Sugar Utilization of Microorganisms Isolated from a Survey of Holly Sugar Beet Factories for 1991 Campaigns

F.M. Steele, I. Ekasari, R.T. Tang,
D.J. Nabben-Schindler, and A.M. Campbell

MICROBIOLOGY SECTION
RESEARCH AND DEVELOPMENT CENTER
COLORADO SPRINGS, COLORADO

AMERICAN SOCIETY OF SUGAR BEET TECHNOLOGISTS
27TH BIENNIAL MEETING
ANAHEIM, CALIFORNIA
MARCH 3-6, 1993

ABSTRACT

The objectives of this project were the determination of sampling procedure from each of Holly Sugar's eight beet factories, sampling, enumeration of microbial contaminants, identification of those contaminants, sugar destruction caused by these microorganisms, and biocide effectiveness against these microorganisms.

The data obtained show a significantly higher microbial population of all microorganism types in diffusion juice sampled from factories operating slope diffusers compared to those operating RT diffusers. Yeast and mold are absent from all RT diffusers in outgoing diffusion juice, with the exception of one factory that operated an RT diffuser. This was also observed with mesophiles and thermophiles, but was not quite as obvious as with the yeast and mold. This observation of overall higher counts in the diffusion juice in slope diffusers is more than likely attributable to the lower operating temperatures in slope diffusers. RT diffusers averaged a total thermophilic count of 10^5 colony forming units (CFU)/ml of diffusion juice. In contrast, slope diffusers averaged a total thermophilic count of 10^7 CFU/ml of diffusion juice.

Organisms obtained from the above mentioned samples were isolated and identified as to their genus and species. The frequency of individual organisms was determined. The predominant organisms belong to the genus *Bacillus*.

Preliminary sugar utilization studies showed the loss of approximately 18% of the total sucrose available in diffusion juice over a period of 15 hours. At 10^5 CFU/ml of diffusion juice either 1% or 18% of the total sucrose available was lost, dependining on the growth stage observed in the growth curve. Further studies will yield an estimate of the economic impact the microorganisms present have on the continuous process.

Factory Microbiological Survey

Introduction

There is an increasing awareness in the sugar industry that the losses due to microbial infections in the process can impose a significant impact on the economics of sugar production. As organisms proliferate in a dilute sucrose solution, catabolizing sucrose into a variety of organic acids, polysaccharides, and other metabolites, the ability to effectively control this system becomes a significant issue. [9,10,11] Bacterial infections in the diffusion process are detrimental in several ways. First, by the catabolism of sucrose; second, by the production of organic acids and other metabolites which either physically interfere with filtration processes or are melassigenic in nature; and third, by reducing indigenous nitrate to nitrite, which in factories using SO_2, leads to imidodisulfonate formation [3,4]

The activity of microorganisms, primarily thermophiles, is typically suppressed by elevated temperatures or the addition of chemical biocides or a combination of the two. Typical factory practice is to operate at a moderately high diffusion temperature in the range of 65-75°C [8] in combination with an approved biocide applied continuously or in shock doses. A reported temperature of greater than 80°C would be necessary to completely inactivate thermophilic fermentation and at these temperatures excessive losses of pulp marc would be unacceptable.[13]

The microbial evaluation of Holly Sugar beet factories has been in progress for almost two years now. The objectives of this project are: the determination of sampling procedure, sampling, enumeration of microbial contaminants, identification of those contaminants, sugar destruction caused by these microorganisms, and biocide effectiveness against these microorganisms.

Sampling procedure determination is a matter of evaluating the available sampling ports within the operation that are amenable to proper microbiological sampling, in other words, those from which a representative

sample is capable of being obtained from the process in an aseptic manner. The area of the operation that is most susceptible to microbial infections is the diffusion loop, ie., anything that enters or exits the diffuser. This area is believed to be the most susceptible area due to the temperatures involved along with the lower concentrations of sucrose, all contributing to a very conducive environment for microbial growth. Naturally, the strength of our ability to isolate such areas is dependent on the number and quality of our samples. Therefore the long term nature of this project.

