

GLUTARALDEHYDE APPLICATION FOR THE CONTROL OF MICROBIAL GROWTH
IN SUGAR BEET DIFFUSION PROCESSES

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ABSTRACT

Glutaraldehyde was recently approved as a biocontrol agent for use in sugar beet diffusion systems. Glutaraldehyde was shown to be an effective bactericide when applied to process waters obtained from sugar beet extraction systems. Application of glutaraldehyde decreased the viable numbers of bacteria in process waters containing the indigenous bacteria. Efficacy was also demonstrated in filter sterilized process waters inoculated with a Leuconostoc spp. that had been isolated from a sugar beet diffusion system. Field trials performed at two midwestern sugar beet factories are also described. Glutaraldehyde was applied when the concentration of L-lactic acid exceeded action limits. The concentration of L-lactate in diffusion juice from two towers decreased significantly following glutaraldehyde addition. Decreases in viable numbers of acid producing thermophilic bacteria were also observed. In these initial field applications, control of the diffusion system was regained by addition of glutaraldehyde when the concentration of L-lactate exceeded action levels. Slug doses of glutaraldehyde when the system was within normal operating parameters did not prevent subsequent increases in microbial activity. Further field trials are in progress to define guidelines for optimum application.

INTRODUCTION

The uncontrolled growth of microorganisms in the sugar beet extraction process results in a number of operating problems such as pluggage, pH depression, and difficulties in clarification and filtration. The proliferation of microorganisms during sucrose extraction has been implicated in product losses with decreases in yields approaching two percent.

Control of microbiological activity in the sugar beet extraction process was accomplished by applying biocides to the extraction system. Application of biocidally active carbamates has been routinely practiced in many factories. However, the use of carbamates alone has not proven effective in preventing periodic upsets due to microbial proliferation and necessitated the addition of formalin to

regain control (1). Formalin, approximately 30 to 37 percent active ingredient (as formaldehyde), was added in response to a decrease in system pH or in response to other criteria used to measure microbial activity. The use of formalin has come under increasing criticism, since exposure to formaldehyde represents a significant health hazard. For reasons of both safety and product quality, suitable alternatives have been sought.

This study reports investigations into the use of glutaraldehyde as an alternative chemistry for the maintenance of biocontrol in the diffusion process. The antimicrobial properties of glutaraldehyde are well established (2,3,4,5). The significant features of the molecule are its two aldehyde moieties. Being a dialdehyde, glutaraldehyde undergoes all of the typical aldehyde reactions. These include oxidation, reduction, and condensation reactions. The reaction of glutaraldehyde with amines dominates its chemistry. Under typical use conditions, glutaraldehyde reacts with free primary amines. It is less reactive with secondary amines and is generally not reactive with tertiary and quaternary amines (2,3). The reaction of glutaraldehyde with proteins primarily involves the lysine residues (3). The reaction is with the primary amine in the side chain of the basic amino acid (positively charged at pH 6.0). Reactivity with the primary amine, combined with the ability to polymerize via aldol condensation reactions, permits glutaraldehyde to efficiently crosslink proteins located at the bacterial cell surface. As a result, the essential functions of these cell surface proteins are disrupted and cell death ensues.

Glutaraldehyde has recently been approved for use in the sugar beet diffusion process at concentrations up to 250ppm, as active ingredient (a.i.), based on the weight of beets sliced per unit time. A number of studies were conducted to determine the parameters that govern the use of glutaraldehyde in sugar beet systems.

Biocidal efficacy was first determined using sugar beet process water samples. The indigenous bacteria served as the test microorganisms. Additional experiments were conducted with pure cultures of a bacterium isolated from a sugar beet processing facility and belonging to the genus Leuconostoc. The bacterium, Leuconostoc mesenteroides is a sucrose-degrading microorganism that is capable of producing copious quantities of a dextran slime. It is often implicated in a number of problems encountered at sugar processing facilities.

The objectives of this work were to define effective dose ranges for glutaraldehyde in this application, examine whether factors inherent to the sugar beet extraction process would limit glutaraldehyde performance, and determine the ability of this chemistry to control microbial activity in actual sugar beet diffusion systems. This represents work in progress, and is an update on the continuing research being performed to introduce and optimize the application of this chemistry in sugar beet factories.

MATERIALS AND METHODS

Bacteria. Biocidal efficacy studies were initially performed using the microbial population present in process water samples. A Leuconostoc bacterium isolated from process samples was isolated on sucrose-gelatin-azide agar and identified by standard microbiological techniques.

Media and cultivation of microorganisms. When performing experiments in process samples employing the indigenous microbial community as the test organisms, total plate counts were typically performed on tryptone-glucose-extract agar (TGE) (Difco Laboratories, Detroit, MI). Plates were incubated at 37°C for 48hr. To enumerate thermophiles, TGE plates were incubated at 55°C.

Specific groups of microorganisms were enumerated on appropriate selective-differential media. Leuconostoc were enumerated on sucrose-gelatin-azide agar incubated at 30°C. Acid producing thermophiles were enumerated on dextrose-tryptone-agar (DTA) (Difco Laboratories, Detroit, MI) with incubation at 55°C.

Determination of glutaraldehyde effectiveness in sugar beet raw juice. Sterile glass screw-cap vials were labelled and glutaraldehyde was added to each vial to achieve the appropriate final concentrations of the biocide. To each vial, sugar beet raw juice was added and mixed thoroughly by inversion or vortexing. A volume of raw juice without biocide was run as a negative control. The test vials were incubated at 30°C and sampled at defined intervals. Surviving microorganisms were enumerated.

Pure culture studies. Experiments were performed with the Leuconostoc isolate to evaluate the antimicrobial effectiveness of glutaraldehyde in sugar beet process waters that had been filter sterilized. The use of process waters as the suspending medium allowed us to determine whether the

activity was biocidal or biostatic under conditions that permitted growth of the test bacterium.

