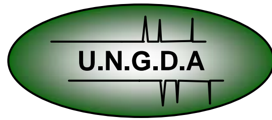

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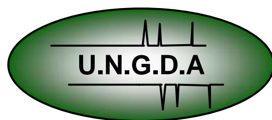
Controlling Bacterial Contamination in Ethanol fermentation Processes

Person to contact : Jean-Paul VIDAL at jpvidal@ungda.com or (33) (0)1.49.65.08.08



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Introduction

Bacterial contamination has been identified as a major problem plaguing the efficient fermentation of sugar- or starch-containing feedstocks in the production of ethanol. More than 500 different bacteria have been isolated and identified to be present at different stages of the ethanol production process. Many bacteria enter the system with the feedstock and more are added with the introduction of contaminated. recycled yeast. For some ethanol producers, bacterial contamination is the greatest obstacle to be overcome in their quest to become more profitable. Following are some of the ways bacteria adversely affect ethanol production.

- Bacteria require food for survival, growth and reproduction, as do all living creatures. The many bacteria that thrive in the ethanol fermentation environment take their nutrition from the fermentation medium, the same source upon which the yeast cells *(Saccharomyces sp.)* depend for their livelihood. Growing and viable yeast cells are essential for the fermentation process. An inadequate level of nutrients for the yeast cells results in stuck or sluggish fermentation. Excessive bacteria can reduce the viability of yeast and, thereby, substantial1y reduce ethanol yields.

- Bacteria metabolize glucose, converting it into many by-products which reduce the amount of ethanol capable of being produced from glucose by yeast and. thereby, reducing ethanol yields.

- In a yeast recycle system, bacterial contamination can cause flocculation of the yeast. Flocculation results in reduced yeast viability and requires more severe acidification of the yeast cream for control. flocculation plugs the centrifuge necessitating frequent disassembly and time-consuming and costly hand cleaning of the equipment

Bacterial contamination of the feedstocks commonly used in ethanol production is unavoidable. The characteristics of the feedstocks that make

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them suitable raw materials for use in ethanol production make them attractive to many bacteria. feedstocks commonly carry sufficient bacteria, (106 per milliliter or more) to be detrimental to the fermentation process. Furthermore, the common practice of reclaiming yeast cream and recycling it back into the fermenters increases bacterial contamination. Although sterilization is the solution to this problem in some other fermentation processes, establishing and maintaining complete sterility in industrial ethanol production is too expensive.

Many different anti-microbials have been used, with varying degrees of success, to control bacteria! contamination in ethanol production. None of the antibiotics used in the past, however, have provided significant long-term bacterial control.

Adjusting the pH of recovered yeast cream to 1.8 to 2.8 with sulfuric acid before recycling it into the fermenters has been reasonably successful in suppressing bacteria while allowing the more pH tolerant yeast to survive. But, when bacterial contamination gets out of control, the viability of the recycled yeast is reduced and the fermentation cycle-time is increased.

The ethanol industry long has been searching for "something" that can be added to the fermentation medium early in the process to control the bacterial population without having any detrimental affect on yeast. With bacterial contamination controlled, the yeast can maintain a high viability required to efficient I y convert the feedstock to ethanol.

KAMORAN® is that "something" the industry has been looking for - "something" that can be used as needed to control bacterial contamination and deliver end-results similar to those provided by sterilization.
Following are brief statements on some of the more important facts about KAMORAN®
.

- In some countries this new product will be named KAMORAN® HJ, and in others it will be known as KAMORAN® Intermediate A. Both are the same pure crystalline formulation of monensin sodium designed specifically and exclusively for use in controlling bacterial contamination in ethanol fermentation processes.

- KAMORAN® has exceptional activity against bacteria indigenous to the ethanol-producing feedstocks that commonly contaminate ethanol fermentation tanks. processing facilities and equipment.

- KAMORAN® remains s1able throughout the process without interfering with the ability of yeast to do its job.

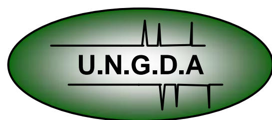
- KAMORAN® provides end-results similar those of sterilization without the extremely high capital expenditure and continuing higher management costs required to establish and maintain sterility throughout the production processes.

- KAMORAN® has been found by some ethanol producers to improve the quality of their product as determined by organoleptic examination (smell and taste}, thereby making a higher percentage of their product at higher prices to high quality users such as the perfume industry.

KAMORAN® can make a difference on your "bottom line."

This manual includes comprehensive information on the product, KAMORAN® , results of laboratory studies and tests in actual commercial ethanol fermentation plants. and guidance for using it in your operation.

Copies of the KAMORAN® package literature and the Material Safety Data sheet are included in the Appendix.)If you have any questions or need clarification of any of the information presented herein, contact **Jean-Paul VIDAL**.



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Physical and Chemical Properties of KAMORAN®

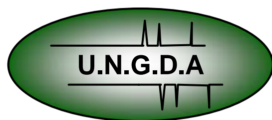
KAMORAN® is a pure crystalline form of Monensin Sodium, an antibiotic discovered and developed by Eli Lilly and Company. KAMORAN® was designed and developed exclusively for use in controlling bacterial contamination in ethanol fermentation. It has exceptional activity against many Gram-positive bacteria that are known to commonly contaminate ethanol fermentation and processing, especially *Lactobacil/us* and *Leuconostoc* species that are particularly destructive to efficient ethanol production.

Normal Physical State, Appearance, Odor: KAMORAN® is a pure crystalline powder with color ranging from off-white to tan. It has a characteristic odor.

pH: (aqueous 50/50): 6-9

Solubility: Slightly soluble in water, soluble in most organic solvents

Melting Point: 267-269° Centigrade (513-515° Fahrenheit)



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Stability of KAMORAN®

Stability In Storage

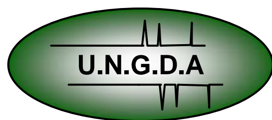
Crystalline KAMORAN® retains its full anti-microbial activity for up to 36 months when properly stored in a cool, dry place and protected from moisture and heat.

Stability In The Fermentation Tank

Tests have shown that KAMORAN® that has been dissolved in ethanol and introduced into the fermentation tank according to use directions remains active for at least 20 days at 33° Centigrade (91° Fahrenheit) under normal commercial ethanol production conditions. For additional information, see Page 13.

Stability In High-Heat Processes

In molasses, KAMORAN® has been found to remain active for 2 hours at 90° C (194° F) and for 1.5 hours at 100° C (212° F). Furthermore, 80 percent of the activity remains after one hour at 110° C (230° F) and 62 percent after one hour at 120° C (248° F).



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Antibacterial Spectrum of KAMORAN®

More than 500 different micro-organisms have been identified as contaminators of ethanol fermentation processes. The most common ones are listed in Table 1, on the next page. Tests in commercial ethanol production facilities have demonstrated that KAMORAN® effectively controls the mixed bacterial populations present in ethanol fermentation operations without affecting the activity of yeast.

Among the early research in the development of any new antimicrobial agent are tests to determine the activity of the compound against pure isolates of a variety of bacteria, particularly some that are pathogenic to humans and farm animals. The Minimum Inhibitory Concentration (MIC) of Monensin Sodium for the individual bacterium on which this data has been determined in the laboratory is included in Table 1. The lack of a MIC value for a bacterium does not indicate that KAMORAN® is not active against it, only that KAMORAN® has not been tested against a pure strain of that specific bacterium in the laboratory.

Bacterial Resistance Development

Several *Streptococcus* and *Staphylococcus* isolates were tested to evaluate the ability of susceptible bacteria to develop resistance to KAMORAN® . In the test, scientists first determined the' antibiotic's activity against the isolates. Subsequently, 12 passages of the isolates were subjected to the highest effective concentration of KAMORAN® . None of the isolates showed any reduced susceptibility to the antibiotic. As with any anti-microbial, the use of KAMORAN® at a bactericidal level is recommended as the best protection against bacterial resistance developing over time with continuous use.

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Table 1 : Bacteria Controlled By KAMORAN® In Ethanol Production with minimum inhibitory Concentration Values For Pure Strains In The Laboratory.

