A COMPARISON OF THREE TECHNIQUES USED IN THE ASSESSMENT OF THE EFFICACY OF BIOCIDES AND SANITIZERS FOR MICROORGANISM CONTROL IN FACTORY DIFFUSERS

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Loss of sugar in factory diffusers due to microbial action is a problem that has continued to plague the sugar industry from its inception. The flora of microorganisms which enter the factory and contribute to loss of sugar originate mainly from the beet fields, and the types of microbes vary with the season and locality in the relative numbers rather than in the species (5).

Carbohydrates are utilized by microorganisms as a source of energy for growth, both in the presence (aerobic) and absence (anaerobic) of oxygen. Therefore a factory diffuser rich in nutrients and with temperatures conducive for growth of microbes is an ideal environment for their multiplication. Microbes possessing the enzyme invertase will degrade sucrose, with increase of biomass and primary and secondary metabolites, such as ethanol, carbon dioxide, lactic, acetic, propionic, or butyric acid or polysaccharides (e.g. dextrose) contributing to sugar loss (3). These losses can be minimized by maintaining temperatures of operational processes above 75°C or by evaporating low brix materials to above 60° Bx with minimal delay. This is not practically feasible in all factory operational processes as high temperatures make pulp pressing less efficient and may increase the activity of high temperature thermophiles.

Alternate methods such as chemical control with use of biocides therefore become necessary. The selection of biocides and amounts used must be governed not only by their effectiveness but also by the residuals which may be carried into the process and indirectly cause losses in sugar, process difficulties, or have toxicity implications. The actual extraction environment is very complex which makes exact laboratory simulation impossible. One alternative then is to work with isolates taken from the system under study (6) and to simulate conditions as far as possible.
A preliminary survey to assess the efficacy of biocides and sanitizers for microorganism control of factory diffusers was carried out by us previously and the technique used was presented at the 1989 25th biennial ASSBT meeting in New Orleans. Biocides were further evaluated using three screening techniques; namely, 1) a modified Kirby Bauer disc diffusion assay technique (2), 2) a shaker technique using diffusion juice, and 3) a microbial count. All three techniques were used simultaneously either for mesophiles (moderate temperature growers) or thermophiles (high temperature growers) so as to assess biocide effectiveness and also the merits and demerits of the three different techniques.

In the first part of testing a disc diffusion assay technique was used. This type of assay has been adapted to test the effectiveness of antiseptics, disinfectants, and chemotherapeutic agents against microorganisms. The technique used was a modification of the Kirby Bauer method (2) used for antibiotic susceptibility testing of microbes. The procedure involves placing a paper disc impregnated with test agent on an agar plate seeded with the bacteria. If the test agent was effective after overnight incubation, a zone of inhibition around the disc should be visible. The diffusion rate of the test agent will depend on depth of medium and amount of bacterial inoculum. These errors were minimized by standardizing the procedures.

In the second part of testing a shaker test was performed. The procedure involved use of diffusion juice as a growth medium for microbial populations found on beets in the factory. The diffusion juice

used in this experiment was produced in the pilot plant at our Research Center and was free of additives such as formaldehyde, sulfur dioxide, or other biocides. The final evaluations of this technique were based on the basis of dry bacterial weight. Therefore this necessitated centrifugation of diffusion juice before using it as a growth medium so as to remove any components that might come down during high speed centrifugations. Measured amounts of biocide (depending on the predetermined range and concentration of biocide) and bacterial inoculum were added to autoclaved and desludged diffusion juice. These flasks were incubated on shaker for 24 hours at respective temperatures for mesophiles or thermophiles. Control flasks with inoculum but no biocide were run on shaker simultaneously with other flasks. After incubation, contents of each flask were centrifuged and bacterial pellet obtained. The pellets were washed and dried overnight in an oven. Percent dry bacterial weights were determined. A decrease in bacterial weight with respect to controls was taken as a measure of biocide effectiveness.

The third part of testing was carried out by means of a microbial plate count. An initial microbial plating was made with standardized inoculum that was used for the first and second parts of testing. After overnight incubation of flasks from second part of testing, a second microbial plating was made on each flask on shaker. A decrease in bacterial count with respect to controls was taken as a measure of biocide effectiveness.