RESULTS AND DISCUSSION

Efficacy studies performed in the laboratory.

Glutaraldehyde was shown to be effective in decreasing the number of viable microorganisms in sugar beet samples obtained from the field when applied at concentrations allowable by FDA guidelines (≤ 250 ppm a.i.)

In the initial study, the indigenous microbial population of sugar beet raw juice was at a cell density of 10^8 CFU/mL. A concentration of 200 ppm of glutaraldehyde was needed to significantly decrease the viable microbial count. The effective dose was defined as the lowest concentration of biocide at which the number of surviving microorganisms decreased by greater than $1 \log_{10}$.

This experiment was repeated with an initial cell density of 10^6 CFU/mL. In this case, significant decreases in viable count were observed at all glutaraldehyde concentrations ≥ 50 ppm (Figure 1), suggesting that the number of microorganisms present at the time of application may be an important parameter. Examination of the results from a number of individual experiments verified that when initial cell densities were high, the effective concentration of glutaraldehyde was also elevated.

Experiments using indigenous microbes as the test population have the potential to be confounded by consortium effects, that is, anomalies which result from changes in the population makeup or due to interactions between the members of a complex population. These effects may interfere in experiments designed to evaluate specific effects of the biocide. In order to obtain meaningful data from a series of bench tests, while minimizing the potential for problems due to differences in microbial population makeup, subsequent bench tests employed an inoculum prepared from a bacterium belonging to the genus *Leuconostoc* that was isolated from sugar beet raw juice.

An experiment was conducted in a mixture of sugar beet raw juice and pulp press water that had been filter sterilized (0.2 μ m pore-size). The results are graphically displayed in Figure 2. A definite dose response was observed. As the concentration of biocide increased, the time of exposure required to produce a significant decrease in viable count was correspondingly shorter.

To determine the effect of background chemistry on glutaraldehyde performance, an inoculum prepared from the Leuconostoc isolate was transferred to filter sterilized raw juice, filter sterilized pulp press water (PPW), and sterile phosphate buffered saline (PBS). PBS does not contain any ingredients that would interfere with glutaraldehyde and served as the control. The relative effectiveness of glutaraldehyde in the three suspending media were compared.

The effective doses required to decrease the viable count by greater than $1 \log_{10}$ CFU/mL in each of the three test liquids are shown in Figure 3. Approximately 10 times as much glutaraldehyde was needed in the raw juice, to achieve the same efficacy, as that needed when the assay was conducted in PBS. The quantities of biocide required to show effectiveness were all ≤ 100 ppm a.i. Initial cell densities were 10^5 CFU/mL.

Field Studies

The laboratory studies indicated that glutaraldehyde exhibited significant activity as an anti-microbial when tested in samples obtained from sugar beet extraction systems. However, the results of laboratory studies alone are not sufficient to predict the efficacy of glutaraldehyde in actual diffusion systems. Results of laboratory studies are obtained under controlled, defined conditions. In contrast, the conditions encountered in actual sugar beet diffusion systems represent an extremely complex and dynamic ecosystem. Therefore, glutaraldehyde was subsequently evaluated in field trials performed at sugar beet factories located in the Midwest.

A number of criteria are used for evaluating the microbiological status of diffusion systems. One factory, at which a trial was conducted, measured the concentration of lactic acid as an indicator of microbial activity, while the other factory monitored the concentration of nitrite. Lactic acid is always present in sugar beet extraction systems, however, rapid increases in lactic acid concentrations are presumed to be due to increased microbial growth. The lactic acid produced by fermenting bacteria may be L-lactate, D-lactate or both isomers. L-lactate is produced by a number of acid producing thermophiles, including Bacillus stearothermophilus. Increases in L-lactic acid concentration, above normally observed levels, can indicate significant proliferation of acid producing thermophilic bacteria. The source of nitrite in these systems is presumed to be due to the reduction of nitrate by

bacteria. When the concentrations of these indicators exceeded a certain value, action was taken.

During these field trials, a number of methods were employed for evaluating the microbiological status of the diffusion systems. These included the monitoring of L-lactate, pH, and nitrite concentrations to indicate changes in microbial activity. During each test period, specific subgroups of bacteria were enumerated using standard culture techniques. The subgroups that were monitored included mesophilic bacteria (i.e. total plate count), Leuconostoc, and thermophiles.

L-lactate concentrations and nitrite concentrations were determined on-site. The enumeration of bacteria was performed at the Nalco corporate laboratory in Naperville, IL. All samples had been shipped cold via overnight courier and were kept refrigerated until analyzed.

Trial 1. This field trial was conducted at a midwest sugar beet factory. This facility operated two BMA diffusion towers at a slice rate of 330 ton/hr. The retention time in the cossette mixer and diffuser was 65 min. The pH in the diffusion tower was pH 5.1 (at midpoint of tower). The concentration of L-lactate in the diffusion juice had risen to a concentration of 760ppm and 750ppm, respectively, for the two diffusion towers. Glutaraldehyde was applied to the cossette mixer and to the hot end of the diffuser as a slug dose to a final concentration of 100ppm (active ingredient, a.i.). Samples were obtained prior to application and at intervals of 10min, 25min, 1hr, 2hr, 4.5hr, and 6hr following application.

Results. Initial numbers of bacteria were determined in samples drawn prior to glutaraldehyde addition. The initial numbers of microorganisms in the raw juice were 6×10^7 CFU/mL as determined by total plate count and 1×10^7 thermophiles were detected. Total plate counts for diffusion juice samples obtained from each of the respective towers were 3×10^7 and 3×10^5 CFU/mL. The numbers of acid producing thermophilic bacteria in these diffusion juice samples were 3×10^7 CFU/mL and 3×10^5 CFU/mL.