Bacterium	MIC (mcg/ml)
Bacillus brevis	
Bacillus cereus	
Bacillus coagulans	
Bacillus megaterium	
Bacillus pumilus	
Bacillus stearothermophilus	
Bacillus subtilis	1.58
Clostridium butyricum	
Lactobacillus acidophilus	
Lactobacillus buchneri	
Lactobacillus brevis 2	
Lactobacillus brevis 3	
Lactobacillus casei alactosos	
Lactobacillus casei casei	0.78
Lactobacillus coryniformis	
Lactobacillus fermentum	
Lactobacillus lindnerii	
Lactobacillus plantarum	
Lactobacillus vaccomptercis	
Lactobacillus yamanashensis	
Leuconostoc acidilactici	
Leuconostoc mesenteroides	
Leuconostoc citrovorium	0.78 – 3.13
Pediococcus pentosaceus	
Staphylococcus aureus	< 0.75- 5.0
Staphylococcus species	3.12 – 6.25
Streptococcus equinus	
Streptococcus faecalis	3.13
Streptococcus viridians	2.5
Streptococcus species	0.78 – 3.12

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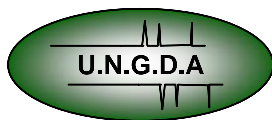
Using KAMORAN®

Indications

KAMORAN® is for use only to control bacterial growth in yeast fermentation of any sugar- or starch-containing feedstock for the industrial production of distilled ethanol. Do not use KAMORAN® in the production of beer, wine or other non distilled beverages.

Directions

When bacterial contamination becomes a problem, dissolve KAMORAN® in 90-100 percent ethanol up to a concentration not to exceed 100 grams per liter. Then introduce this KAMORAN® -ethanol solution into the fermenter at not less than 1.0 nor more than 3.0 parts per million (PPM). To achieve optimum control of the contaminating bacteria, the bactericidal level of 3 ppm is recommended. Always wear protective clothing, respirator,
goggles or face shield and rubber gloves when handling KAMORAN® . Wash thoroughly with soap and water after handling. See Page 33 for additional information on the safe handling of KAMORAN® and what to do if accidental contact occurs.



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Effectiveness of KAMORAN® proved in tests

Although sterilization is sometimes thought to be the only way to totally control bacterial contamination in fermentation processes, it is impractical, if not impossible, in today's commercial ethanol operations. Furthermore, initial capital for the necessary equipment and the greatly increased level of management required to maintain sterility is too costly in relation to today's market for industrial ethanol. It is appropriate, nevertheless, to compare the effects of KAMORAN® versus sterilization on ethanol production. The only practical way to do this with adequate control of the variables is in the laboratory. The most important tests of KAMORAN® were conducted under normal working conditions in several commercial ethanol fermentation plants. Both laboratory and commercial plant studies are summarized in this manual.

Laboratory studies

North American Study

In a study conducted to compare the effects of KAMORAN® versus sterilization, uninoculated starch-based-waste feedstock was collected from a commercial fermenter. Seven hundred grams of the broth were added to each of seven 1-liter Erlenmeyer flasks. Two of the flasks of feedstock were used as non-treated controls; one was sterilized at 121° Centigrade for 20 minutes, the other was not sterilized. The other five flasks were not sterilized and each was inoculated with one of five levels of KAMORAN® ranging from 0.5 ppm to 2.5 ppm. All flasks were inoculated with one milliliter of yeast solution (18 grams of yeast to 101.56 grams of feedstock) and 0.126 milliliter of enzyme solution (700 grams per 378,500 liters).

Results: The data presented in Table 2, on Page 11, were recorded after 46 1/2 hours of fermentation. The sterilized control run produced a 10 percent higher ethanol concentration than the unsterilized control.

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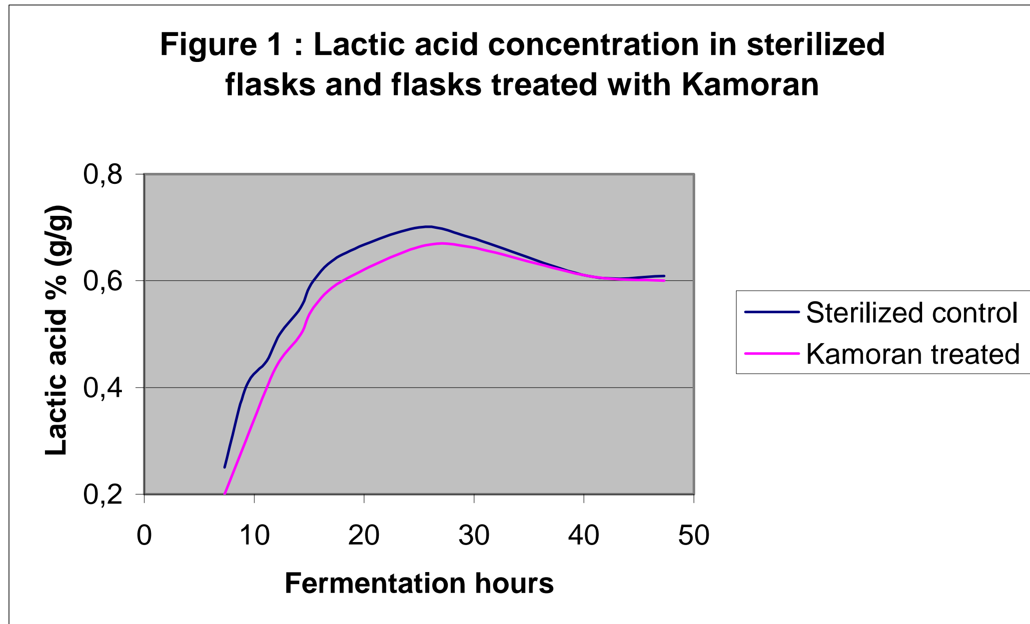
Lactic acid concentration, acidity and final pH were lower in the sterilized controol corn pa red to the unsterilized control. The effects of administering KAMORAN® were similar to those resulting from sterilization of the one control. Lactic acid concentrations and ethanol yields were essentially equivalent as illustrated graphically in Figures 1 and 2, below.



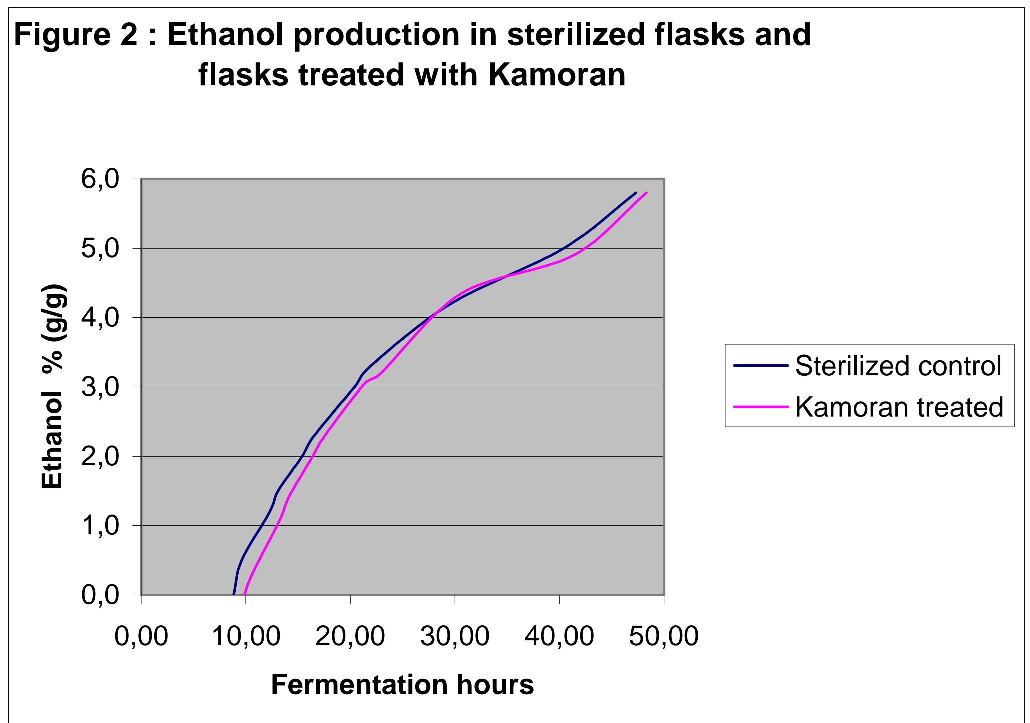


Table 2 : The effects of sterilization and different levels of KAMORAN® on ethanol production measurements.