MATERIALS AND METHODS

1. Mixed Aerobic Mesophilic and Thermophilic Microbial Populations from Beets
   Frozen beets coming into the factory were collected off the picking table. The beets were peeled with sterile implements and placed in two separate clear plastic bags washed with sterile water. One and one half (1½) L of m-plate count broth was added to each bag, tied, shaken, and incubated at 35°C (for mesophiles) and at 55°C (for thermophiles). After overnight incubation bags at each temperature were shaken and streaked on a number of plate count agar slants which were incubated at respective temperatures for mesophiles and thermophiles. After 24 hrs incubation the agar slants were washed down with sterile physiological saline and made equivalent to the turbidity of 0.5 McFarland's Standard. The standardized mesophilic and thermophilic cultures were then used as inoculum for seeding of plates or flasks. These microbial populations were maintained throughout the experiment.

2. Disc Diffusion Assay (A modified Kirby Bauer Technique)

   (a) Turbidity Standard used was 0.5 McFarland's Standard.

   (b) Preparation of Inoculum - A culture of mesophiles or thermophiles obtained from beets and grown on plate count agar for 24 hrs was washed down with physiological saline to obtain a turbidity equivalent to 0.5 McFarland's Standard.

   (c) Preparation and inoculation of plates - Petri plates of sterile media (plate count agar) of 4 mm thickness were dried completely to eliminate moisture. Plates were then streaked with bacterial inoculum made equivalent to 0.5 McFarland's Standard so as to cover the entire sterile agar surface. The plates were streaked successively with sterile cotton swabs in three different directions to obtain an even inoculum and then allowed to dry. A single sterile paper disc impregnated with test agent was placed on the center of the seeded plate. The plates were incubated overnight at 35°C for mesophiles and 55°C for thermophiles and observed the following day for zones of inhibition. The diameter of the inhibitory zones was measured on duplicate plates and averaged.

3. A Shaker Test

(a) Preparation of Diffusion Juice - Diffusion juice without any additives (biocide or SO_2) from pilot plant runs was frozen in 2½ L jugs and kept for later use. A jug of this diffusion juice was thawed overnight in refrigerator and desludged in centrifuge at 20,000 rpm and 25°C for 10 min. The supernatant was passed through cheese cloth and the filtrate collected.

(b) Running of Samples on Shaker - Aliquots of 190 mL of desludged and filtered diffusion juice were added to 500 mL baffle flasks and autoclaved. Next 10 mL of standardized inoculum from beet peels incubated at 55°C or 35°C was added to separate flasks with sterile diffusion juice. A number of flasks were set up in this manner. The range and concentrations of biocide to be tested were predetermined. Measured aliquots of biocide depending on concentration required were added to flasks with diffusion juice and inoculum. Flasks were run in duplicate for each concentration of biocide being evaluated for mesophiles or thermophiles. All flasks inoculated with the biocide and mixed bacterial culture were clamped on orbital shaker and rotated at 150 rpm for 24 hrs at 55°C for thermophiles or 35°C for mesophiles. A set of control flasks with sterile diffusion juice, inoculum, and no biocide was run simultaneously on shaker in duplicate at different temperatures. Flasks were removed from shaker after 24 hrs and refrigerated.

(c) Obtaining Bacterial Pellet - The contents of each flask were put into several tubes and centrifuged at 20,000 rpm at 5°C for 10 min. The supernatant was carefully removed from each tube and the bacterial pellets resuspended in a small volume of sterile water. All bacterial pellets obtained from one flask were pooled into one tube. More distilled water was added so as to wash out bacterial cells thoroughly. The pooled pellet was resuspended, and cells were recentrifuged at 20,000 rpm and 5°C for 10 min. The supernatant from the washed single bacterial pellet was removed and resuspended in 8 mL of distilled water.

(d) Determination of Bacterial Weight - The bacterial suspension from step (c) was added to an ashed, cooled, and weighed crucible. The samples were dried overnight at 105°C, cooled in desiccator, weighed, and % dry bacterial weight determined.

