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(54) **UTILISATION D'UREE-PEROXYDE D'HYDROGENE POUR LA PRODUCTION D'ALCOOL**

(54) **USE OF UREA HYDROGEN PEROXIDE IN FUEL ALCOHOL PRODUCTION**

(57) A process is described for the production of alcohol in which a starch- or sugar-based aqueous fermentation medium is prepared and to this fermentation medium is added urea hydrogen peroxide in an amount sufficient to substantially reduce the level of bacterial contaminants in the fermentation medium. The urea hydrogen peroxide is left in contact with the fermentation medium for a time of at least one hour and sufficient to substantially reduce the level of bacterial contaminants. Thereafter the fermentation medium is inoculated with yeast wherein the yeast produces catalase enzyme which degrades liberated hydrogen peroxide to water and oxygen. This oxygen is needed by the yeast for membrane sterol and unsaturated fatty acid synthesis. Both urea and oxygen are supplied in near optimum amounts for growth subsequent to the suppression of bacterial contaminants by urea hydrogen peroxide. The fermentation continues to produce alcohol, in particular fuel or industrial alcohol, at the highest possible yields.

Abstract

A process is described for the production of alcohol in which a starch- or sugar-based aqueous fermentation medium is prepared and to this fermentation medium is added urea hydrogen peroxide in an amount sufficient to substantially reduce the level of bacterial contaminants in the fermentation medium. The urea hydrogen peroxide is left in contact with the fermentation medium for a time of at least one hour and sufficient to substantially reduce the level of bacterial contaminants. Thereafter the fermentation medium is inoculated with yeast wherein the yeast produces catalase enzyme which degrades liberated hydrogen peroxide to water and oxygen. This oxygen is needed by the yeast for membrane sterol and unsaturated fatty acid synthesis. Both urea and oxygen are supplied in near optimum amounts for growth subsequent to the suppression of bacterial contaminants by urea hydrogen peroxide. The fermentation continues to produce alcohol, in particular fuel or industrial alcohol, at the highest possible yields.

USE OF UREA HYDROGEN PEROXIDE IN FUEL ALCOHOL PRODUCTION

Background of the Invention

Field of the Invention

The present invention relates to the use of hydrogen peroxide or urea hydrogen peroxide in alcohol production, and in other fermentations where catalase-producing microbes are employed or where catalase enzyme could be added.

Description of the Prior Art

Bacterial contamination is a major cause for reduction in yeast growth, yeast viability and ethanol yield during the fermentation of starch-derived or sugar-based feedstocks by *Saccharomyces cerevisiae*. Among the contaminants encountered, lactic acid bacteria are the most persistent because of their tolerance to ethanol, low pH and high temperature and their ability to therefore survive the alcoholic fermentation. Predominant isolates from distilleries and fuel alcohol plants belong to the genus *Lactobacillus*. This microbe is able to ferment carbohydrates for growth and metabolism - the latter leading to production of lactic acid that causes a reduction in yeast growth, yeast viability, and subsequently ethanol yield. The management of these bacterial contaminants is often achieved in the fuel alcohol industry by using antibiotics like penicillin G and tetracycline. Recently, it has been shown that virginiamycin may be a better choice, since this antibiotic, unlike penicillin, retains its activity at lower pH values. However, antibiotics are costly, and the concept of indiscriminate usage of medically important antibiotics for such an industrial process is in question in spite of absence of antibiotic residues in spent grains subsequent to distillation. Antibiotic-resistant bacteria are found in this industry after continued use of the antibiotics.

