically added in an amount of 50 ppm to 500 ppm, by weight of the quaternary ammonium compound, based on the total weight of the feedstock, wherein all amounts herein expressed as ppm are on a weight/weight basis. Preferably, peroxide compound is added in an amount of 100 ppm to 1000 ppm, more preferably 100 ppm to 500 ppm, and most preferably 100 ppm to 200 ppm by weight of peroxide compound, based on the total weight of the feedstock. Preferably the quaternary ammonium compound is added in an amount to provide a concentration in the feedstock of 100 ppm to 500 ppm of quaternary ammonium compound, by weight, based on total weight of the feedstock, more preferably 100 ppm to 200 ppm of quaternary ammonium compound, by weight, based on total weight of the feedstock.

In this method, PC and QAC are contacted with a carbohydrate feedstock in an effective amount to protect the carbohydrate from the growth of undesirable microorganisms and thus to prevent deterioration of the feedstock. Deterioration of the feedstock can be determined by the populations of contaminant microorganisms present, or the concentration of microbial metabolites, such as organic acids, that generally indicate unintended and undesirable microbial activity in the feedstock. Microorganisms are thus substantially prevented from proliferating in the stored or transported feedstock following the addition of the PC and QAC.

Surprisingly, the carbohydrate feedstock treated according to this invention remains stable for at least one month. By "stable", it is meant herein the addition of PC and QAC preserves the carbohydrate feedstock, where "preserve" is defined hereinabove as preventing reaction of or consumption of carbohydrate by contaminant microorganisms. A stable carbohydrate feedstock does not undergo an increase in the microbial population in the feedstock of more than 1 log<sub>10</sub> CFU/ml or 1 log<sub>10</sub> CFU/g. CFU, an abbreviation for colony forming unit, is a measure of microbial population in

the feedstock. CFU is used to determine the number of viable microbial cells in a sample per unit volume or per unit mass, or the degree of microbial contamination in samples. A second measure of change is pH of the preserved feedstock. The pH of a properly preserved feedstock should not change by more than 0.5 pH units. However,  
5 as previously stated, pH change may not be sufficient under all circumstances to monitor preservation of a carbohydrate feedstock.

The carbohydrate feedstock is defined hereinabove.

In the present invention, the combination of PC and QAC is used as a preservative for carbohydrate feedstocks to impede contaminant microorganism  
10 activity and subsequent deterioration of the carbohydrate feedstock as a step in providing a fermentable sugar. Contaminant microorganisms include bacteria as disclosed in WO 2007/149450 and contaminant yeast as disclosed in U.S. Patent Application Serial No. 12/467,728, filed May 18, 2009. The combination inhibits growth of certain bacteria that cause undesired decomposition of carbohydrates such as  
15 simple sugars to deleterious acids and also selectively to reduce the activity of contaminant yeasts.

## **EXAMPLES**

### **Example 1**

A fermentation broth was prepared by diluting 1 part molasses with 3 parts  
20 water as is commonly practiced in molasses-based fermentation processes. A cocktail of three lactic acid bacteria (LAB) species (*Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus paracasei*) was concurrently prepared by growing an overnight culture of each bacteria in deMann-Rogosa- Sharpe (MRS) broth (available from Difco Laboratories, Inc., Sparks, MD) at 32°C in a shaking incubator, then  
25 combining the broth to make up a mixed culture. A separate overnight culture of *Saccharomyces cerevisiae* (inoculant yeast) was prepared by growing an overnight culture in Yeast Peptone Dextrose (YPD) broth (available from Difco Laboratories Inc., Sparks, MD). A portion of the molasses medium was inoculated with the cocktail of LAB to result in approximately  $1 \times 10^6$  colony forming units (CFU) per ml of medium.  
30 A separate portion was inoculated with the yeast to result in approximately  $6 \times 10^5$  CFU/ml. Portions of the inoculated molasses medium were treated with hydrogen

peroxide (HP), quaternary ammonium compound (didecyl dimethyl ammonium chloride) or combinations of the two as outlined below:

**LAB**

1. Control (No treatment)
- 5    2. 100 ppm QAC
3. 150 ppm QAC
4. 75 ppm HP
5. 100 ppm HP
6. 75 ppm HP/100ppm QAC
- 10    7. 75 ppm HP/ 100ppm QAC
8. 100ppm HP/ 100ppm QAC
9. 100ppm HP/ 150ppm QAC



**Inoculant yeast (*S. cerevisiae*)**

1. Control (No treatment)
2. 100 ppm QAC
3. 150ppm QAC
- 5 4. 100ppm HP/ 100ppm QAC

Treated samples were incubated at 32°C in a shaking incubator, following which serial dilutions were made in phosphate buffered saline (PBS) before plating the dilutions on MRS agar and Potato Dextrose Agar (PDA) plates for viable count determination. The plates were incubated at 32°C for 48hrs. The results are shown below:

<b><u>LAB</u></b>	
<b><u>Treatment</u></b>	<b><u>Viable Population (CFU/ml)</u></b>
15 1. Control (No treatment)	$1.11 \times 10^6$
2. 100 ppm QAC	$1.19 \times 10^6$
3. 150 ppm QAC	$1.16 \times 10^6$
4. 75 ppm HP	$4.5 \times 10^5$
5. 100 ppm HP	$8.1 \times 10^4$
20 6. 75 ppm HP/100ppm QAC	$2.3 \times 10^4$
7. 75 ppm HP/ 150ppm QAC	ND**
8. 100ppm HP/ 100ppm QAC	ND
9. 100ppm HP/ 150ppm QAC	ND

25 \*\* - Counts were below detectable limits

***S. cerevisiae* (Inoculant yeast)**

<b><u>Treatment</u></b>	<b><u>Viable Population (CFU/ml)</u></b>
1. Control (No treatment)	$5.7 \times 10^5$
30 2. 100 ppm QAC	$5.0 \times 10^5$
3. 150ppm QAC	$3.5 \times 10^5$
4. 100ppm HP/ 100ppm QAC	$4.0 \times 10^5$

The data shows that the combination of QAC and HP was more effective in reducing the population of LAB in molasses medium than each individual treatment on its own. For example, the combination of 100 ppm HP and 100 ppm QAC resulted in a reduction in LAB to undetectable levels; 100 ppm QAC by itself did not reduce the LAB population, while 100 ppm HP by itself reduced the viable LAB population by only about 1 log CFU/ml. Similar concentrations of QAC and HP did not have any significant effects on the viability of the inoculant yeast even when both treatments were combined.

## 10 Example 2

In this example, hydrogen peroxide (HP) and a quaternary ammonium chloride compound were used in a checkerboard assay to determine whether their interaction was synergistic against *Lactobacillus fermentum*. The checkerboard assay method is used to analyze interactions between compounds by mixing them in increasing concentrations to determine whether the compounds act synergistically, additively or antagonistically. (See, M. Najjar, D. Kashtanov, M. Chikindas, Letters in Applied Microbiology, vol. 45 (2007) 13-18.) *L. fermentum* was cultured overnight in MRS broth (deMan Rogosa and Sharp broth, Difco Laboratories, Inc., Sparks, MD) at 32°C. The culture was then diluted and resuspended in fresh MRS broth adjusted to pH 4.8. The number of bacteria in the resuspended culture was determined using standard plate counts. Samples were diluted (1:10) in sterile phosphate-buffered saline (Sigma-Aldrich Co., St. Louis, MO) and plated onto the surface of MRS plates. The resuspended culture (150 µl) was then used to fill the wells of a 96 well plate (Becton, Dickinson and Co., Franklin Lakes, NJ). Hydrogen peroxide (HP) (VWR Scientific Products, Bridgeport, NJ) and didecyl dimethyl ammonium chloride (DDAC) quaternary ammonium compound (Bardac 2250 DDAC, Lonza Inc., Allendale, NJ) were then added at the concentrations shown in the “checkerboard” plate diagram below. The plate was covered and incubated for 24 hours at 32°C. Following incubation, the plate was removed and the optical density of each well was determined using a plate reader at 600 nm (PowerWave, BioTek Instruments Inc., Winooski, VT). Based on the optical density, each well was scored to determine whether there was no inhibition of growth (NI), partial inhibition (PI), or complete inhibition (CI). Results are provided in Table 1.