Following application of glutaraldehyde (100ppm a.i.), the numbers of viable mesophilic bacteria in the raw juice showed little change. The number of thermophiles in the raw juice were lowered by more than $2 \log_{10}$ CFU/mL. Mesophiles in one of the diffusion towers decreased from 3×10^7 to 3×10^5 CFU/mL (Figure 4). The mesophile numbers in the second

diffusion tower did not change significantly, remaining at 10^5 CFU/mL. Enumeration of thermophilic bacteria showed that the numbers of viable thermophiles decreased nearly $3 \log_{10}$, from 3×10^7 to 7×10^4 CFU/mL in diffusion tower 1 (Figure 5), while thermophile numbers in diffusion tower 2 showed little change. These data indicate that diffusion tower 1 had a significantly greater level of microbial activity than tower 2 before biocide addition.

The most dramatic results were the changes in L-lactate concentrations following glutaraldehyde application. The L-lactate concentrations exhibited progressive decreases in all of the samples for a period of 6.5hr following biocide addition (Figure 6). The decreases in L-lactate concentration were 380ppm and 400ppm, respectively, for diffusion juice samples obtained from the two diffusion towers. Nitrite measurements followed the same general trend as the lactate measurements (data not shown).

When taken together, these data demonstrated a significant beneficial effect that could be correlated to the application of glutaraldehyde. In this case, glutaraldehyde was added when action levels of lactic acid were exceeded. The lactic acid concentrations decreased to acceptable levels in response to biocide addition, demonstrating that microbiological control was regained by application of glutaraldehyde to the diffuser.

Trial 2.

This trial was conducted at a factory located in the upper Midwest. This facility operated one BMA diffusion tower at a throughput of 242 ton/hour of sliced beets. Retention time in the cossette mixer and diffuser was 60 min. The pH in the tower ranged from pH 5.6 to pH 6.3 during this trial. The concentration of L-lactic acid in the diffusion juice was 396 ppm. Samples were obtained hourly, beginning one hour prior to addition of glutaraldehyde. A second application of glutaraldehyde was performed after three hours. The specific groups of bacteria which were enumerated included Leuconostoc, total thermophiles and acid producing thermophiles.

Results. Initial L-lactate concentrations were low, but increased steadily following application of the biocide to the diffusion system (Figure 7). The increases in L-lactic acid concentrations for samples obtained from the mixed cossettes, the diffusion juice and the mid-bay of the diffuser, roughly correlated with decreases in pH. For raw

juice samples, however, pH measurements were lower than expected based on the L-lactate concentration. The presence of Leuconostoc at 10^5 to 10^6 CFU/mL may have contributed to the pH depression. These bacteria produce only D-lactate, an isomer of lactic acid that would not be detected by the YSI instrument we used during this study. This analyzer detects only L-lactic acid.

Total numbers of mesophilic bacteria showed little change (Figure 8). Leuconostoc were detected only in the raw juice samples and showed a general decrease of nearly $2 \log_{10}$ CFU/mL over the course of the two applications (Figure 9).

The number of thermophilic bacteria, specifically, the acid producing thermophiles, appeared to decrease following the first biocide application (Figure 10). However, one hour following the second application of glutaraldehyde, a rapid increase in thermophilic numbers was detected in the raw juice, mixed cossettes, and diffusion juice. The number of thermophiles in the mid-bay of the diffuser were at or near the lower level of detection used for these analyses (LDL of $<10^3$ CFU/mL for samples obtained prior to application and 10^2 CFU/mL for samples obtained post-application). Total plate counts for the pulp press water samples were all less than 10^3 CFU/mL.

At this factory, microbial numbers in the range of 10^4 to 10^5 CFU/ml were not unusual and their system was considered to be in control. The only samples in which microbial numbers consistently exceeded this level were raw juice samples. Application of glutaraldehyde in this trial did not produce a decrease in lactic acid concentrations, as it did in the first trial. However, in this case, the lactic acid concentrations at the time of biocide addition were considerably lower than in trial 1.

Data from Trial 1 indicated that the benefit from glutaraldehyde application was realized when it was applied in response to upset conditions resulting from uncontrolled microbial growth and thus provided a means to return the microbial activity to normal levels. Data from the second trial suggested that slug dose application of this biocide to a system that was in control by established criteria did not prevent subsequent increases in microbial activity from occurring.

Alternative explanations for the observed differences in performance are being considered and will be addressed in future studies.

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SUMMARY

Glutaraldehyde was shown to be an effective biocide in sugar beet process samples. When applied to actual sugar beet diffusion process samples, it effectively decreased the number of viable bacteria in the indigenous population. The initial bacterial cell density at the time of application was shown to be a factor in defining the concentration of product that was required to achieve a significant decrease in viable microbial numbers.

Field trial results are promising, yet still considered to be preliminary at this time. At one site the beneficial effect of the biocide was clearly evident, while application at a second field trial site yielded less conclusive information. At this time, field investigations are continuing. Introduction of this chemistry into sugar beet processes will require the identification of a number of undetermined variables in the process that influence glutaraldehyde performance. However, with the optimizing of biocide application, glutaraldehyde is likely to become an important tool for minimizing product loss due to microbial proliferation.