Methodology

Sampling of the designated areas was carried out in a manner that presumably gave the best picture of what occurs microbiologically within the system. The area of sampling was limited to the diffuser area of each beet factory. Sampling was done at each of the diffuser cell ports that were operable, diffusion supply water, diffusion juice, flume water, pulp press return, and cossettes as they entered the diffuser. Samples were collected in sterile sample bottles and stored at 4 to 7°C until all samples were collected at each location. Samples were then packed in ice and shipped overnight to the R&D facility.

From the samples collected, the organisms present were then quantified using standard methods[1,2]. Microbial counts were determined for mesophiles (growth at intermediate temperatures, 37°C), thermophiles (growth at 55°C), yeast and mold, and anaerobic organisms. This was done to give a general idea of the scope of the "problem" and also the general location of its source. The organisms were then grouped according to general growth characteristics. From the samples collected, the organisms present were isolated and identified. Identification of these organisms allowed the use of reported characteristics of known organisms rather than researching fundamental characteristics independently. Identification was based on a specific battery of biochemical tests utilizing a Vitek Jr system (BioMerieux; Hazelwood, MO). This system allowed for rapid identification and matching of known organisms from the database associated with the Vitek system.

Identified organisms from the process were then screened by a novel method as to their ability to utilize sucrose as a nutrient source[6]. Growth rate of the organisms was determined utilizing electrical impedance detection times as obtained from a Bactometer system (BioMerieux; Hazelwood, MO) and then screened relative to their observed detection times. The destruction of sucrose via metabolic processes was monitored in a 2.5 liter batch fermenter (New Brunswick Scientific; Edison, NJ). The subsequent production of metabolites from selected organisms was measured through chromatographic methods (Dionex Corporation; Sunnyvale, CA). The end-products produced have also been shown to have a significant negative impact on the loss of sugar being carried into the molasses[7,12].

Results

Sample Collection

Successful sampling of any system is largely dependent on the design of the system being capable to yield the data necessary. To obtain a representative sample of the system is of paramount importance. Ports along the diffuser itself need to be sampled in order to obtain a profile across the diffuser. Also, the difficulty in obtaining a representative sample from a half-inch valve sampling a flow of thousands of gallons of diffusion juice is fairly obvious.

The ability to put in more sampling ports at critical locations is one option in improving sampling capabilities, and additional ports have been put in at several factories to aid in obtaining a better representation of the distribution of microorganisms within the entire diffusion loop process.

Microbial Enumeration

Microbial counts from each of the factories can be seen in Figures 1-3 for total mesophiles, thermophiles, and yeast and mold. Microbial counts are graphed for each group of microorganisms present from the five sampling areas that were common to each of the factories. Cossettes coming into the system, flume water, diffusion supply water, diffusion juice, and pulp press

return are all represented on the respective graphs. Individual cells within the diffuser were not available at all of the factories to allow for a factory to factory comparison. Therefore, a composite of all RT diffusers and the samples collected within each RT is represented in Figure 4, titled Average Microbial Counts Across the Diffuser. This figure is only an extrapolation from the pooled data, but, it is believed, a valid representation of what occurs in the RT diffusers with respect to mesophilic and thermophilic microbial growth.

The data show a significantly higher microbial population of all microorganism types in diffusion juice sampled from factories operating slope diffusers compared to those operating RT diffusers. Factories A through E all operate RT diffusers. Factories F,G, and H operate slope diffusers. Yeast and mold are absent from all RT diffusers in outgoing diffusion juice, with the exception of Factory F. This was also observed for mesophiles and thermophiles, but was not quite as obvious as in the yeast and mold counts. This observation of overall higher counts in the diffusion juice in slope diffusers is more than likely attributable to their lower temperatures in the diffusion juice. The causes for the relatively higher counts observed at Factory F are not obvious, but do show a definite area for study and improvement.