Apart from bacterial contamination, "stuck" or sluggish fermentations are a cause for reductions in ethanol yield. Stuck fermentations are most often caused by inadequate levels of yeast nutrients that lead to a cessation of yeast growth

with a concomitant reduction in ethanol yield. Two such nutrients are usable (assimilable) nitrogen and oxygen. Yeasts used in alcohol production are not proteolytic and can use only low molecular weight nitrogenous compounds such as inorganic ammonium ion, urea, amino acids or dipeptides. Urea and liquid ammonia are used in the fuel alcohol industry as inexpensive sources of nitrogen for yeast. Diammonium phosphate is often added in wine making. In addition to a source of usable nitrogen, oxygen is required in small quantities by yeast for the synthesis of unsaturated fatty acids and sterols which are both essential components of the yeast cell membrane. Unfortunately, oxygen is not available at optimal levels due to industrial practices and due to its lower solubility in mashes at fermentation temperatures. Nutrient deficiencies in usable nitrogen and oxygen affect the ethanol tolerance of the yeast.

Hydrogen peroxide has been known as an antibacterial agent for more than one hundred years, and may be found in pharmaceutical preparations, mouthwashes, dentifrices, etc. It has been widely used as a topical antiseptic. Jacobs et al. U.S. Patent 5,667,753 describes the use of hydrogen peroxide vapour for the sterilization of medical instruments.

Based on the physiological differences between yeast and *Lactobacilli*, hydrogen peroxide could be thought of as an effective agent for managing these bacteria in mashes used for alcoholic fermentations. However, hydrogen peroxide is not a desirable reagent for a large scale industrial operation such as fuel or industrial alcohol production. Hydrogen peroxide is typically marketed as a 30% to 60% w/v solution which is a reactive oxidizing material that is corrosive and expensive both to ship and to store. It is also not very stable. The present inventors have found hydrogen peroxide to be an effective antimicrobial agent in alcohol fermentation mashes, particularly when a source of assimilable nitrogen is also added. However, this involves equipment for adding both a nitrogen source, such as urea (solid or in solution) or ammonia, and the corrosive hydrogen peroxide solution.

It is an object of the present invention to provide an improved means for suppressing growth of contaminating bacteria and improving yields in the production of alcohol, particularly fuel and industrial alcohol.

Summary of the Invention

According to this invention, it has been found that a highly effective and very practical agent for suppressing bacterial contamination that may occur during the production of ethanol, particularly fuel and industrial alcohol, is urea hydrogen peroxide. This is a stabilized form of hydrogen peroxide having the formula $CO(NH_2)_2.H_2O_2$. It is typically stored and shipped in a solid (crystalline) form, and is much more stable at room temperature than is hydrogen peroxide.

The urea hydrogen peroxide is typically added to a saccharification tank or a fermentation tank used in the production of fuel or industrial alcohol from a starch- or sugar-based aqueous fermentation medium. In order for the urea hydrogen peroxide to have maximum bactericidal activity against *Lactobacilli*, it is added to the fermentation medium a substantial period of time before yeast inoculation. Thus, the urea hydrogen peroxide is added at least one hour, and preferably at least two hours, before yeast inoculation.

The urea hydrogen peroxide not only exhibits excellent bactericidal activity against *Lactobacilli*, but also has the important advantage of providing the fermentation yeast with usable nitrogen in the form of urea and with oxygen, both of which are essential nutrients to stimulate yeast growth and fermentation rate. This serves to prevent "stuck" fermentations that would lead to reduction in alcohol yields.

When urea hydrogen peroxide is added to the fermentation medium, the hydrogen peroxide released acts as a bactericidal agent and the urea component is available as a nitrogen source. Also after yeast inoculation, the hydrogen peroxide is decomposed by yeast catalase yielding free oxygen for use in the fermentation. It has furthermore been found that the urea hydrogen peroxide is fully consumed when added to the

fermentation medium; no residues are left, and the pH of the mash is not affected when ammonium salts are employed. Thus, urea hydrogen peroxide has been found to be an ideal additive for use in the production of fuel or industrial alcohol, satisfying the three most important requirements which promote contaminant-free and vigorous fermentation performance by the yeast.