REFERENCES

1. Beet-Sugar Technology, R. A. McGinnis (ed.), Copyright 1971, Beet Sugar Development Foundation, Fort Collins, CO.
2. Eager, R. J. Jr., J. Leder, and A. B. Theis. 1986. Glutaraldehyde: factors important for microbiocidal efficiency. Presented at the Third Conference on Progress in Chemical Disinfection, Binghamton, NY.
3. Gorman, S. P., A. D. Russell, and E. M. Scott. 1980. Antimicrobial activity, uses, and mechanism of action of glutaraldehyde. *J. Appl. Bacteriol.* 48:161-190.
4. Rubbo, S. D., Joan F. Gardner, and R. L. Webb. 1967. Biocidal activities of glutaraldehyde and related compounds. *J. Appl. Bacteriol.* 30:78-87.
5. Thomas, S., and A. D. Russell. 1974. Studies on the mechanism of the sporocidal action of glutaraldehyde. *J. Appl. Bacteriol.* 37:83-92.

FIGURE 1.
EFFECTIVENESS OF GLUTARALDEHYDE
IN RAW JUICE DILUTED 1:100

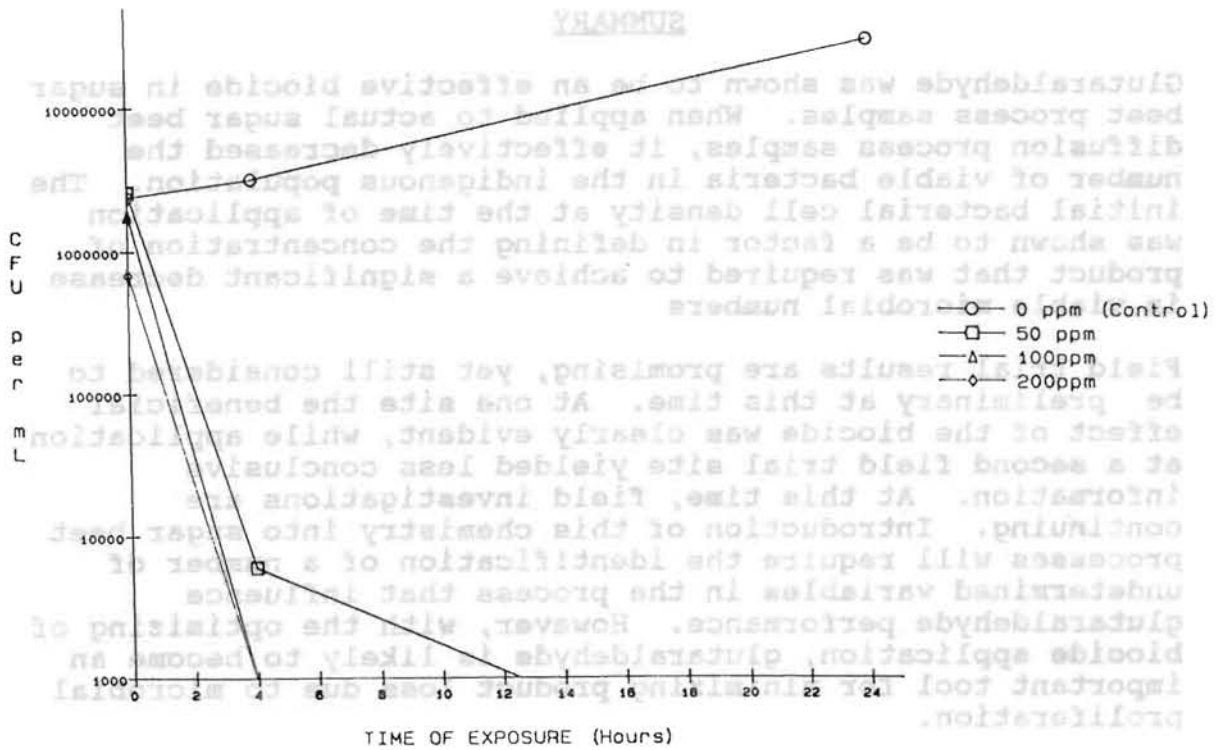


FIGURE 2.
GLUTARALDEHYDE EFFECTIVENESS
Test Organism: *Leuconostoc* spp.
Test Medium: 1:5 Mixture of Raw Juice and Pulp Press Water