Microbial Identification

Organisms obtained from the above mentioned samples were isolated and identified as to their genus and species. The organisms identified are listed in Table 1. The relative frequency of individual species is designated by the order of listing. Table 1 indicates the predominant organisms belong to the genus *Bacillus*.

Bacillus species are Gram positive, spore-forming microorganisms which are either aerobic or facultative anaerobes (able to grow in low oxygen environments)[15]. The primary reservoir for *Bacillus* species is the soil. This is consistent with the high ($>10^8$ CFU/g) mesophilic count observed throughout the samples of the cossettes going into the process. *Bacillus* species are also very adaptable to thermophilic temperature ranges either through a direct adaptation or through sporulation initiation. The reported maximum temperature for vegetative (non-spore state) growth ranges from

about 25°C to above 75°C[14]. Spore formation in this genus is a mechanism for survival brought on by harsh environmental conditions, ie. temperature, drying, increased osmotic pressure, etc.

An interesting note on *Bacillus* species is their great diversity. In the fermentation of glucose to specific metabolites, there are several variants: *B. coagulans* produces exclusively lactic acid, *B. subtilis* and *B. lichenformis* and *B. cereus* form 2,3-butanediol and glycerol as major products, *B. polymyxa* forms 2,3-butanediol, ethanol, and H_2 as the main products, and *B. macerans* produces chiefly ethanol, acetone, acetic acid and formic acid[15]. This is quite interesting considering the fact the predominant method presently used for determining microbiological problems is either L-lactate analysis or pH.

Sucrose Metabolism

A selected *B. subtilis* strain was grown and monitored as shown in Figure 5 for growth as well as metabolites produced. Approximately five percent of the total sugar present was utilized after 10 hours of growth at 45°C. After 16 hours almost 18% of the total sucrose available was utilized. At 10^5 CFU of *B. subtilis*/ml of diffusion juice as observed at 2-3 h, only about 1% of the total sucrose available was lost. However, as the microbial population declines in late stationary phase (16-24h), approximately 18% of the total sucrose available has been utilized. This illustrates a deficiency inherent in predicting the course of microbial growth in a continuous system from data obtained from a batch fermentation. Sucrose degraded was converted to primarily lactic, acetic, and succinic acid as measured by ion chromatography. Glucose and fructose concentrations remained relatively constant. This is probably due to their immediate utilization as they are produced from inversion of the sucrose. A significant increase in the metabolites produced was observed between the hours of 11 to 13. This correlates well with the decrease in pH and can probably be attributed to the change in growth cycle from log to stationary phase. At this point in the growth cycle organisms tend to switch energy requirements from growth and multiplication efforts to that of metabolizing and sustaining the population.

Future Work

Sugar utilization studies will yield a dollar figure as an estimate of the economic impact the microorganisms present have on the process. The rate of growth of these isolated organisms will continue to be evaluated as well as their metabolites produced. Metabolite analysis will also enable us to look at some possible alternatives for monitoring microbial infection in contrast to what is currently being done in the factories (pH and/or lactate).

Biocides will also be evaluated as to their respective effectiveness at controlling the organisms in a specific system. A cost benefit analysis will be done to measure the economics of biocides currently being used as well as others that are currently available and approved for the process. Alternative methods of microorganism control will also be evaluated.

Summary

The need for improved sampling of the diffusion loop area of beet factories must be stressed to obtain information relating to the microbiological status of the given system. It is in the best interest of all involved to work to implement new and better sampling schemes. At one factory we had new sampling ports put in the diffuser at regular intervals (approximately every 2 to 3 cells). Sampling ports were also installed at each pulp press and at strategic locations along the pulp press return lines. These new ports have enabled us to obtain a more representative sampling of the specific area that was targeted from the data as it has been presented in this report.