The urea hydrogen peroxide is typically used in an amount of less than about 4 g/L of fermentation medium. Where the fermentation medium is a grain-based unclarified mash, the urea hydrogen peroxide is preferably used at a concentration of about 2.0 to 4.0 g/L. For particulate-free or clarified fermentation media, e.g. when starch slurries or molasses are used, lower concentrations are appropriate.

It is also within the scope of this invention to utilize hydrogen peroxide for suppressive bacterial contamination. When the hydrogen peroxide is used, the nutrient value of the urea is absent and a further source of nitrogen must be included. Thus, it is possible to separately add hydrogen peroxide and a further nutrient such as urea or liquid ammonia.

Description of the Preferred Embodiments

Example 1

A series of laboratory tests were conducted to determine the concentration of urea hydrogen peroxide required to kill *Lactobacilli* found in fermentation mashes. Fifty grams of a normal gravity wheat mash containing about 21 g/100 ml dissolved solids were added into sterile, 250 ml screw-capped Erlenmeyer flasks. A strain of *Lactobacillus paracasei* isolated from a commercial alcohol factory was used as the bacterial contaminant in this study since it is well adapted to fermentation conditions and tolerant to concentration of ethanol over 10% (v/v). The bacterial inoculum was grown in MRS broth at 30°C, harvested at 4°C by centrifugation at 10,300 x g for 15 minutes and resuspended in sterile 0.1% w/v peptone water. The slurry was stored in ice. Appropriate quantities

of this bacterial suspension were added to the mashes so that the bacterial numbers corresponded to approximately 10^7 CFU/ml.

Six different concentrations of urea hydrogen peroxide were tested in triplicate. Urea hydrogen peroxide, obtained from Sigma Chemical Co. was used to prepare a 40% w/v solution in deionized water. For lab studies only, this was filter-sterilized through a 0.2 μm membrane filter and dispensed into the flasks in predetermined amounts.

The flasks were then incubated at 30°C and shaken at 150 rpm in an orbital shaker. After 48 hours, samples were withdrawn from the flasks, centrifuged at 10,200 x g for 30 minutes and the supernatant was analysed for lactic acid by high performance liquid chromatography (HPLC). Normally, a linear relationship between final lactic acid concentration and initial viable bacterial numbers in a mash have been observed as described by Narendranath et al., Effects of lactobacilli on yeast-catalyzed ethanol fermentation. Appl. Environ. Microbiol. 63:4158-4163. Lactic acid concentrations found when different levels of urea hydrogen peroxide were used are shown in Table 1 below. These results show that at a concentration of 21.3 mmoles/L (2 g/L) of urea hydrogen peroxide there is a definite effect on the growth and metabolism of the bacterial contaminant. At a concentration 32.1 mmoles/L (3 g/L) urea hydrogen peroxide was fully effective and lactic acid was totally suppressed.

Table 1 - Concentration of lactic acid produced by 48 hours by *L. paracasei* inoculated at appropriately 10^7 CFU/ml to 50 g samples of wheat mash at 30°C in the presence of urea hydrogen peroxide at various concentration.

Urea Hydrogen Peroxide (mmoles/L)	Lactic Acid produced (% w/v)[1]
0	1.14±0.03
2.1	1.10±0.01
5.4	1.05±0.010
10.7	1.01±0.03
21.3	0.57±0.03
32.1	0.00±0.00
42.6	0.00±0.00

[1] Average of triplicate samples

Example 2

When urea hydrogen peroxide is added to the mash, it begins to break down into urea and hydrogen peroxide. It has been found that if yeast is added immediately, the hydrogen peroxide liberated from urea hydrogen peroxide decomposes through the action of yeast catalase into water and oxygen resulting in the loss of the bactericidal effect of hydrogen peroxide on the contaminating *Lactobacilli*. It is therefore necessary to incubate uninoculated mash with urea hydrogen peroxide for a period of time prior to addition of yeast. This may be done during the saccharification of the mash or post-saccharification in the fermentor prior to yeast addition. A series of tests were carried out to determine the times required for the pre-incubation of mash with urea hydrogen peroxide.