4. Determination of Microbial Count

An initial microbial plating on the standardized inoculum of the mixed aerobic culture of mesophiles or thermophiles from beet peels was made on the day that the modified disc diffusion assay and shaker test were performed. The next day after overnight incubation of flasks (shaker test) a second microbial plating was carried out. A 1 mL aliquot of sample was removed from each flask before centrifugation of rest of contents. Serial dilutions were made up in buffered distilled water, and decimal dilutions were plated. A pour plate technique with duplicate plates at each dilution was used in both determinations. Plating media used was plate count agar (Difco). Plates were incubated at 35°C for mesophiles and 55°C for thermophiles for 48 hrs, and total plate counts were made in each case.

DISCUSSION

Different microbiological methods have been used in the evaluation of diffuser biocides. Osweiler & Sisler (7) in their evaluations used two microbial count techniques, namely an agar plate method (purple agar base), a cell count method, and a redox indicator method (resazurin). Here the microbial counts were made only for thermophiles, and the purple agar base used in the plate count method accounts only for the total lactic acid bacteria per ml of sample; but the cell count method used would account for total thermophilic viable cells. The third method employed assessed the microbiological activity by means of oxidation reduction indicators. This method gives only an approximation of growth since

the rapidity of color change is directly related to the concentration and growth rate of organisms present but may be inaccurate as the relation between reduction time and bacterial numbers is not reliable for less than 100,000 organisms/mL.

The total microflora present in beet sugar extraction is large (4); but in spite of the large number and variety of organisms present, only a few species are able to grow and become dominant in the system. The interest devoted to mesophilic bacteria could seem excessive bearing in mind that the temperatures inside the diffusers are notably higher than the optimum required for the growth of these microbes. Also at certain points the temperature is higher than 70°C, in which case their growth would be inhibited (1). However, Haska and Nystrand (4) have pointed out that the majority of bacteria present in cossettes do not enter the diffuser but are washed away by the raw juice leaving the diffuser without being subjected to high temperatures. If this were the case, mesophiles could become adapted to the environment and increase in biomass contributing to sugar loss. It must be noted, however, that the actual extraction environment is very complex which makes exact laboratory simulation almost impossible. Therefore with the use of a microbial inoculum obtained from beets off the picking table and the use of diffusion juice in the shaker technique, an attempt was made to simulate conditions in the factory diffusion process as far as possible.

In our biocide and sanitizer evaluations very consistent results were obtained in the three different techniques for the mesophiles. In the case of biocides such as the thiocarbamates, sodium metabisulfite, formalin, and chlorine dioxide, to mention a few, the microbes grew in the control flasks in shaker test and counts were higher than inoculum. On addition of biocide, there was a reduction in bacterial numbers as shown in the plate count method, a reduction in bacterial weight as shown in the shaker test, and an increase in zone of clearing in the modified disc diffusion assay with increasing concentration of biocide (Annexes I, II, & III). In the screening of metabisulfite and formalin for mesophiles, the disc test did not seem to be sensitive to insignificant changes in counts but did show zones of inhibition when significant decrease in counts were seen. Although the results in shaker technique were consistent with other tests, the decrease in weight with increase in biocide concentration was minor in comparison with changes in other two methods. Therefore the modified disc diffusion assay and the plate count method were found to be more suitable, sensitive, and apt to less error for determining of biocide effectiveness on mesophilic microbes.

Although in general the three techniques worked well for mesophiles and gave consistent results, the thermophiles were more difficult to work with. These microbes did not grow well in diffusion juice and died out on incubation giving counts in control flasks less than that of the inoculum. This led to inconsistencies in results especially in the shaker or weight test and in some cases in the microbial count test as well. Therefore the most sensitive and reliable test especially for the thermophiles was found to be the modified disc diffusion assay technique (Annexes I & II).

An interesting aspect of this testing was seen when screening was carried out for mesophiles using hydrogen peroxide. In this case no significant change in weight or microbial count was obtained with increase in concentration of biocide. This was probably due to microbial catalase activity which decomposed the hydrogen peroxide before it could have any biocidal effect. However the disc test worked well giving increase in zones of inhibition with increase in concentration of biocide (Annex III).