Treatment	Ethanol (g/g)	Lactic acid (g/g)	Acidity (ml *)	pH
Unsterilized control	5.0	0.8	3.6	5.0
Sterilized control	5.5	0.5	2.0	5.8
KAMORAN® 0.5 ppm	5.3	0.6	2.8	5.4
KAMORAN® 1.0 ppm	5.3	0.5	2.3	5.4
KAMORAN® 1.5 ppm	5.3	0.5	2.1	5.8
KAMORAN® 2.0 ppm	5.4	0.4	1.9	5.8
KAMORAN® 2.5 ppm	5.2	0.4	1.9	5.7

* : milliters of NaOH solution (0.1 N) required to neutralize filtrate-water solution (1 part filtrate to 4 parts distilled water)

European Study :

In this laboratory study, fermentation feedstock derived from sugar beet molasses was placed in petri dishes. Initial acid content was one gram per liter and the pH was 5.6. Each petri dish was inoculated with 2x1 06 cells per milliliter of *Lactobacillus buchneri.* KAMORAN® was introduced into the inoculated molasses at concentrations of 0, 0.5, 1.0, 1.5, 2.0,2.5, and 3.0 parts per million (ppm) and the petri dishes were incubated at 33° Centigrade. Bacterial counts, acid content and pH were recorded after 24 hours and 48 hours of incubation.

Results: The lowest level of KAMORAN® , 0.5 ppm, provided only limited control of the growth of the bacterial population compared *ta* that which occurred in the non treated control petri dish. The higher levels of KAMORAN® were bactericidal as indicated by reduced numbers of contaminating bacteria. Final acidity was reduced and pH was increased equally by all KAMORAN® levels tested compared to those measurements recorded for the non treated control. See Table 3, below.

Table 3 : The effects of KAMORAN® on *Lactobacillus buchneri,* acid content and pH in sugar beet molasses.

Treatment	Bacterial counts after incubation periods of		pH	Final acidity g/l	Acidity change g/l
	24 hours	48 hours			
Control	4.10^6	10^9	4.5	5	4
KAMORAN® 0.5 ppm	8.10^5	2.10^6	5.6	1	0
KAMORAN® 1.0 ppm	10^4	2.103	5.6	1	0
KAMORAN® 1.5 ppm	$<10^3$	$<10^3$	5.6	1	0
KAMORAN® 2.0 ppm	2.10^2	$<10^3$	5.6	1	0
KAMORAN® 2.5 ppm	2.10^2	$<10^3$	5.6	1	0
KAMORAN® 3.0 ppm	2.10^2	$<10^3$	5.6	1	0

* : milliters of NaOH solution (0.1 N) required to neutralize filtrate-water solution (1 part filtrate to 4 parts distilled water)

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Studies in commercial Ethanol production plants

Trials have been conducted in Europe, North America and South America in established commercial plants under normal ethanol production conditions. Descriptions of the trials and their results are summarized here.

European Continuous-Process Trial 1

This trial was conducted in a typical continuous-run ethanol fermentation plant under normal operating conditions. KAMORAN® was introduced at a concentration of one part per million (1 ppm) into diluted molasses juice with a bacterial count of 106 bacteria per milliliter during a 24-hour period. The won had an acid content of 1 gram per liter and was maintained at 33° Centigrade.

Results : The bacterial population began declining upon the introduction of KAMORAN® and continued to decline during the 20-day trial period as shown in Figure 3, below. This trial demonstrated the effectiveness of KAMORAN® in controlling bacterial contamination and its stability in the fermentation environment for at least 20 days.

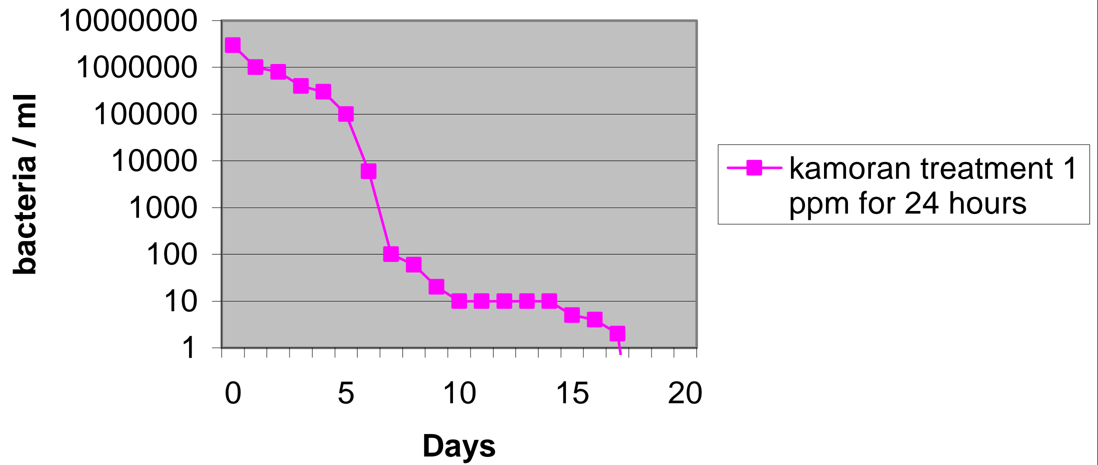
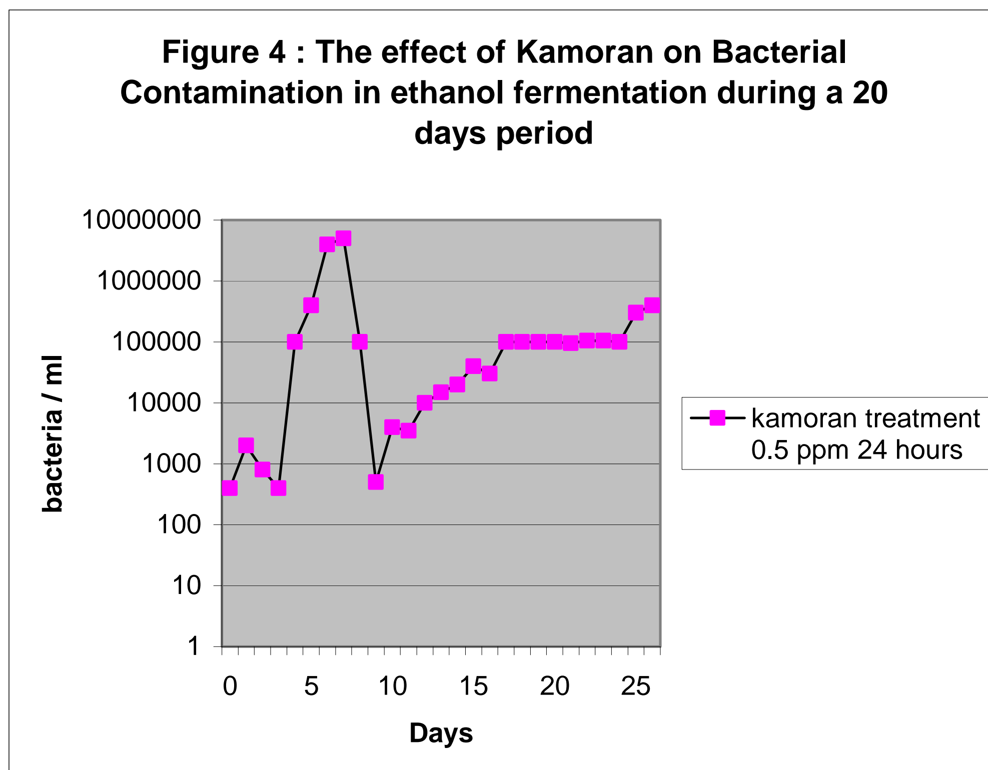


Figure 3 : The effect of Kamoran on Bacterial Contamination in a continous process ethanol fermentation operation
bacteria / ml
10000000
1000000
100000
10000
1000
100
10
1
0
5
10
15
20
Days
kamoran treatment 1 ppm for 24 hours

European Continuous-Process Trial 2

This trial was conducted in a commercial plant with a continuous ethanol fermentation process. The trial protocol called for the introduction of KAMORAN® into the fermenter and the feedstock at a concentration of 0.5 parts per million for 24 hours when the bacterial count exceeded 106 organisms per milliliter. That degree of bacterial contamination occurred on Day 7 and the 24-hour KAMORAN® treatment began on Day 8.