Another area that we will be investigating will be the metabolic end products that are being produced as these organisms utilize sucrose. It is believed that there may be alternative methods to determine the extent of a microbiological infection compared to what is currently being used (pH and lactate). This could very well be an indication of only a fraction of the microbial activity. De Bruijn *et al.* concluded that approximately 70% of the fermented sucrose is converted to lactate, and subsequently felt that lactate was a good tracer for microbial activity. In contrast, approximately 50% of the sucrose destroyed in our trials was converted to lactate. This is an area in which we will be concentrating in the future.

References

1- American Public Health Association. 1984. Compendium of methods for the microbiological examination of foods. M.L. Speck (ed.) Washington, DC.

2- Association of Official Analytical Chemists. 1992. FDA Bacteriological Analytical Manual (7th Edition), Arlington, VA.

3- Carruthers, A., P.J. Gallagher, and J.F.T. Oldfield. 1958. Nitrate reduction by thermophilic bacteria in sugar beet diffusion systems. Eleventh Technical Conference, British Sugar Corp.

4- Carruthers, A., J.F.T. Oldfield, and A.E. Wootton. 1958. Studies in the chemistry of sulfitation. Part III. Eleventh Technical Conference, British Sugar Corp.

5- Carruthers, A. and J.F.T. Oldfield. 1955. The activity of thermophilic bacteria in sugar beet diffusion systems. Eighth Technical Conference, British Sugar Corp.

6- Coppola, K. and R. Firstenberg-Eden. 1988. Impedance based rapid method for detection of spoilage organisms in UHT low-acid foods. J. Food Sci. 5:1521-1524.

7- De Bruijn, J.M., P.W. van der Poel, R. Heringa, and M. van den Bliek. 1991. Sugar degradation in sugar beet extraction--a model study. Zuckerindustrie 8:729-732.

8- Ebell, H.O. and M. Storz. 1982. Diffusion. In R.A. McGinnis(ed.). Beet-Sugar Technology (3rd Edition), Beet Sugar Development Foundation, Denver, CO.

9- Field, P.J. 1992. Chemical and microbiological loss and control. Sugar Industry Technologists, Inc. 1992 Technical Meeting Brussels, Symposium-Section IV.

10- Gurha, S., and K.A. Prabhu. 1992. Chemical control of bacterial contamination in sugars. Int. Sugar J. 1119:57-60.

11- Holland, G., M.E. Buckley, and S. Frampton. 1990. Experiences in the use of biocides for microbiological control in the raw factory. British Sugar Technical Conference-Eastbourne 1990.

12- Nystrand, R. 1984. Microflora in sugar extraction. Dissertation, University of Göteborg, Sweden.

13- Oldfield, J.F.T., J.V. Dutton, D. Grierson, R.K. Heaney, and H.J. Teague. 1968. Comparison of sulphur dioxide and formaldehyde in diffusers. Nineteenth Technical Conference, British Sugar Corp.

14- Priest, F.G. 1989. Isolation and identification of aerobic endospore-forming bacteria. In C.R. Harwood (ed.). Bacillus. Plenum Press, New York, NY.

15- Sneath, P.H.A. 1986. Endospore-forming Gram-positive rods and cocci. In J.G. Holt (ed.). Bergey's Manual of Systematic Bacteriology (Vol 2). Williams and Wilkins, Baltimore, MD.

Table 1
Factory Microbiological Survey
Identification

● Major Species	● Minor Species
Bacillus subtilis	*Bacillus spp.*
Bacillus licheniformis	*Leuconostoc spp.*
Bacillus pumilus	*Streptococcus spp.*
Bacillus megaterium	*Enterococcus spp.*
Bacillus coagulans	
Unknown *Bacillus spp.*	
Aspergillus spp.	*Rhizopus spp.*
Penicillium spp.	*Trichoderma spp.*
Fusarium spp.	
Mucor spp.	
Candida spp.	*Rhodotorula spp.*
Saccharomyces spp.	*Trichosporon spp.*
Geotrichium spp.	Unknowns