From Example 1, doses of 32.1 and 42.6 mmoles of urea hydrogen peroxide per liter of mash were chosen. Five hundred mL quantities of normal gravity (about 21 g/100 ml dissolved solids) wheat mash were filled into one liter jacketed, glass fermentors. The mashes were inoculated with the chosen *L.*

paracasei strain at approximately 10^7 CFU/ml, mixed, and 0 hour samples were withdrawn. This was followed by the addition of urea hydrogen peroxide at desired levels. The treatments were: control (no urea hydrogen peroxide), urea hydrogen peroxide at 32.1 mmoles/L (which decomposes to form 32.1 mmoles/L of hydrogen peroxide and 32.1 mmoles/L urea), and 42.6 mmoles/L urea hydrogen peroxide (corresponding to 42.6 mmoles/L of hydrogen peroxide and 42.6 mmoles/L urea).

Samples were withdrawn at 0, 1, 2, 4 and 6 hours, and analysed in triplicate for viable bacterial numbers by the membrane filtration technique. MRS agar plates with overlayed membranes were incubated in a CO_2 incubator at 30°C for 48 hours. Colony forming units (CFUs) were also recorded from the same Petri plates after 96 hours to determine whether the growth of the organisms was only slowed down by the urea hydrogen peroxide. The results are shown in Table 2 below. The results indicate that there is some reduction in bacterial cell numbers after 1 hour of preincubation and that a preincubation period of about 2 hours with urea hydrogen peroxide is required prior to yeast inoculation for maximum reduction of bacterial cell numbers. The results also show that the reagent is bactericidal and not bacteriostatic since no increased growth on the plates after 48 hours was observed.

Table 2:- Survival of *L. paracasei* (in CFU/ml) inoculated at ~10^7 CFU/ml wheat mash in the presence and absence of urea hydrogen peroxide at 30°C.

Time (h)	No urea hydrogen peroxide control	Urea H_2O_2 concentration	
		32.1 mmoles/L	42.6 mmoles/L
0	1.20×10^7	1.15×10^7	1.07×10^7
1	2.23×10^7	8.33×10^4	9.67×10^3
2	4.43×10^7	8.67×10^2	3.00×10^2
4	6.03×10^7	8.33×10^2	2.67×10^2
6	7.70×10^7	7.67×10^2	2.33×10^2

Example 3

The effect of urea hydrogen peroxide in controlling the lactic acid bacterium, *L. paracasei* during yeast-catalysed fermentation of wheat mash at 30°C was studied. This was carried out as follows:

Normal gravity (~21 g/100 ml dissolved solids) wheat mash was prepared and distributed into 1L jacketed glass fermentors in 500 mL quantities. The fermentors were connected through a circulating waterbath which was maintained at a temperature of 30°C throughout the fermentation. The treatments were: 1) control with yeast at about 10^6 CFU/ml 30 mmoles urea/L added but no bacteria; 2) yeast at ~10^6 CFU/ml, bacteria inoculated at about 10^7 CFU/ml and 30 mmoles urea/L (no antimicrobial agents); 3) yeast at about 10^6 CFU/ml, with urea hydrogen peroxide corresponding to 30 mmoles H_2O_2/L and 30 mmoles urea/L and no bacteria; 4) yeast at about 10^6 CFU/ml, bacteria inoculated at about 10^7 CFU/ml and urea hydrogen peroxide (30 mmoles/L); 5) yeast at about 10^6 CFU/ml and separate additions of 30 mmoles H_2O_2/L and 30 mmoles urea/L (no bacteria); and 6) yeast at about 10^6 CFU/ml, bacteria inoculated at about 10^7 CFU/ml and separate additions of 30 mmoles H_2O_2/L and 30 mmoles of urea per L mash.