Results: The bacterial population declined rapidly upon the introduction of KAMORAN® and continued for 24 hours after the treatment was discontinued. The composition-of the bacterial contamination was not deter- mined; but, because of the effectiveness of the very low level of KAMORAN® (0.5 ppm), it is suspected that principally bacteria that are highly sensitive to KAMORAN® were present. With KAMORAN® eliminated from the broth, bacte- rial growth resumed at a slow pace. Because KAMORAN® had great I y re- duced the bacterial population, the contamination level was manageable for the next 15 days after cessation of treatment. See Figure 4, below.



North American Batch-Process Study

This study was conducted to evaluate the effectiveness of KAMORAN® in reducing lactic acid production and increasing ethanol yield in a commercial, batch fermentation plant using starch-based feedstock. Four fermenter lots were used as non-treated controls. Two fermenters were treated during fill with 1.0 ppm or 1.5 ppm of KAMORAN® , each. After yeast was introduced, samples were periodically taken from each fermenter and analyzed for acidity, solids and ethanol, glucose, lactic acid and acetic acid concentrations.

Results: KAMORAN® reduced lactic acid production as indicated by lower lactic acid concentrations, lower acidity, and higher pH values for the fermenters treated with the product. Also, the final ethanol concentration was reached more quickly in the fermenters treated with KAMORAN® . Solids conversion rates were equal in the control and treated fermenters.

The differences in ethanol yield, lactic acid, acidity and pH between the treated and untreated fermenters are shown graphically in Figures 5-8, on Pages 16 and 17.

The averages of the measurements recorded during 4-hour time periods following introduction of yeast into the fermenters are presented in Table 4, on Pages 18 and 19. Nat all of the fermenters were tested for all of the measurements during each 4-hour time segment, consequently, "NM" in the table indicates the value was not measured. The values listed for Controls are averages of the values from the four non treated control fermenters.

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Figure 5 : The effect of Kamoran on ethanol yield in a North American Starch based batch fermentation study

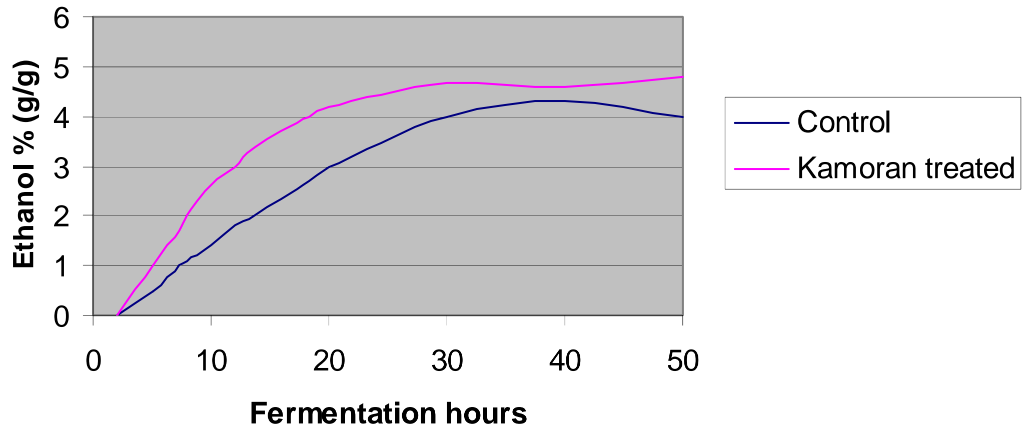
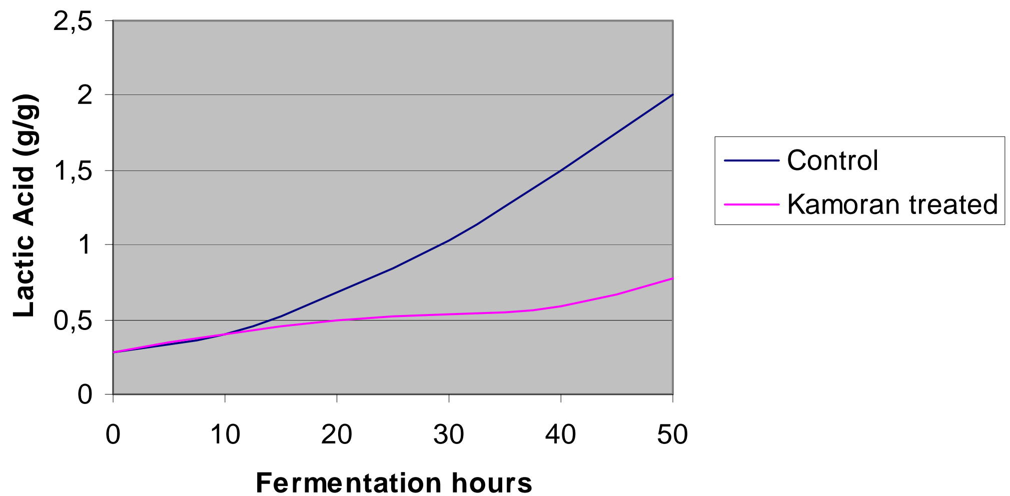


Figure 6 : The effect of Kamoran on Lactic Acid production in a North American Starch based batch fermentation study



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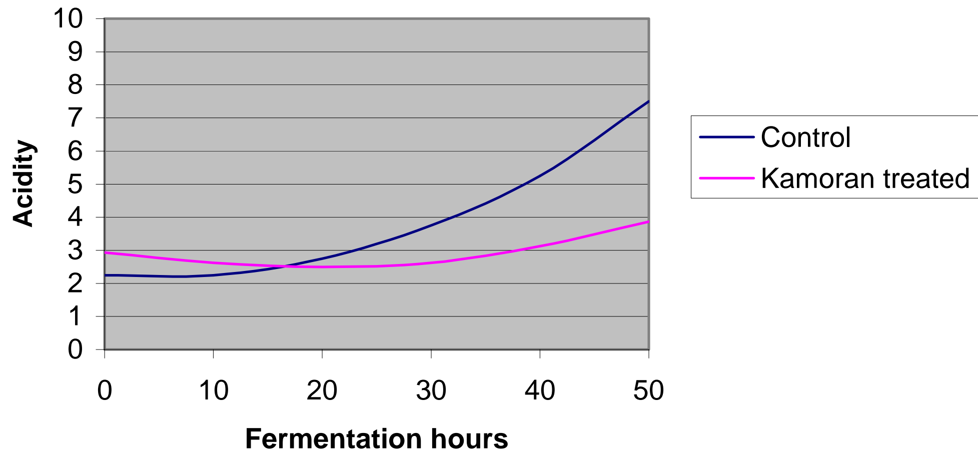