Figure 1--Microbial Population

Mesophiles

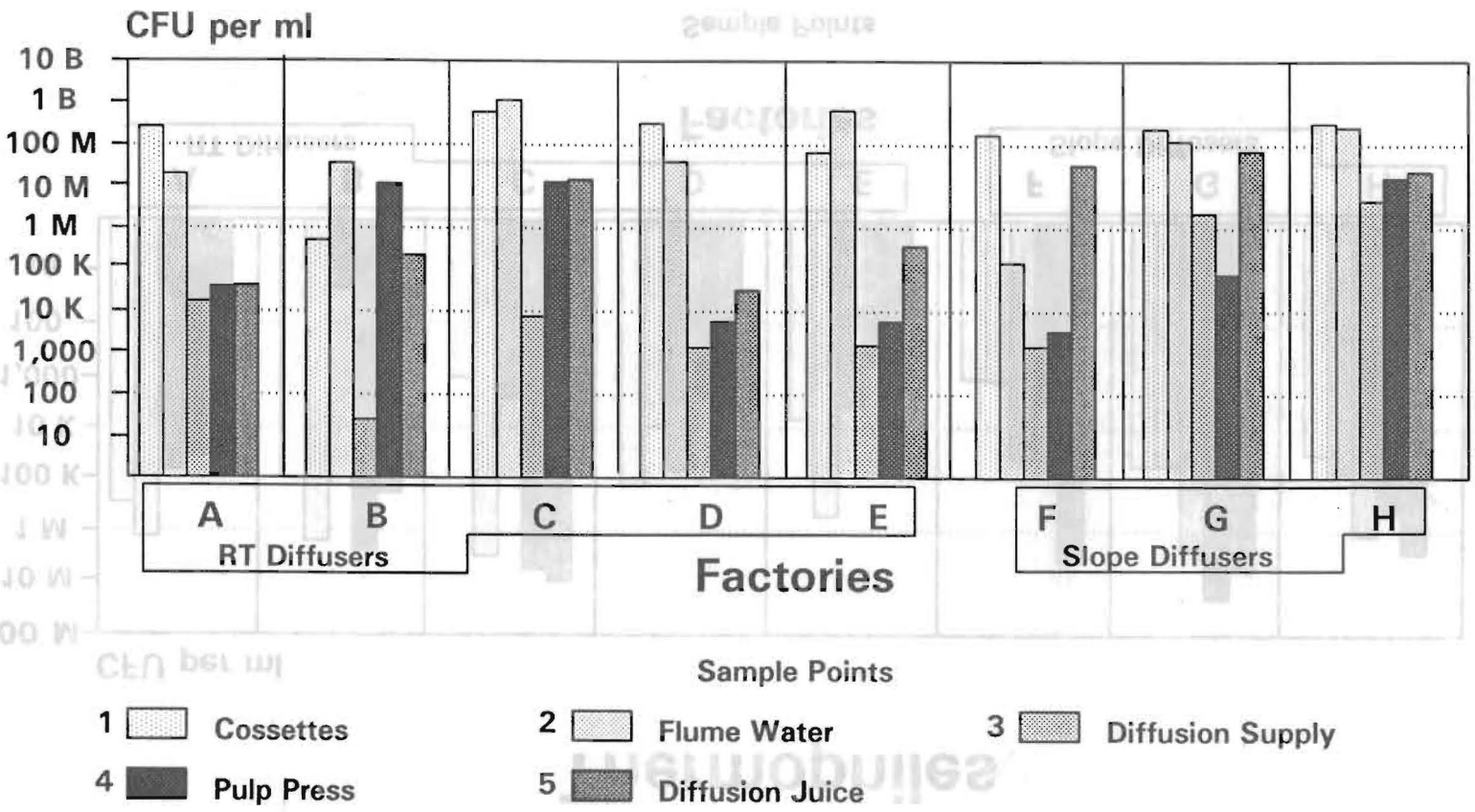