L. paracasei was grown in MRS broth, harvested by centrifugation (10,200 x g at 4°C) and resuspended in sterile 0.1% w/v peptone water (slurry was stored on ice). An initial bacterial load of approximately 10^7 CFU/ml was added to treatments 2), 4) and 6). Urea hydrogen peroxide at 30 mmoles/L mash was added to treatments 3) and 4) (30 mmoles/L). Hydrogen peroxide was added to treatments 5) and 6) (final concentration of 30 mmoles/L). Urea was added to treatments 1), 2), 5) and 6) at 30 mmoles/L. Samples were taken from 2), 4) and 6) and analysed for initial viable numbers of bacteria by the membrane filtration technique. After 90 min, glucoamylase (0.4 ml) was added to all the fermentors for dextrin saccharification. Exactly 30 minutes after the addition of glucoamylase, yeast was added to all the fermenters at approximately 10^6 CFU/ml (so that there had been an incubation period of 2 hours for the urea hydrogen peroxide

in treatments 3) and 4), and for hydrogen peroxide in treatments 5) and 6) before yeast inoculation). Then, samples were withdrawn at 0 h, 12 h, 24 h, 36 h, 48 h and 72 h. The samples were centrifuged (10,3000 x g) and the supernatant was analysed for concentration of dissolved solids. A portion of each supernatant was frozen for later analysis of alcohol and lactic acid concentrations. Ethanol and lactic acid were estimated by HPLC (HPX-87H BioRad column). Samples were also drawn at these intervals for enumerating viable numbers of yeast and bacteria by the membrane filtration technique. Plating was done in triplicate and results were expressed as CFU/ml. Results are shown in Tables 3 through 7 below.

The results in Table 3 show that the fermentation completed in about 24-36 hours in all treatments. This was because of the increased availability of assimilable nitrogen and oxygen. As grain mashes are deficient in usable nitrogen, yeast growth and fermentation rate benefitted from added urea whether it is added as free urea or as urea hydrogen peroxide. Urea hydrogen peroxide at the levels needed for antibacterial action is almost perfect as a supplier of assimilable nitrogen and oxygen-supplied at the perfect time to the yeast as a growth stimulant.

As can be seen in Table 4, the viable bacterial numbers in mash dropped significantly from ~10^7 CFU/ml to ~2 x 10^2 CFU/ml in the first 2 hours when urea hydrogen peroxide or hydrogen peroxide were used. Once the yeast was inoculated, remaining bacteria (not yet killed) resumed growth because the yeast decomposed residual hydrogen peroxide in the medium using the enzyme catalase. In the treatments where urea hydrogen peroxide was used, its increased stability led to a less rapid release of hydrogen peroxide; the growth rate of bacteria was somewhat slower than in the treatment where equal quantities of urea and hydrogen peroxide were added separately.

Numbers of viable yeast cells reached a maximum during the first 24 hours as shown in Table 5. The yeast viable numbers were higher in samples treated with urea hydrogen

peroxide and with hydrogen peroxide than in the control where no antibacterial agents were used. The viable number of yeast cells were the lowest in samples treated with bacteria where no antibacterial agents were added. This is due to the competition for nutrients by the bacteria and the production of lactic acid which at levels approaching 1 % w/v begins to stress the yeast and suppress yeast growth.

In all treatments most of the ethanol production occurred within 36 hours, as can be seen in Table 6. By that time, the lactic acid in the medium where urea hydrogen peroxide was used was still the same as in the treatment with yeast alone without bacteria (0.03 % w/v), as seen in Table 7. In the treatment where hydrogen peroxide was used, only 0.05% (w/v) lactic acid was detected. But, in the medium where neither urea hydrogen peroxide nor hydrogen peroxide was used, 0.9% (w/v) lactic acid was found. This level of lactic acid (0.9%) was enough to affect yeast metabolism and growth, and to reduce final alcohol levels.

In the treatment that had neither urea hydrogen peroxide nor hydrogen peroxide to kill the *L. paracasei*, a 5.64% reduction in the maximum ethanol produced was observed compared with the control with yeast alone and no agents added. The maximum ethanol produced in all other treatments were not significantly different from one another. This relatively routine level of ethanol yield reduction represents a significant loss of revenue in this industry.