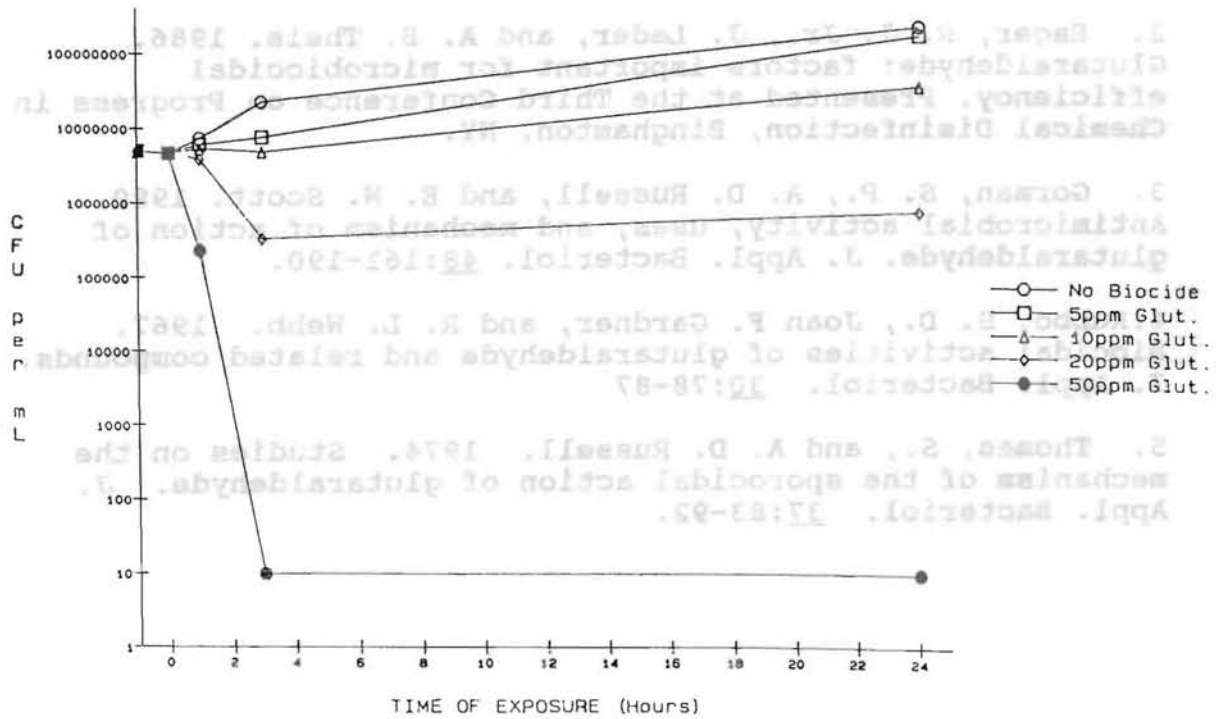


FIGURE 3.
EFFECTIVE DOSES OF GLUTARALDEHYDE
A Comparison of Effective Concentrations
in PBS, Sugar Beet Raw Juice, and Pulp Press Water
Effective Dose = Decrease of $>1\log_{10}$ CFU/mL

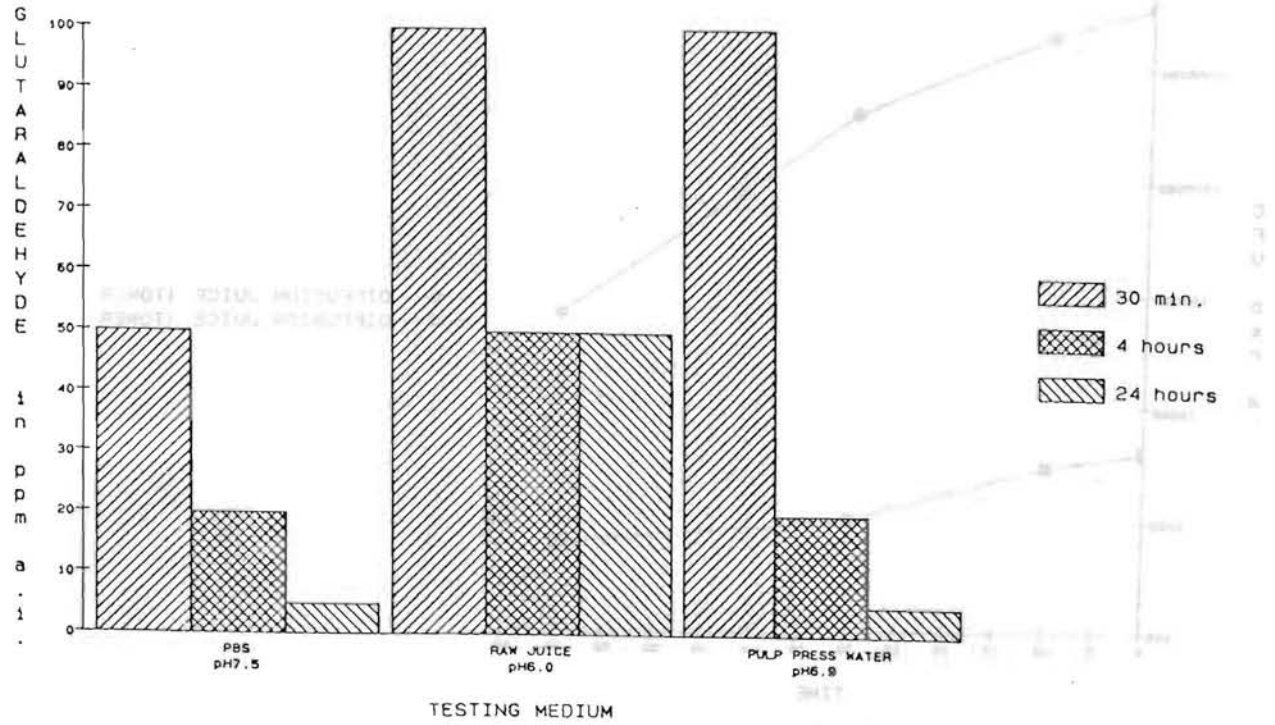


FIGURE 4.
FIELD TRIAL 1.
TOTAL PLATE COUNT
(Mesophiles @ 37C)