Figure 2--Microbial Population

Thermophiles

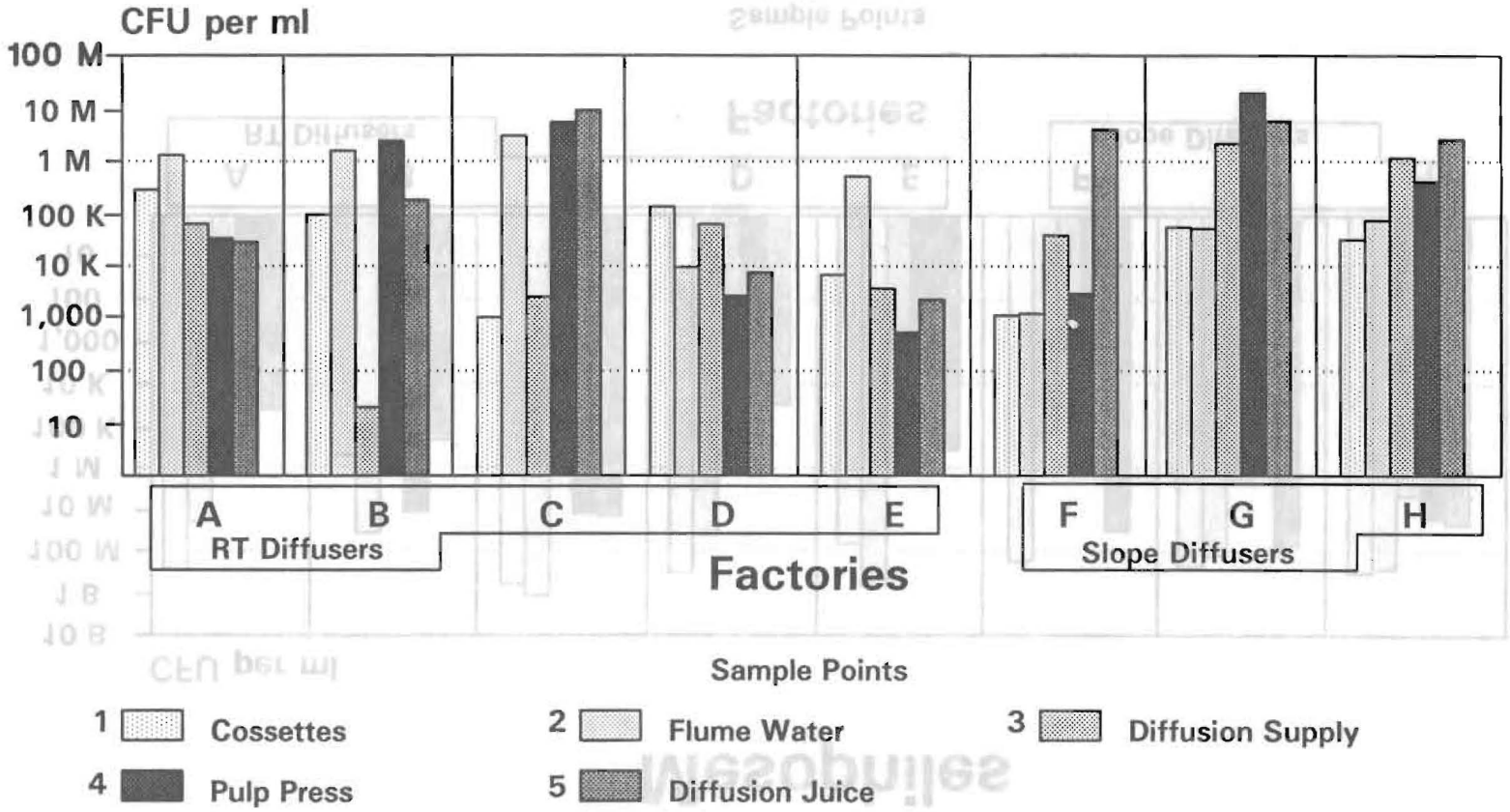

Figure 3--Microbial Population