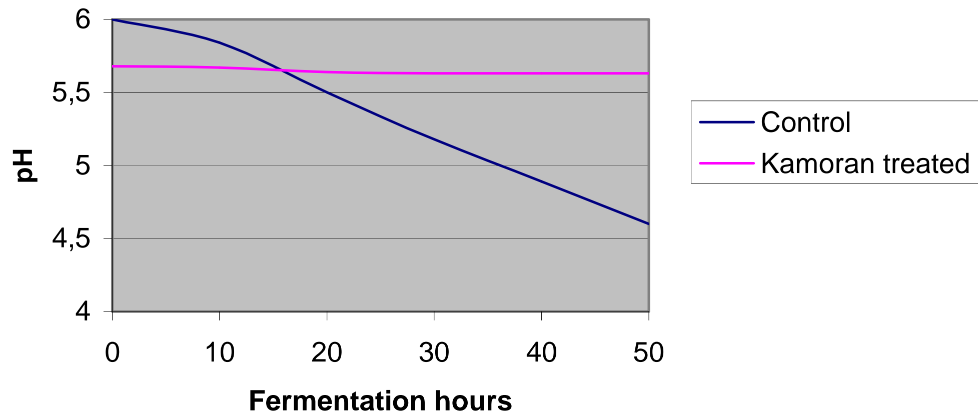

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Table 4 : The effects of KAMORAN® treatments on production measurements in a North America Batch Fermentation Study

Hours after yeast treatment	Concentration [1]					Acidity ml [2]	pH
	Glucose	Lactic Acid	Acetic Acid	Ethanol	Solids		
3-6							
Controls[3]	2.07	0.23	0.13	0.33	13.9	3.07	6.00
KAMORAN® 1.0 ppm	1.8	0.6	0.2	NM	NM	NM	NM
KAMORAN® 1.5 ppm	2.25	0.4	0.2	0.35	12.9	1.6	6.20
7-10							
ControlsNM[3]	0.9	0.25	0.2	0.7	14.6	3.1	5.72
KAMORAN® 1.0 ppm	NM	NM	NM	0.8	12.4	2.7	5.65
KAMORAN® 1.5 ppm	0.0	0.5	0.2	1.0	11.9	3.2	5.80
11-14							
Controls[3]	0.2	0.43	0.23	1.37	NM	2.23	5.59
KAMORAN® 1.0 ppm	1.1	0.3	0.1	2.8	10.1	2.7	5.73
KAMORAN® 1.5 ppm	0.0	0.5	0.6	1.5	14.6	2.9	5.74
15-18							
Controls[3]	0.6	0.53	0.1	2.73	8.3	2.68	5.59
KAMORAN® 1.0 ppm	NM	0.4	NM	3.9	NM	2.5	NM
KAMORAN® 1.5 ppm	NM	NM	NM	NM	NM	NM	NM
19-22							
Controls[3]	0.2	0.7	0.2	3.35	11.0	3.1	5.46
KAMORAN® 1.0 ppm	0.0	NM	NM	3.3	NM	NM	NM
KAMORAN® 1.5 ppm	0.0	1.3	0.25	3.1	8.0	2.6	5.74
23-26							
Controls[3]	0.0	0.7	0.2	3.43	8.3	3.18	5.3
KAMORAN® 1.0 ppm	NM	NM	NM	NM	NM	NM	NM
KAMORAN® 1.5 ppm	NM	NM	NM	NM	NM	NM	NM
27-30							
Controls[3]	0.0	0.93	0.35	3.4	7.62	3.13	5.30
KAMORAN® 1.0 ppm	0.45	0.5	0.1	4.95	6.16	2.85	5.56
KAMORAN® 1.5 ppm	0.4	0.5	0.1	3.7	7.54	2.5	5.76
31-34							
Controls[3]	0.2	1.65	0.3	3.85	7.68	4.1	5.03
KAMORAN® 1.0 ppm	0.4	0.4	0.0	4.8	7.18	2.8	5.54
KAMORAN® 1.5 ppm	0.2	0.9	0.2	4.1	7.25	2.3	5.63
35-38							
Controls[3]	0.0	1.4	0.2	4.0	7.36	9.1	4.50

KAMORAN® 1.0 ppm	NM	NM	NM	NM	NM	NM	NM
KAMORAN® 1.5 ppm	NM	NM	NM	4.1	NM	NM	5.70
39-42							
Controls[3]	0.17	1.47	0.28	4.45	6.25	5.15	4.70
KAMORAN® 1.0 ppm	1.1	0.5	0.0	5.1	6.72	2.6	5.70
KAMORAN® 1.5 ppm	NM	NM	NM	NM	NM	NM	NM
43-46							
Controls[3]	0.0	1.85	0.4	4.45	6.67	6.8	4.40
KAMORAN® 1.0 ppm	0.4	0.6	0.0	5.2	6.72	2.8	5.55
KAMORAN® 1.5 ppm	0.3	0.8	0.2	4.9	6.49	2.5	5.38
47-50							
Controls[3]	0.0	NM	0.3	4.3	6.75	5.35	4.35
KAMORAN® 1.0 ppm	NM	NM	NM	NM	NM	NM	NM
KAMORAN® 1.5 ppm	0.2	0.7	0.1	5.0	6.82	NM	5.17

1 : g/g **2:** milliters of NaOH solution (0.1 N) required to neutralize filtrate-water solution (1 part filtrate to 4 parts distilled water) **3 :** averages of measurements from four non treated fermenters

South American Batch-Process Trial 1

Two trials were conducted in a commercial batch fermentation plant that uses sugar cane feedstock. The plant consists of two identical units of fermenters that receive the same feedstock from a single sugar cane crushing and preparation facility.

For Trial 1, feedstock going into fermenters of Unit 1 was treated -with 3 ppm of KAMORAN® beginning during Day 2 and continuing into Day 3 cover- ing 2.4 fermentation cycles of a 7-day test period. Feedstock going into the fermenters of Unit 2, was not treated. Bacterial contamination, aciorty, yeast viability and cycle time were measured in both units for each batch.

Results : In Trial 1 , bacterial contamination was quite high when the KAMORAN® treatment was initiated; 9.0x107 and 9.8x107 in Units 1 and 2, respectively. KAMORAN® had a positive effect on each of the parameters measured in comparison to the nontreated fermeters in Unit 2. Bacterial contamination, acidity and fermentation cycle time were reduced and yeast viability was increased by KAMORAN® . Feedstock contained greater than 106 bacteria per milliliter throughout the trial period. The reduction of bacterial contamination is illustrated in Figure 9, below. The data collected in this trial are summarized in Table 5, on the next page.

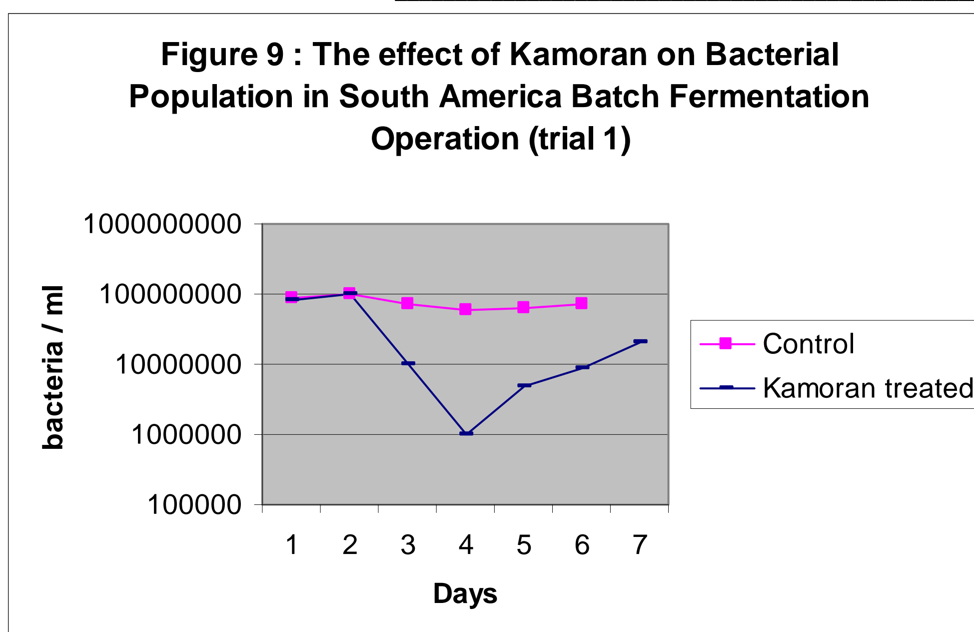


Figure 9 : The effect of Kamoran on Bacterial Population in South America Batch Fermentation Operation (trial 1)