In this study a total of ten biocides and four sanitizers were evaluated, but only a few results have been presented here. Hence from the foregoing it is clear that the modified disc diffusion assay developed by us seems to be applicable and more sensitive to a wide spectrum of microbes than the other two methods. The Kirby Bauer disc diffusion assay has been in use for a long time in medical bacteriology to assess the susceptibility of microbes to antibiotics and is the basis of diagnosis and therapy used in all leading hospitals here in the U.S. and the world. The diffusion rate of the test agent depends on depth of medium and amount of bacterial inoculum. These errors were minimized by standardizing the procedures. Also this technique is simple, quick, has stood the test of time, and is certainly not outdated and seems to be the most applicable for assessment of the effectiveness of

biocides and sanitizers at levels recommended for usage. However, since the microbial population entering the factory is changing continuously, this screening should be carried out on several different microbial populations in the factory for more accurate assessments.

Future work will involve the screening of anaerobic microflora as well.

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EVALUATION OF BIOCIDES

Biocide Concentration	Shaker Technique % Dry Bacterial Weight	Disc Test Avg. Zone of Inhibition (mm)	Microbial Count cfu/g
Effect of Sodium Metabisulfite (pH 5-6) on Mesophiles (Aerobic Conditions)			Inoculum = 1.04×10^8
a) Control (no biocide)	2.09	Absent	4.47×10^8
b) 100 ppm	1.07	Absent	2.59×10^8
c) 500 ppm	0.12	13.5 mm (v. slight partial zone of clearing)	7.3×10^3
d) 1000 ppm	0.13	24.0 mm (partial zone of clearing	1.32×10^3
e) 2000 ppm	0.15	Inner zone = 18.5 mm (partial clearing but clearer than outer zone) Outer zone = 33.75 mm (partial zone of clearing)	4.0×10^1
Effect of Sodium Metabisulfite (pH 5-6) on Thermophiles			Inoculum = 1.88×10^6
a) Control (no biocide)	0.14	Absent	2.0×10^1
b) 100 ppm	0.12	Absent	2.18×10^2
c) 500 ppm	0.19	Absent	1.5×10^2
d) 1000 ppm	0.16	Absent	2.23×10^2
e) 2000 ppm	0.38	Absent	2.1×10^2

Biocide Concentration	Shaker Technique % Dry Bacterial Weight	Disc Test Avg. Zone of Inhibition (mm)	Microbial Count cfu/g
Effect of Formalin on Mesophiles			Inoculum = 1.89×10^{8}
a) Control (no biocide)	1.92	Absent	6.2×10^{8}
b) 200 ppm	1.72	Absent	2.67×10^{4}
c) 400 ppm	0.36	17.0 mm (hazy zone)	5.45×10^{3}
d) 500 ppm	0.15	18.25 mm (hazy zone)	8.0×10^{1}
e) 1000 ppm	0.20	19.5 mm (inner clear zone) 29.25 mm (outer hazy zone)	<10
Effect of Formalin on Thermophiles			Inoculum 1.66×10^{6}
a) Control (no biocide)	0.17	Absent	1.25×10^{2}
b) 200 ppm	0.17	21.25 mm*	2.0×10^{1}
c) 400 ppm	0.14	24.5 mm*	<10
d) 500 ppm	0.19	25.0 mm (inner zone)* 35.0 mm (outer zone)*	<10
e) 1000 ppm	0.18	36.0 mm (inner zone)* 45.0 mm (outer zone)*	<10

[* Zones no complete clearing - just lesser growth]

Biocide Concentration	Shaker Technique % Dry Bacterial Weight	Disc Test Avg. Zone of Inhibition (mm)	Microbial Count cfu/g
Effect of Hydrogen Peroxide on Mesophiles			Inoculum = 7.6×10^7
a) Control (no biocide)	1.55	Absent	2.11×10^8
b) 100 ppm	1.59	Absent	1.20×10^8
c) 400 ppm	1.33	17.0 mm (hazy zone)	1.32×10^8
d) 500 ppm	1.35	21.0 mm (19 mm - hazy zone) (23 mm - clear zone)	1.55×10^8
e) 1000 ppm	1.55	21.5 mm (clear zone)	2.15×10^8
f) 1500 ppm	1.53	26.5 mm (clear zone)	1.94×10^8