Table 3 - Concentration of dissolved solids (g/100 ml) during the fermentation of wheat mash by yeast at 30°C.

Time (h)	Urea only 30 mmoles/L		Urea hydrogen peroxide[1]		Urea and H_2O_2 (each at 30 mmoles/L)	
	Yeast alone [2]	Yeast[2] + Bacteria[3]	Yeast alone[2]	Yeast[2]+ Bacteria[3]	Yeast alone[2]	Yeast[2]+ Bacteria[3]
0	19.85	19.82	19.74	19.72	19.77	19.82
12	17.21	17.18	17.65	17.73	17.96	17.28
24	1.10	0.84	2.08	2.06	2.32	2.06
36	0.00	0.00	0.00	0.00	0.00	0.00

[1] 30 mmoles urea hydrogen peroxide/L yields 30 mmoles urea and 30 mmoles hydrogen peroxide/L mash

[2] Yeast inoculated at ~10^6 CFU/ml,

[3] *L. paracasei* inoculated at ~10^7 CFU/ml.

Table 4 - Growth of *L. paracasei* in fermenting wheat mash at 30°C in the presence of urea hydrogen peroxide or hydrogen peroxide.

Time (h)	No agents	Urea hydrogen peroxide[1]	H_2O_2 (30 mmoles/L)
-2	$1.11x10^7$	$1.10x10^7$	$1.10x10^7$
0	$2.23x10^7$	$1.33x10^2$	$2.00x10^2$
12	$1.42x10^9$	$2.47x10^3$	$4.23x10^3$
24	$1.44x10^9$	$2.17x10^5$	$3.07x10^5$
36[2]	$1.29x10^9$	$1.34x10^6$	$2.27x10^6$
48	$1.21x10^9$	$9.93x10^6$	$1.32x10^7$
72	$9.10x10^8$	$1.00x10^8$	$1.38x10^8$

[1] In media, 30 mmoles urea hydrogen peroxide/L yields 30 mmoles of urea and 30 mmoles of hydrogen peroxide/L.

[2] Fermentations are effectively completed at 36 hours. Bacteria continue to grow using non-carbohydrate nutrients and lytic products of yeast.

All the treatments were inoculated with yeast at ~10^6 CFU/ml at time 0 hour. Bacteria remaining viable at 0 time begin to grow when hydrogen peroxide levels are reduced by yeast catalase action.

Table 5 - Growth of yeast during the fermentation of wheat mash at 30°C.

Time (h)	Urea only 30 mmoles/L		Urea hydrogen peroxide[1]		Urea and H_2O_2 (each at 30 mmoles/L)	
	Yeast alone [2]	Yeast[2] + Bacteria[3]	Yeast alone[2]	Yeast[2]+ Bacteria[3]	Yeast alone[2]	Yeast[2]+ Bacteria[3]
0	$1.86x10^6$	$2.13x10^6$	$1.38x10^6$	$1.63x10^6$	$1.07x10^6$	$1.59x10^6$
12	$2.47x10^7$	$2.13x10^7$	$2.53x10^7$	$2.03x10^7$	$1.83x10^7$	$2.37x10^7$
24	$2.07x10^8$	$1.37x10^8$	$2.20x10^8$	$2.07x10^8$	$2.00x10^8$	$2.33x10^8$
36[4]	$1.58x10^8$	$6.00x10^7$	$1.70x10^8$	$1.67x10^8$	$1.57x10^8$	$1.28x10^8$
48	$1.37x10^8$	$1.23x10^7$	$1.54x10^8$	$1.51x10^8$	$1.49x10^8$	$1.56x10^8$
72	$1.21x10^8$	$6.67x10^6$	$1.43x10^8$	$1.39x10^8$	$1.45x10^8$	$1.45x10^8$

[1] In mash, 30 mmoles urea hydrogen peroxide/L yields 30 mmoles urea and 30 mmoles hydrogen peroxide/L

[2] Yeast inoculated at ~10^6 CFU/ml,

[3] *L. paracasei* inoculated at ~10^7 CFU/ml.