To determine whether any synergistic activity occurred between HP and the quaternary ammonium compound, the fractional inhibitory concentration (FIC) was determined using the formula:

$$\text{FIC} = [\text{HP}]/\text{MIC}_{\text{HP}} + [\text{DDAC}]/\text{MIC}_{\text{DDAC}}$$

- 5 where [HP] and [DDAC] are the concentrations of HP and Bardac 2250 that result in partial inhibition and  $\text{MIC}_{\text{HP}}$  and  $\text{MIC}_{\text{DDAC}}$  are the minimum inhibitor concentration of HP and DDAC, respectively. Synergy between the two antimicrobials is determined when the resulting FIC is <1.0.

		<i>Hydrogen Peroxide</i>							
		0 ppm	50 ppm	75 ppm	100 ppm	150 ppm	200 ppm	250 ppm	300 ppm
DDAC	0 ppm	NI	PI	PI	PI	PI	PI	PI	CI
	4 ppm	PI	PI	PI	PI	PI	PI	CI	CI
	8 ppm	PI	PI	PI	PI	PI	PI	CI	CI
	12 ppm	PI	PI	PI	CI	CI	CI	CI	CI
	16 ppm	CI	CI	CI	CI	CI	CI	CI	CI

10

- For the checkerboard assay above, the FIC is determined to be 0.42, indicating a synergistic effect for the interaction between HP and the DDAC quaternary ammonium compound against *Lactobacillus fermentum*. Thus, Example 2 demonstrates that the combination of hydrogen peroxide and quaternary ammonium compound will enable a reduction in the dose of the components required to inactivate bacteria in a fermentation process.

15

## CLAIMS

What is claimed is:

1. A method for controlling growth of contaminant microorganisms in a fermentation process comprising adding the components (a) peroxide compound and  
5 (b) a quaternary ammonium compound, to one or more steps of a fermentation process, wherein the fermentation process comprises (i) providing a fermentation vessel; (ii) providing an inoculant, a fermentable sugar and process water; (iii) introducing separately, or in any combination, the inoculant, fermentable sugar and process water into a fermentation vessel to provide a fermentation broth; and (iv) contacting the  
10 inoculant with the fermentable sugar in the fermentation vessel at a temperature at which the inoculant converts the fermentable sugar to ethanol.
2. The method of claim 1 further comprising adding nutrients to one or more of the inoculant, fermentable sugar and process water or directly to the fermentation vessel.
- 15 3. The method of claim 2 wherein the peroxide compound and quaternary ammonium compound are added separately to one or more steps of the fermentation process.
4. The method of claim 2 further comprising contacting the peroxide compound and quaternary ammonium compound prior to adding the peroxide  
20 compound and quaternary ammonium compound to one or more steps of the fermentation process.
5. The method of claim 2 wherein peroxide compound or quaternary ammonium compound is added to the inoculant, fermentable sugar or process water prior to introducing the inoculant, fermentable sugar or process water into the  
25 fermentation vessel.
6. The method of claim 1 wherein the inoculant is a yeast inoculum produced by contacting a yeast starter culture and a nutrient composition in a propagation tank.
7. The method of claim 5 wherein the fermentation process is a batch  
30 fermentation, and the peroxide compound and quaternary ammonium compound are

added to the fermentation vessel prior to adding the fermentable sugar to the fermentation vessel.

8. The method of claim 5 wherein the fermentation process is a continuous fermentation process and peroxide compound and quaternary ammonium compound are combined and added as a single composition to the yeast propagation step to produce the yeast inoculum or during the contacting step (iv).