Yeast and Mold

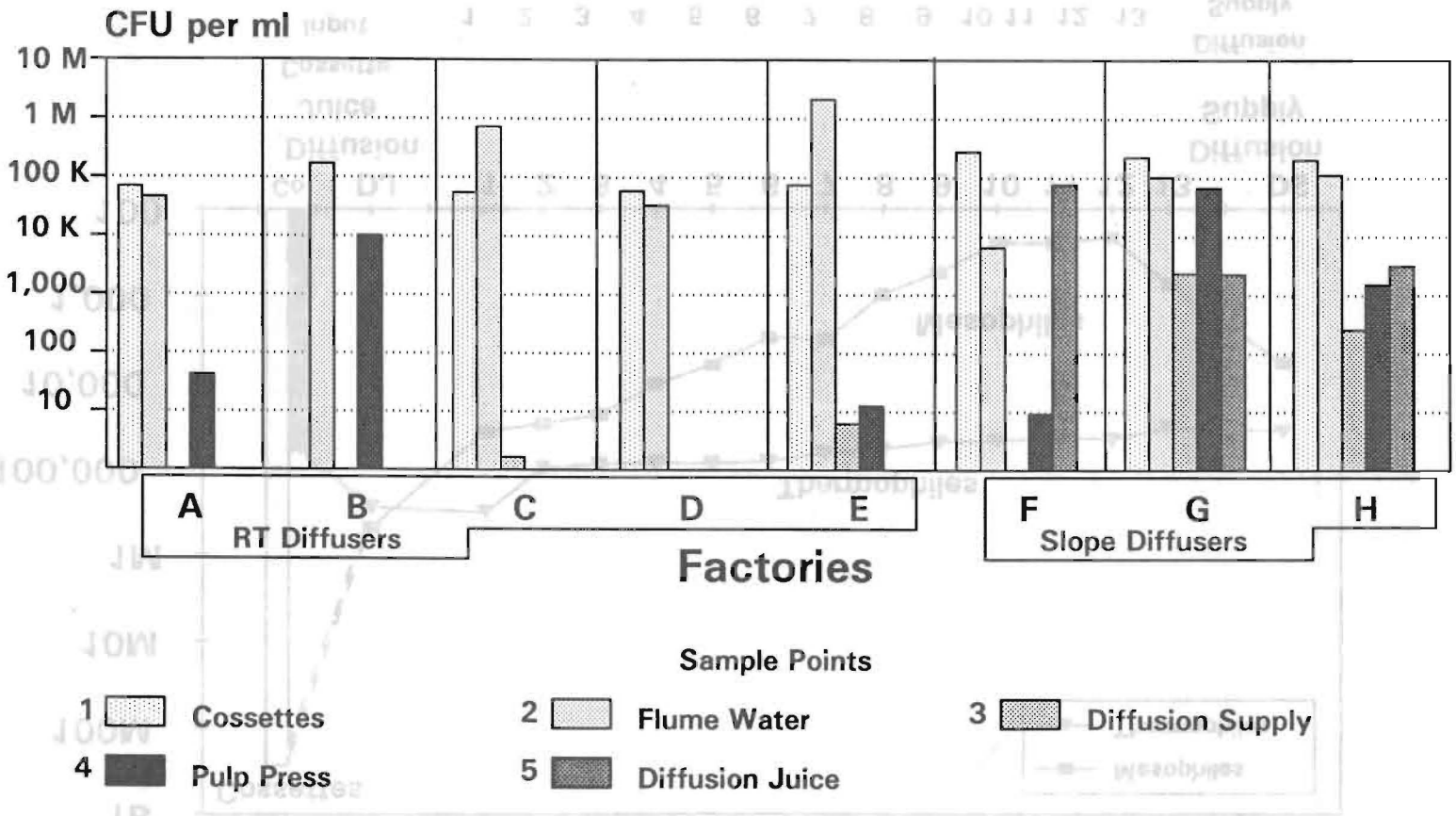

Figure 4--Average Microbiological