[4] Fermentations are effectively completed at 36 hours. Yeasts continue to die due to acid and alcohol stress over time in untreated, bacteria-infected samples.

Table 6 - Ethanol (% v/v) produced during the fermentation of wheat mash by yeast at 30°C.

Time (h)	Urea only 30 mmoles/L		Urea hydrogen peroxide[1]		Urea and H_2O_2 (each at 30 mmoles/L)	
	Yeast alone [2]	Yeast[2] + Bacteria[3]	Yeast alone[2]	Yeast[2]+ Bacteria[3]	Yeast alone[2]	Yeast[2]+ Bacteria[3]
0	0.00	0.00	0.00	0.00	0.00	0.00
12	1.04	1.01	0.88	0.81	0.80	0.85
24	8.11	7.66	8.82	8.59	8.70	8.78
36[4]	10.26	9.66	10.28	10.25	10.27	10.23
48	10.28	9.68	10.29	10.27	10.27	10.23
72	10.28	9.70	10.34	10.30	10.29	10.25

[1] In medium, 30 mmoles urea hydrogen peroxide/L yields 30 mmoles urea and 30 mmoles hydrogen peroxide/L

[2] Yeast inoculated at ~10^6 CFU/ml,

[3] *L. paracasei* inoculated at ~10^7 CFU/ml.

[4] Fermentations are effectively completed at 36 hours. No further increase in alcohol is seen. Where bacteria were not controlled, ethanol yields were decreased.

Table 7 - Lactic acid (% w/v) produced by *L. paracasei* in the presence and absence of urea hydrogen peroxide or hydrogen peroxide in fermenting wheat mash at 30°C.

Time (h)	Urea only 30 mmoles/L		Urea hydrogen peroxide[1]		Urea and H_2O_2 (each at 30 mmoles/L)	
	Yeast alone [2]	Yeast[2] + Bacteria[3]	Yeast alone[2]	Yeast[2]+ Bacteria[3]	Yeast alone[2]	Yeast[2]+ Bacteria[3]
0	0.00	0.00	0.00	0.00	0.00	0.00
12	0.00	0.56	0.00	0.00	0.00	0.00
24	0.00	0.78	0.00	0.00	0.00	0.00
36[4]	0.03	0.90	0.04	0.03	0.03	0.05
48	0.04	0.95	0.04	0.05	0.03	0.06
72	0.03	0.96	0.03	0.15	0.03	0.17

[1] In medium, 30 mmoles urea hydrogen peroxide/L yields 30 mmoles urea and 30 mmoles hydrogen peroxide/L

[2] Yeast inoculated at ~10^6 CFU/ml,

[3] *L. paracasei* inoculated at ~10^7 CFU/ml.

[4] Fermentations are effectively completed at 36 hours. Lactic acid does not increase in untreated bacteria-infected fermentors due to depletion of fermentable carbohydrate.

Example 4

The survival of four other selected ethanol tolerant, *Lactobacillus* contaminants in wheat mash in the presence of the same dose of urea peroxide was studied as follows:

Normal gravity (~21 g/100ml dissolved solids) wheat mash was distributed in 500 g quantities into eight, one liter jacketed, sterile glass fermentors. The mashes were inoculated with *L. plantarum, L. rhamnosus, L. fermentum* and *Lactobacillus* #3 at approximately 10^7 CFU/ml followed by the addition of a 40% w/v solution of urea hydrogen peroxide to give a final concentration of 32 mM immediately after the 0 hour sample was withdrawn.

The treatments were: *L. plantarum* + urea hydrogen peroxide; *Lactobacillus* #3 + urea hydrogen peroxide; *L. rhamnosus* + urea hydrogen peroxide; and *L. fermentum* + urea hydrogen peroxide. All tests were done in duplicate.