Table 5 : The effects of KAMORAN® on batch production of ethanol from sugar cane feedstock in a South American Operation (trial 1)

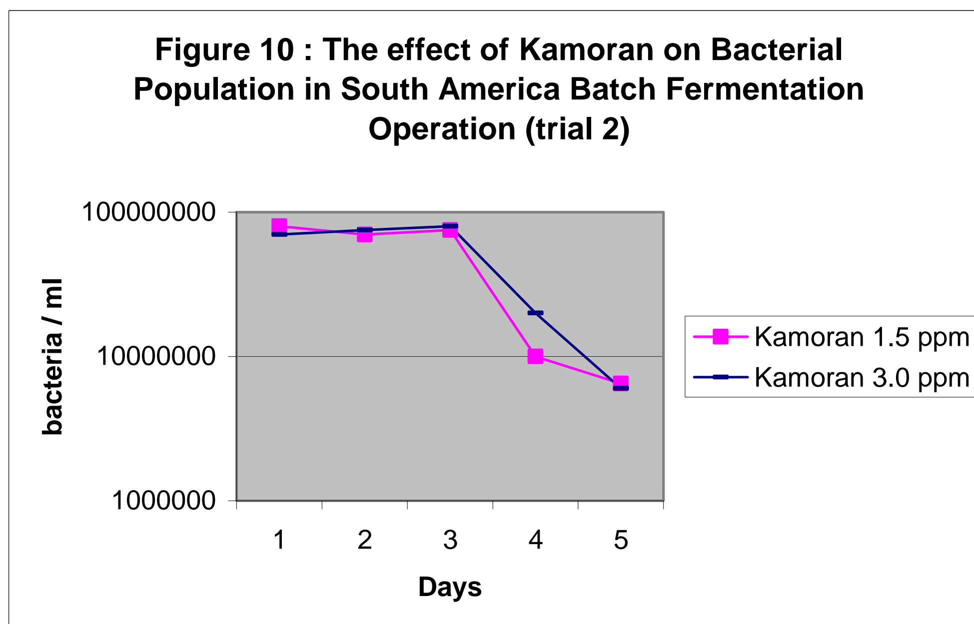
Day	Bacterial contamination / ml		Acidity		Yeast viability %		Fermentation cycle, time hours	
	Control	KAMORAN®	Control	KAMORAN®	Control	KAMORAN®	Control	KAMORAN®
1	$5.6.10^7$	$7.8.10^7$	2.2	2.1	84	80	8.4	7.1
2[1]	$9.8.10^7$	$9.0.10^7$	2.1	2.1	78	81	8.0	7.0
3[2]	$5.4.10^7$	$1.0.10^7$	2.0	1.8	74	74	7.4	7.0
4	$4.1.10^7$	$0.1.10^7$	2.0	1.9	78	81	7.5	7.3
5	$5.5.10^7$	$0.3.10^7$	1.9	1.6	79	83	7.6	7.4
6	$6.1.10^7$	$0.8.10^7$	2.0	1.8	78	82	8.6	7.0
7	$8.1.10^7$	$1.5.10^7$	2.2	1.8	79	81	8.1	7.2

1 : 2.4 cycle KAMORAN® treatment initiated during day 2
2 : 2.4 cycle KAMORAN® treatment concluded during day 3

South American Batch-Process Trial 2

In the South American Trial 2, the fermenters in Unit 1 were treated with 3 ppm of KAMORAN® and the fermenters in Unit 2 were treated with 1.5 ppm of KAMORAN® . Bacterial contamination was measured daily and treatments were initiated on Day 3 of a five-day test period.

Results: In Trial 2, on Day 1, the .bacterial contamination in Unit 1 was less than that in Unit 2, however, on Day 3, when the KAMORAN® treatments were initiated, contamination was greater in Unit 1. When the final measurement was made on Day 5, the3.ppm level of KAMORAN® in Unit 1 had reduced the bacterial contamination to slightly below that found in the fermenters of Unit 2 which were treated with 1.5 ppm of KAMORAN® . Feed- stock contained greater than 106 bacteria per milliliter, therefore, that was the lowest possible level of bacteria throughout the trial period. The results are summarized in Figure 10, on Page 22.



South American Continuous-Process Study

This trial was conducted in a commercial, continuous-process plant using sugar cane feedstock. On Day 1 of a 15-day test period, bacterial contamination, pH, yeast viability, ethanol yield and fermentation efficiency measurements were recorded. KAMORAN® , at a level of 3 ppm, was introduced on Day 2 and was added at that level continuously throughou1 the test period.

Results : KAMORAN® reduced the level of bacterial contamination, increased the viability of the yeast, and increased pH, ethanol yield, and fermentation efficiency compared to the measurements of those parameters recorded before KAMORAN® was introduced. The data for each of the 15 days during the test period are summarized in Table 6 and the positive effects of KAMORAN® on bacterial contamination and yeast viability are illustrated in Figure 11, on Page 23.

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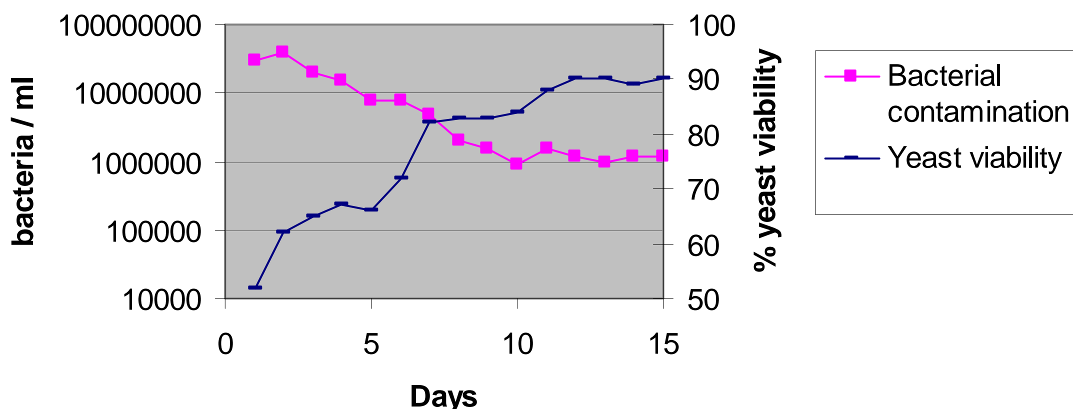
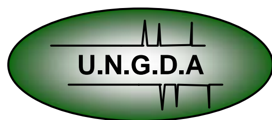
Table 6 : The effects of KAMORAN® on continuous production of ethanol from sugar cane feedstock in a South American Operation.

Day	Bacteria/ml	pH	Yeast viability %	Ethanol %	Fermentation efficiency
1[1]	$2.5.10^{7}$	3.9	52.7	8.74	91.54
2	$2.7.10^{7}$	4.2	61.7	8.60	91.93
3	$2.0.10^{7}$	4.2	64.0	8.80	91.80
4	$1.7.10^{7}$	4.4	66.0	8.40	92.84
5	6.2.106	4.2	65.0	9.00	92.49
6	6.0.106	4.3	73.0	10.00	92.97
7	$3.5.10^{6}$	4.4	82.0	9.60	91.20
8	$1.9.10^{6}$	4.1	84.0	9.50	91.20
9	1.6.106	4.7	84.0	8.60	92.66
10	$8.0.10^{5}$	4.9	85.0	8.30	91.33
11	1.9.106	4.7	89.0	8.70	92.10
12	1.4.106	4.5	91.0	9.10	93.37
13	$1.0.10^{6}$	4.3	91.0	9.10	92.71
14	1.4.106	4.3	89.0	9.40	92.97
15	$1.4.10^{6}$	4.6	91.0	8.80	92.62

1 : not treated with KAMORAN®

Figure 11 : The effect of Kamoran on Bacterial Contamination iand Yeast Viability in Continuous Production of ethanol from sugar-cane feedstock





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Safety of KAMORAN®

The safety of Monensin Sodium, the active ingredient in KAMORAN® , has been tested in many laboratory and farm animals as well as common wildlife species. Also, its effects on the environment have been extensively studied. The results of these studies demonstrate the safety of Monensin Sodium, or KAMORAN® , when it is used as recommended for approved purposes. Following are summaries of studies pertinent ta the use of KAMORAN® in commercial ethanol fermentation.