9. The method of claim 2 wherein the fermentable sugar is derived from corn using a dry grind process.

10. The method of claim 2 wherein the fermentable sugar is derived from corn using a wet mill process.

11. The method of claim 2 wherein the fermentable sugar is sugarcane- or sugar beet- or molasses-based fermentable sugar.

12. The method of claim 1 wherein peroxide compound is added in an amount to provide a concentration of the peroxide compound of 10 ppm to 1000 ppm by weight, based on the total weight of the fermentation broth and the quaternary ammonium compound is added in an amount to provide a concentration of 10 to 1000 ppm, based on the total weight of the fermentation broth.

13. The method of claim 1 wherein the peroxide compound is selected from the group consisting of a hydroperoxide, organic peroxide, inorganic peroxide or peroxygen-releasing compound.

14. The method of claim 13 wherein the peroxide compound is a hydroperoxide.

15. The method of claim 14 wherein the peroxide compound is hydrogen peroxide.

16. The method of claim 1 wherein the quaternary ammonium compound is selected from the group consisting of alkyl dimethyl benzylammonium chloride; dioctyl-dimethylammonium chloride; didecyl dimethyl ammonium chloride; diallyl-dimethylammonium chloride; di-n-decyl-dimethylammonium chloride; cetylpyridinium chloride; benzethonium chloride; and mixtures thereof.

17. The method of claim 16 wherein the peroxide compound is hydrogen peroxide.

18. A method to control growth of at least one contaminant microorganism in a carbohydrate feedstock which comprises contacting the feedstock with peroxide  
5 compound and quaternary ammonium compound, wherein the carbohydrate content of the feedstock is at least 1% by weight, based on the total feedstock weight.

19. The method of claim 18 wherein the feedstock is a sugar-based feedstock.

20. The method of claim 18 wherein the feedstock is a cellulose feedstock.

10 21. The method of claim 18 wherein the peroxide compound is added in an amount of 10 ppm to 1000 ppm of peroxide compound, based on total weight of the feedstock, and the quaternary ammonium compound is added in an amount of 50 ppm to 500 ppm of the quaternary ammonium compound, based on the total weight of the feedstock.

15 22. A method according to claim 1 wherein the fermentation process further comprises after step (iv) , adding PC and QAC to the product of the contacting step which product comprises ethanol.

23. A method according to claim 1 wherein the fermentation process further comprises after step (iv), separating the ethanol from the product of the contacting step  
20 which product comprises ethanol to provide a remaining product, wherein the remaining product is fed, into a beer-well or a thin stillage tank and adding the PC and QAC to the beer-well or thin stillage tank.

# INTERNATIONAL SEARCH REPORT

International application No

PCT/US2011/066311

## A. CLASSIFICATION OF SUBJECT MATTER

INV. C12P7/06 A01N33/12 A01N59/00  
ADD.

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

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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, WPI Data, COMPENDEX, FSTA, BIOSIS, EMBASE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CA 2 300 807 A1 (UNIVERSITY OF SASKATCHEWAN TECHNOLOGIES INC.) 18 September 2000 (2000-09-18)	1-5, 7-17,22, 23
A	page 3, line 1 - page 4, line 21 page 6 - page 15; examples 2-4 page 17 - page 18; claims 1-12 -----	6,18-21
X	DATABASE FSTA [Online] INTERNATIONAL FOOD INFORMATION SERVICE (IFIS); SLYUSARENKO, T.P. & VITUKEVICH, E.R.: "Cetylpyridinium chloride as antimicrobial agent in alcohol production from molasses", XP002674567, Database accession no. FS-1970-07-H-0764	1-5, 7-17,22, 23
A	abstract -/-	6,18-21



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

25 April 2012

Date of mailing of the international search report

09/05/2012

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
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Authorized officer

Fuchs, Ulrike

# INTERNATIONAL SEARCH REPORT

International application No

PCT/US2011/066311

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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