Samples were withdrawn at 0, 2 and 4 hours and analysed for viable bacterial numbers in triplicate by the membrane filtration technique. MRS agar plates with membranes were incubated in a CO_2 incubator at 30°C for 48 hours for the counts. Bacterial counts were also recorded after 96 hours (to see if the growth of these organisms were only slowed down by the urea hydrogen peroxide). No increase in colony forming units (CFUs) was observed. The results are given in Table 8.

The results indicate that urea hydrogen peroxide is an effective bactericidal agent to prevent the growth of yield-reducing bacterial contaminants in mashes in the fuel alcohol industry. This provides more carbohydrate to the yeast, which otherwise would have been used for the production of lactic acid by bacteria, and also increases ethanol yield by

preventing stuck fermentations through provision of the nutrients urea and (ultimately) oxygen.

Table 8 - The survival of various lactobacilli in wheat mash at 30°C in the presence of urea hydrogen peroxide. The numbers are expressed as CFU/ml.

Time (h)	*L. plantarum*	*L. paracasei*	*Lactobacillus #3*	*L. rhamnosus*	*L. fermentum*
0	$1.09x10^7$	$1.15x10^7$	$0.88x10^7$	$0.85x10^7$	$0.83x10^7$
2	$5.67x10^2$	$8.67x10^2$	$7.67x10^2$	$3.00x10^2$	$7.50x10^2$
4	$5.83x10^2$	$8.33x10^2$	$7.67x10^2$	$3.33x10^2$	$7.00x10^2$

The results for *L. paracasei* were obtained from Example 2. All values are means of duplicate samples. Plating was done in triplicate.

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Claims:

1. A process for the production of ethanol which comprises preparing a starch- or sugar-based aqueous fermentation medium, adding to the fermentation medium urea hydrogen peroxide in an amount sufficient to substantially reduce the level of bacterial contaminants in the fermentation medium, leaving the urea hydrogen peroxide in contact with the fermentation medium for a time sufficient to substantially reduce the level of bacterial contaminants and thereafter inoculating the fermentation medium with yeast and continuing fermentation to form ethanol.

2. A process according to claim 1 wherein the ethanol comprises an alcohol intended for fuel or industrial purposes.

3. A process according to claim 2 wherein the urea hydrogen peroxide is left in contact with the fermentation medium for a period of at least one hour before inoculating with yeast.

4. A process according to claim 3 wherein the urea hydrogen peroxide is left in contact with the fermentation medium for a period of at least two hours before inoculating with yeast.

5. A process according to any one of claims 1 to 4 wherein the urea hydrogen peroxide is added in an amount of up to about 4 g/l of fermentation medium.

6. A process according to claim 5 wherein the urea hydrogen peroxide is added during saccharification of the mash.

7. A process according to claim 5 wherein the urea hydrogen peroxide is added to a fermentation mash after saccharification.

8. A process according to claim 6 wherein the mash is a grain-based or sugar-based mash.

9. A process according to claim 8 wherein the mash is a grain-based mash.

10. A process according to any one of claims 1 to 9 wherein the fermentation medium is a grain-based unclarified mash and the urea hydrogen peroxide is added in an amount of about 2.0 to 4.0 g/L.

11. A process according to claim 5 wherein the fermentation medium is a starch-based or sugar-based clarified mash and the urea hydrogen peroxide is added in an amount of less than 2 g/L.

12. A process for the production of fuel or industrial alcohol which comprises preparing a starch- or sugar-based aqueous fermentation medium, adding to the fermentation medium urea or liquid ammonia together with hydrogen peroxide in an amount sufficient to substantially reduce the level of bacterial contaminants in the fermentation medium, leaving the hydrogen peroxide in contact with the fermentation medium for a time sufficient to substantially reduce the level of bacterial contaminants and thereafter inoculating the fermentation medium with yeast and continuing fermentation to form fuel or industrial alcohol.