Toxicological Studies

Acute Toxicity in Laboratory and farm Animals :

The acute oral toxicity of Monensin Sodium has been evaluated in many species. The LD_{50}'s and LD_0's for several species are listed in Table 7.

Table 7 : Monensin LD 50's and LD 0's for several species.

Species	LD50+/- SE (mg monensin activity/kg body weight)	LD0 (mg monensin activity/kg body weight)
Mouse		
Male	70.0 +/- 9.0	
Female	98.0+/- 12.0	
Rat		
Male	40.1+/- 3.0	
Female	28.6+/- 3.8	
Dog		20
Male	> 20.0	
Female	> 10.0	
Rabbit	41.7+/- 3.6	
Monkey		>160.0
Cattle	26.4	10
Sheep	11.9+/- 1.2	3
Goat	26.4 +/- 4.0	10
Pig	16.7 +/- 3.57	4
Horse	2.3 (estimated)	
Guinea fowl	95 +/- 11	Approx. 28

Aerosolization Study In Rats :

Ten male and 10 female Wistar rats were fasted overnight prior to a one- hour "head only- exposure to a solid particulate aerosol of Monensin Sodium. The total gravimetric exposure concentration was 2.53 ::t: 0.39 (S.D.) milligrams per liter. The trial duration was 14 days.

Results : All rats survived the exposure. Males and females exhibited hypoactivity immediately after exposure. All animals appeared normal within 24 hours post exposure and remained 50 for the duration of the 14- day study. Mean body weights for bath male and female rats increased over the course of the trial. -

Subchronic Inhalation Toxicity of Monensln Sodium in Beagle Dogs :

Male and female beagle dogs were exposed to a sub-80 sieve fraction of mycelial monensin six hours per day, five days per week for 90 days for a total of 65 exposures. Monensin activity levels tested were 0, 0.08, 0.15, and 0.84 micrograms per liter. Measurements included clinical signs, mortality, body weight, organ weights, serum chemistry, hematology, electrocardiography, gross pathology and Monensin blood levels.

Results : Monensin had no effect on mortality, organ weight or body weight. No clinical signs were observed in dogs exposed to the low and middle levels of Monensin. No treatment related pathological lesions occurred. Clinical signs observed with the higher exposure included ocular irritation, bloody diarrhea, salivation and hypoactivity. Hematology tests revealed the dogs exposed at the high level of monensin had elevated mean platelet counts. Serum chemistry showed elevated ALT, AST, CPK and LDH. The low and middle level of exposure had no effect on electrocardiography results. The high level, however, caused tachycardia, R wave suppression, altered T waves and premature ventricular repolarization. Blood levels of monensin sodium were not related to exposure level, and at times during the study were not detectable. The 0.84 microgram I eve I was identified as a toxic level to the dogs and 0.15 microgram was identified as a no effect level.

Cardiovascular Effects of Monensin Sodium In Dogs:

To evaluate the cardiovascular effects of Monensin Sodium, doses of 0.035 and 0.69 milligrams per kilogram of body weight were administered intravenously to anesthetized dogs.

Results : Both intravenous doses tested resulted in increased left ventricular contractility, blood pressure, heart rate and coronary artery flow. They also caused premature ventricular contractions and ventricular tachycardia.

In another test, conscious dogs received intravenous injections of 0.0069 to 0.138 milligrams per kilogram of body weight, or 0.138 to 1.38 milligrams per kilogram of body weight orally. Blood pressure, left anterior descending coronary artery blood flow and heart rate were measured.

Results : There were no biologically important changes in dogs that received 0.0345 milligrams per kilogram, or less, intravenously. In- creased coronary blood flow occurred in dogs that received monensin intravenously at 0.069 milligrams per kilogram, or higher. The intravenous dose of 0.138 milligrams per kilogram increased blood pressure. The heart rate was not affected by intravenous doses of less than 0.138 milligrams per kilogram.

Following oral administration, no significant changes in coronary flow, mean blood pressure or heart rate were observed at doses of 0.138 and 0.345 milligrams per kilogram of body weight. Heart rate and blood pres- sure did not change significantly following oral doses up to 1.38 milligrams per kilogram. Coronary flow, however, increased following doses of 0.69 and 1.38 milIigrams per kilogram.

Acute Dermal Toxicity in Rabbits :

To evaluate the dermal toxicity of M0nensin Sodium in rabbits, the fur was clipped from the backs of two groups of six New Zealand albino rabbits (three per sex). The exposed skin of all animals was abraded with a stiff nylon brush. One group of rabbits received an application of 2 grams of Monensin Sodium per kilogram that was held on the animal's bad{ under occlusion for 24 hours. Following removal of the occlusive dressings, the treated skin was rinsed with tap water and dried. Rabbits wore felt collars

throughout the study to inhibit licking of the treatment site. The controls wore occlusive dressings and were washed and collared. Signs of toxicity, including dermal irritation, were monitored daily for two weeks. All animals were weighed daily and submitted for grass necropsy at trial end.

Results : Mean body weight data indicated an initial loss among animals receiving the Monensin treatment that reversed after several days. The weight gains for the controls and the rabbits treated with Monensin over the two weeks were similar. Slight transient dermal irritation was observed in all of the animals treated with Monensin Sodium.

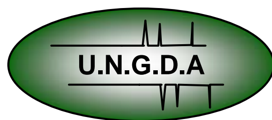
Toxicity Studies in Wildlife Species :

A number of toxicity tests have been conducted with representative wildlife species to evaluate the effects of exposure to Monensin Sodium. The results of these studies are summarized in Table 8. below.

Table 8 : Toxicity of mycelial monensin in representative wildlife species.

Study number	Test animal	Route of exposure	Observation period	Median effect monensin sodium Concentration/dose	No observed effect monensin sodium Concentration/dose
F10082	Bluegill (*Lepomis macrochirus)*	Water	96 hours	LC_{50} = 16.9 mg/L[a]	3.1 mg/l[a]
F10182	Rainbow trout (*Salmo gairdneri)*	Water	96 hours	LC_{50} = 9.0 mg/L[a]	0.70 mg/l[a]
CO2382	*Daphnia magna*	Water	48 hours	EC_{50} = 10.7 mg/L[a]	4.2 mg/l[a]
W01082	Earthworm	Soil	14 days	LC_{50} >100 mg/Kg[b]	22.5 mg/Kg
A03680	Bobwhite	Oral	14 days	LD_{50} = 85.7 mg/Kg[b]	ND[c]
A01882	Bobwhite	Oral	14 days	LD_{50} > 45.0 mg/Kg[b]	27.5 mg/Kg
A03780	Bobwhite	Diet	8 days	LC_{50} = 0.109 %	ND[c]
A01982	Bobwhite	Diet	8 days	LC_{50} = 0.0365 %[b]	0.01 %
A01782	Mallard	Diet	8 days	LC_{50} = 0.5 %[b]	0.062 %

a : based on analyzed monensin sodium concentrations in exposure solutions
b : these concentrations/doses represented the highest levels tested
c : the no observed effect was not determined.



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Environmental Safety Studies

Many studies have been conducted to evaluate the possible effects of the commercial use of Monensin Sodium on the environment and on workers who handle it regularly. Following are summaries of important studies.

Degradation of Monensin Sodium in Soil

Studies have been conducted to determine the degradation of Monensin Sodium in soil. In greenhouse soil flats, Monensin was added to the soil at an initial level of approximately one ppm, with and without animal feces.

Results : Assay results reveal that Monensin Sodium begins to degrade quickly in the soil. In the samples with feces, only about 22 percent of the initial assay value was detectable after five days in the soil and no Monensin activity was present after 12 to 14 days. About 50 percent of the initial Monensin assay values of samples without feces were present after five days in the soil and no activity was found at 28 days.