Counts Across Diffuser

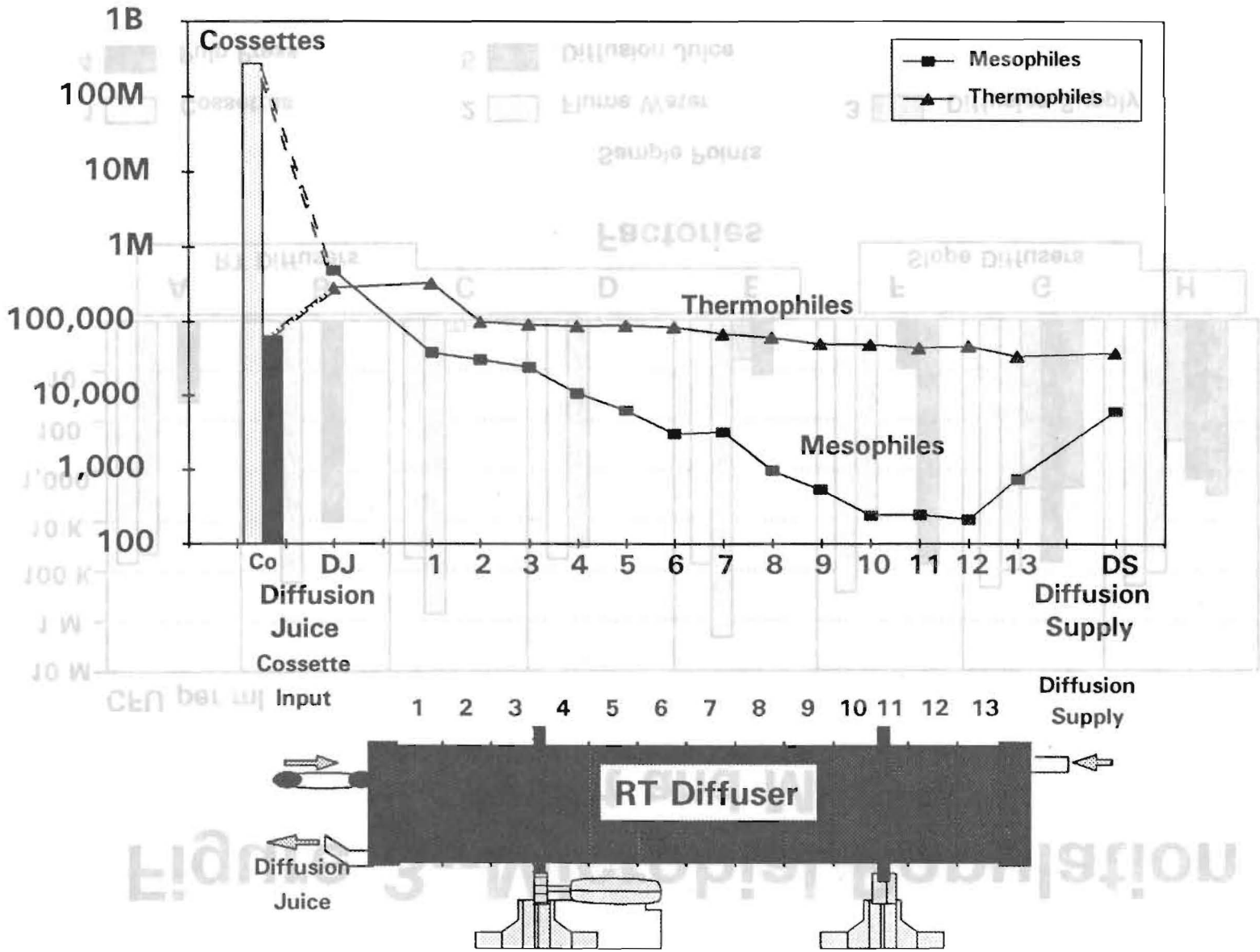

Figure 5--Sucrose Destruction