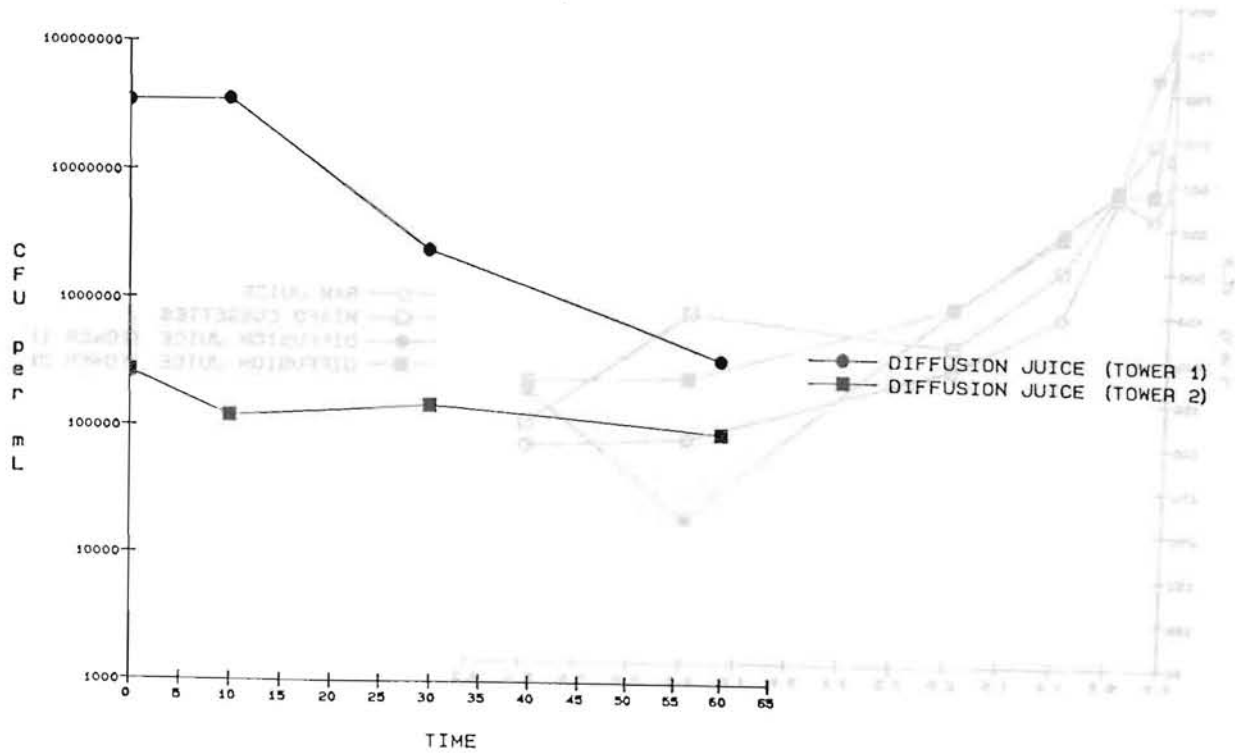


FIGURE 5.
FIELD TRIAL 1.
THERMOPHILIC ACID PRODUCING BACTERIA
(55C)