Phytopathology :

Two experiments were conducted to evaluate the effects of soil incorporated crystalline Monensin Sodium on a variety of plants.

The test plants included :

Cotton	Sugar Beet P	Tomato	Alfafa
Peppers	Cucumbers	Soybeans	Wheat
Barley	Rice	Corn	Ky.31 fescue
Oats	Sorghum		

Results : Crystalline Monensin Sodium incorporated into the soil is relatively non phytotoxic at rates of 1.12 to 2.24 kilograms per hectare. Moderate to severe plant injury was observed on several plants at rates of 4.48 to 8.96 kilograms per hectare.

Soil Leaching Study

A soil column leaching study was conducted in the laboratory to ascertain the leaching characteristics of Monensin Sodium in four types of soil- sandy, sandy loam, loam and silty clay loam.

Results : The application of the equivalent of 63.5 centimeters of rain caused substantial leaching of Monensin Sodium from sandy and sandy loam soil, but there was

little leaching from the loam and silty clay loam soils. Substantial losses of Monensin, presumably due to degradation, were observed during the leaching process with greater losses occur- ring in soils requiring longer time periods for leaching.

Exposure hazards

The following information, obtained from animal toxicological studies. is provided to aid you in the safe use of KAMORAN® .

Acute Exposure

Eyes : In rabbit. a 24 percent Monensin Sodium mixture was corrosive but permanent damage was prevented by rinsing the *eye* with water immediately after the exposure.

Skin : In rabbit. a 24 percent Monensin Sodium mixture applied to the skin at the level of 500 milligrams per kilogram of body weight was non irritating and no toxic effects or deaths occurred.

Inhalation : In rat, a 24 percent Monensin Sodium mixture administered by inhalation at the level of 370 milligrams per cubic meter of air for one hour did not cause any deaths.

Ingestion : In rat, the median lethal dose of ingested Monensin Sodium was determined to be 34 milligrams per kilogram of body weight. Decreased food consumption, reduced activity, skeletal muscle weakness, incoordination, diarrhea, decreased weight gain and delayed death were observed.

Sensitization : In Guinea pig, a 24 percent mixture of Monensin Sodium was not a contact sensitizer**.**

Chronic exposure

The following effects of Monensin Sodium were reported in chronic, teratogenic, and reproductive toxicity studies in laboratory animals with experimental dosage levels and durations of exposure in excess of those likely to occur in humans.

Chronic Toxicity : Heart and skeletal muscle lesions (degenerative and reparative). Decreased body weight gains. Increased kidney, heart, thyroid, adrenal, prostate, testes, liver, and spleen weights. Electro- cardiogram effects. Congestive heart failure. Elevated blood enzymes.

Teratology and Reproduction : animal studies.

No effects of were identified in

Mutagenicity :
Monensin sodium was found to be not mutagenetic in bacteria cells.

Carcinogenicity :
Based upon the results of lifetime studies, Monensin Sodium is not considered to be carcinogenic.

Effects of exposure

While there are laboratory animal studies indicating that Monensin Sodium at exaggerated levels of exposure may cause cardiac and skeletal muscle damage, it has been concluded that KAMORAN® does not present a hazard when recommended handling procedures are followed.

Signs and Symptoms of Exposure

Skin rash and irritation resulting from exposure to Monensin Sodium have been reported. Based on the results of animal studies, Monensin Sodium may cause burns or permanent tissue damage to eyes unless rinsed with water immediately. Nausea, dizziness, nasal congestion/irritation, diarrhea, muscular discomfort, chest heaviness or pain, and difficulty in breathing have occurred infrequently. Rare or singular events reported

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include headache, puffiness of face, abdominal pain/cramps, coughing up blood, nosebleed, eye swelling/irritation/redness, allergic reactions, nervousness, and increased pulse rate.

Medical Conditions Generally Aggravated by Exposure

Persons with a history of allergies, contact dermatitis, or chronic rashes should use special precautions to avoid skin contact with Monensin sodium or exposure to dust. When a bolus injection of Monensin Sodium is administered to laboratory animals, cardiovascular changes such as increased heart rate and elevated blood pressure occur. There is no corroborative information available to establish that exposure to Monensin Sodium aggravates any medical condition in humans.

Primary Routes of Entry Inhalation and skin contact.

Keep KAMORAN® out of reach of children. KAMORAN® is hazardous to humans and animals and is not to be consumed by humans or animals. It is fatal (poisonous) if swallowed or inhaled. Don not use KAMORAN® in the production of beer, wine or other non-distilled beverages.

Operator Protection

Do not breathe dust of KAMORAN® . KAMORAN® is corrosive and can cause eye damage and skin irritation. Do not get in eyes, on skin or on clothing. Wear protective clothing, respirator, goggles or face shield and rubber gloves when handling KAMORAN® . After handling, wash thoroughly with soap and water. If accidental eye contact occurs, immediately rinse with water. High levels of exposure may cause impairment to the skeletal and heart muscles.

Spill Handling

A1ways wear protective c1othing white hand1ing KAMORAN® . Use ails or water to contro1 dust then sweep or vacuum the spi11ed product. Residues may be f1ushed with water. Prevent spil1ed material from flowing onto adjacent land or into streams, ponds or lakes.

Exposure Guidelines

Although neither Permissable Exposure limit or Threshold Limit Value has been established for KAMORAN® , the Manufacturer Exposure Guideline is 0.015 milligrams per cubic meter TWA for 12 hours.

Use of Yeast and Non-volatile Residues

When KAMORAN® has been used in the production of distilled ethanol. do not use yeast or non-volatile residues for human consumption or in products intended for human consumption nor feed them to non-ruminant animals.

Fire and explosion hazard data

Auto-ignition Temperature: No ignition up to 262 ° Centigrade *(504°* F).

Flashpoint: Not applicable.

Explosive limits

Lower Explosive limit (LEL) -0.115 oz/cu ft. Upper Explosive limit (UEL) -Not established

Fire and Explosion Hazards: As a finely divided material, KAMORAN® my form dust mixtures in air which could explode if subjected to an ignition source. KAMORAN® is relatively non combustible and continuous ignition is required to support flames.

Fire Fighting Information: Use wa1er, carbon dioxide, dry chemical, foam, of Halon. Do not allow water run-off from the tire site to enter streams, ponds or lakes. Keep containers of KAMORAN® cooled with water spray.

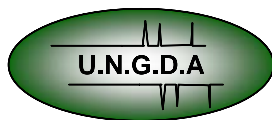
Safe storage and container disposal

Storage

Always store KAMORAN® in a cool dry place where it is protected from moisture and heat.

Container Disposal

Completely empty fiber drum by shaking and tapping sides and bottom to loosen clinging particles. Empty residue into manufacturing equipment. Crush or puncture and dispose of container in a sanitary landfill or by incineration if allowed by local regulations.



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First aid - practical treatment for exposure to KAMORAN®

Eyes

Hold eyelids open and rinse eyes with a steady, gentle stream of water for 15 minutes. Immediately have eyes examined by an opthamologist (eye doctor) or other physician. Failure to thoroughly rinse the eyes can result in possible damage.

Skin

Remove contaminated clothing. Wash all exposed areas of skin with plenty of soap and water. Get medical attention if irritation develops. Do not wear any contaminated articles of clothing until they have been thoroughly cleaned or laundered.

Inhalation

Move the individual to fresh air. Get professional medical assistance if breathing difficulty occurs. If the individual has stopped breathing, provide artificial respiration (mouth-to-mouth) and call a physician immediately.

Ingestion

Call a physician immediately. Victim should drink one or two glasses of water and take 1 to 2 tablespoons (15-30 milliliters) of syrup of ipeca to induce vomiting. Do not attempt to give anything by mouth or induce vomiting if the person is unconscious. Immediately transport the person to a medical facility for examination and treatment by a physician.

Appendix

Items proposed to be inserted in the Appendix include .Package literature
.Material Safety Data sheet
.List of appropriate affiliate ad dresses, contacts, etc.