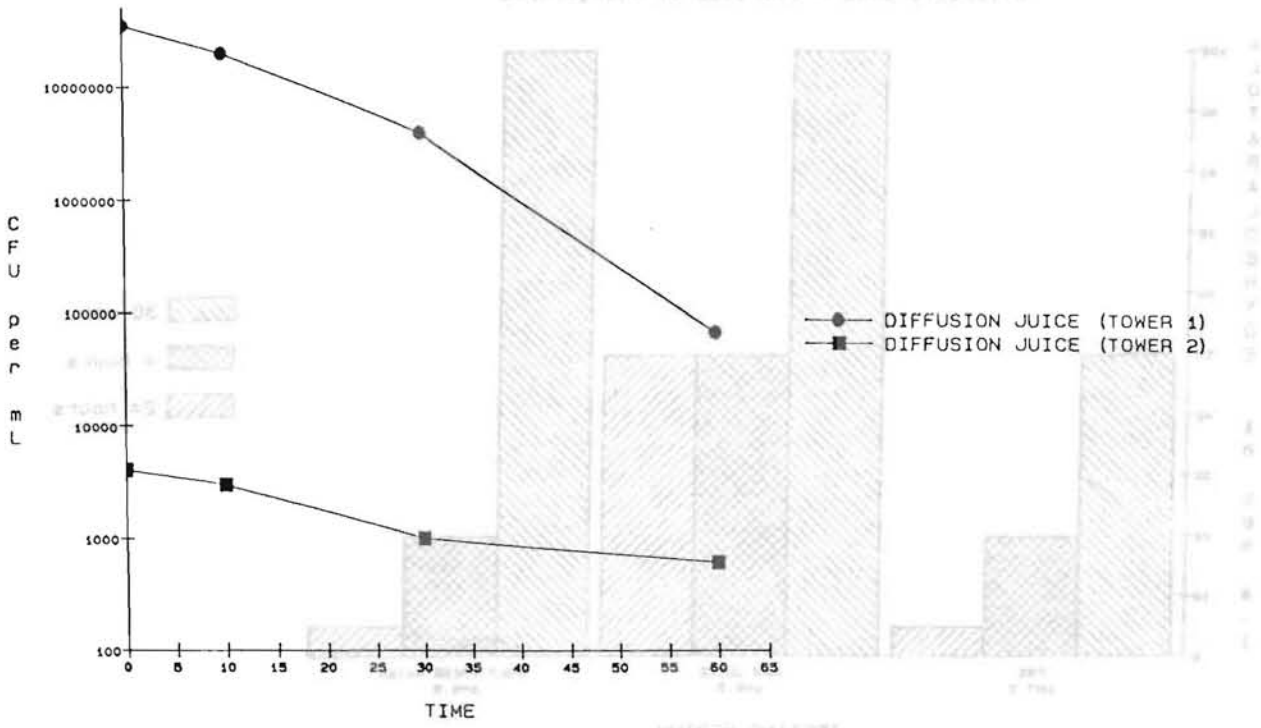


FIGURE 6.
FIELD TRIAL 1.
L-LACTIC ACID CONCENTRATIONS

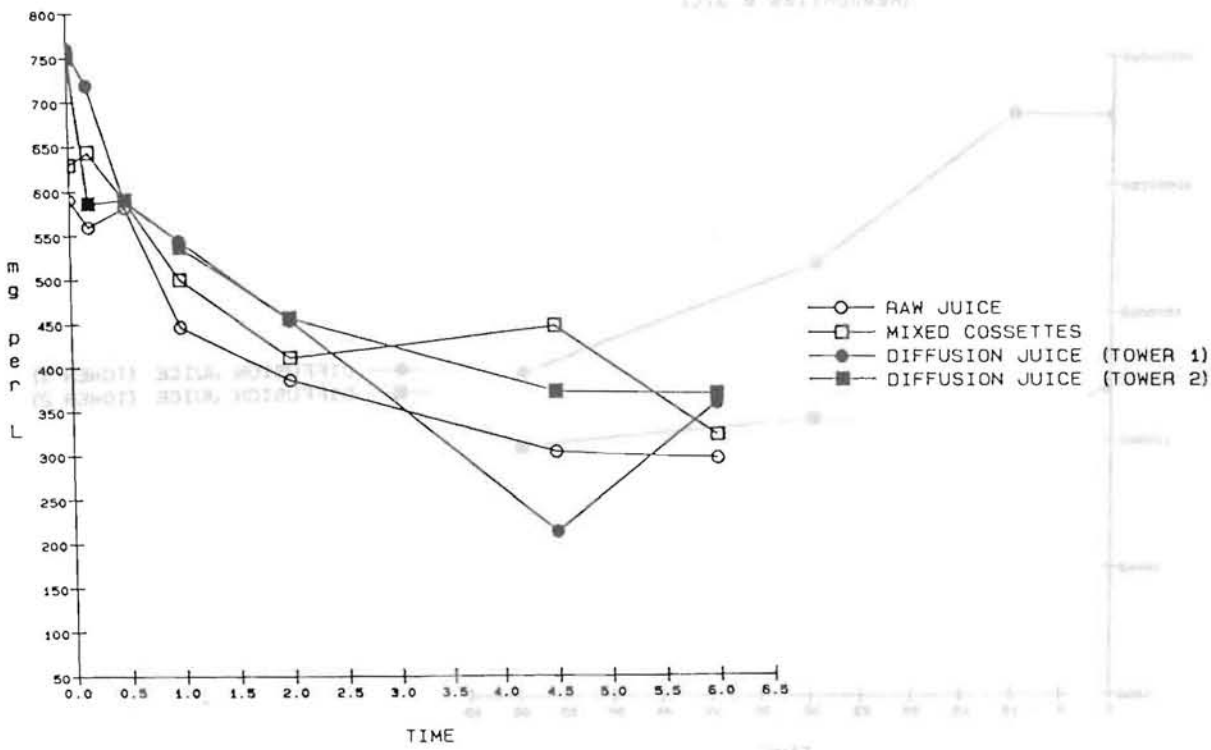


FIGURE 7.
FIELD TRIAL 2.
L-LACTIC ACID CONCENTRATIONS

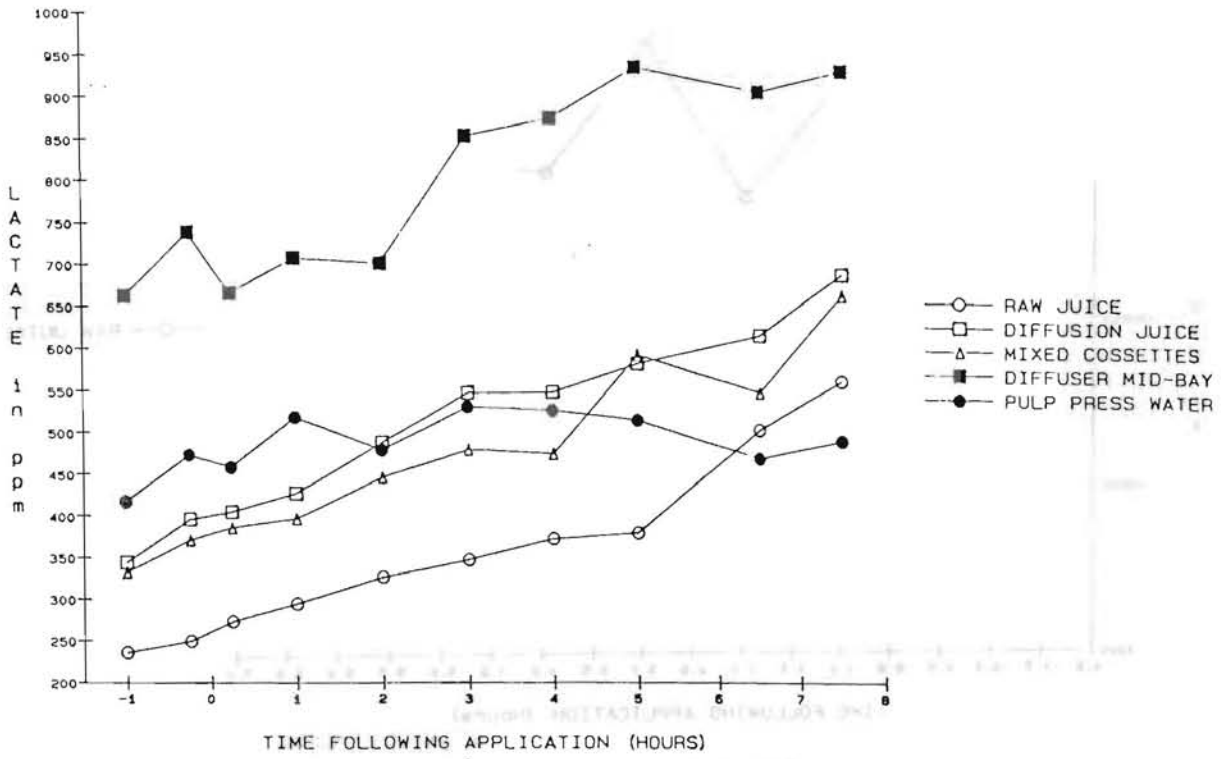


FIGURE 8.
FIELD TRIAL 2.
TOTAL PLATE COUNT
(Mesophiles @ 37C)

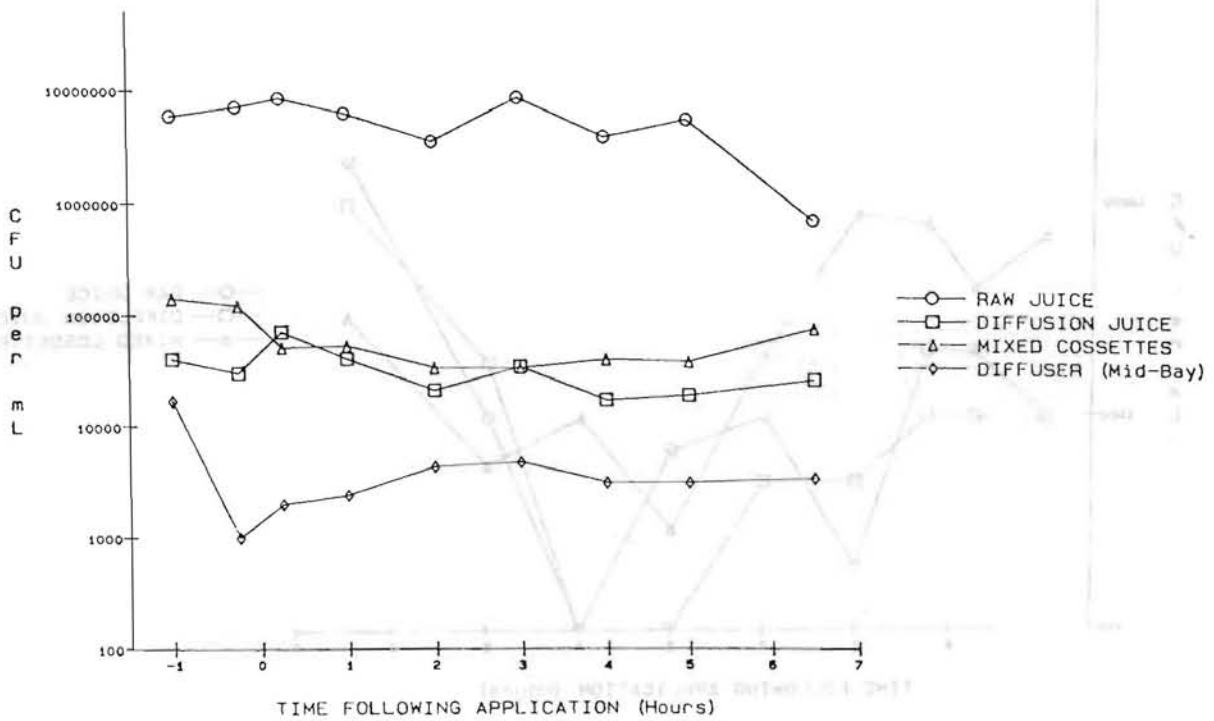


FIGURE 9.
FIELD TRIAL 2.
LEUCONOSTOC

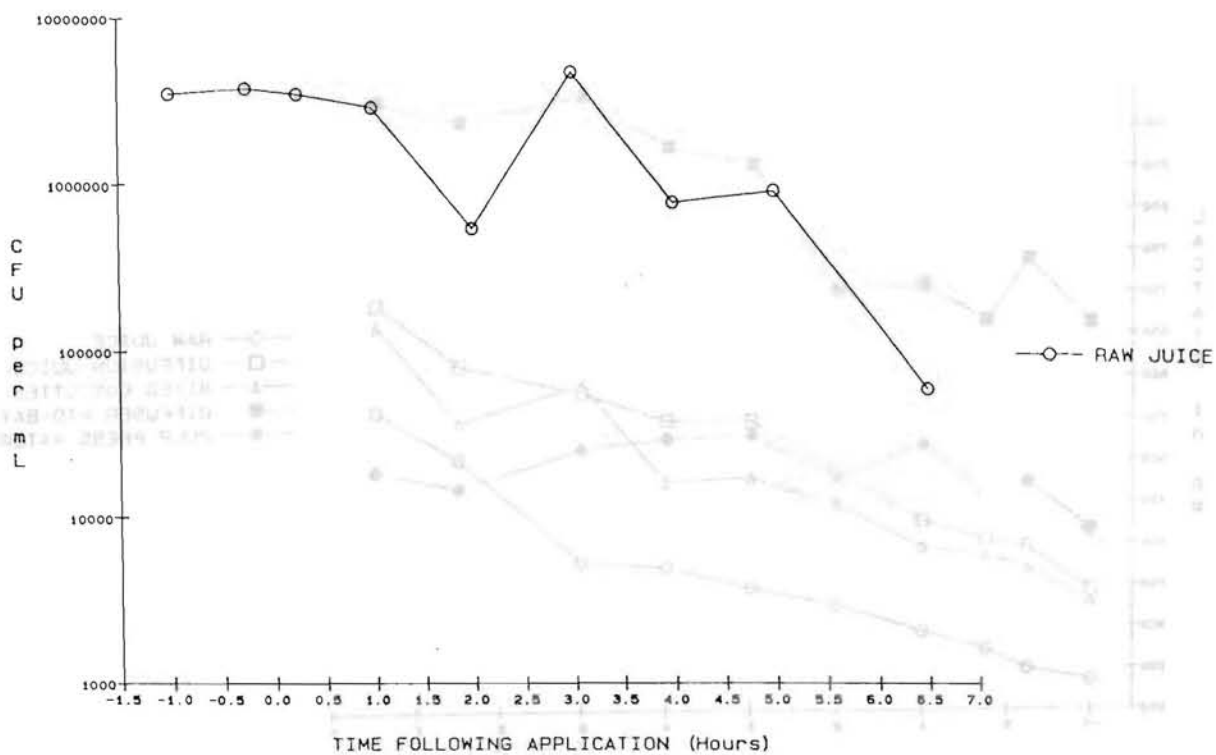


FIGURE 10.
FIELD TRIAL 2.
ACID PRODUCING THERMOPHILES
(55C)

