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Microbial Containment in Conventional Fermentation Processes

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An evaluation of the engineering controls in enzyme fermentation processes was conducted by researchers from the National Institute for Occupational Safety and Health (NIOSH) to identify effective controls applicable to processes involving process microorganisms, processing chemicals, and biologically active products and intermediates. The collected data and evaluation will help to establish a baseline of information on the equipment (and related safety and health programs and practices) currently used in enzyme fermentation operations. Walk-through surveys were conducted at six fermentation plants, and in-depth surveys were conducted at three enzyme plant sites selected from among the six. The various control measures were evaluated primarily by collecting environmental air samples for the microorganisms involved in the manufacturing processes. Approximately 200 bioaerosol samples were collected, using an Andersen two-stage cascade impactor, from each plant around fermentation processes suspected as being potential emission sites, including the laboratory, seed tank, fermentor tank, and filtering operations. Identification of the plant production strain was made on randomly selected sample populations using a standardized microbiological test method combined with a sugar utilization profile. Sample concentrations around unit processes were compared to background (locations away from the influence of unit processes—indoor and/or outdoor) concentrations to ascertain the degree of containment of those processes. Comparisons were also made between similar unit processes from plant to plant to determine which controls or combination of controls minimized emissions. At one plant, the geometric mean of total viable microorganisms at a filtering operation (filter press) was 5626 CFU/m^3 with the predominant strain being the process microorganism. At a second plant, utilizing a different filtering process (rotary vacuum drum filter with local exhaust ventilation), the geometric mean was 216 CFU/m^3 where the production strain was identified in very low quantity on less than ten percent of the sample plates. The results of the study indicate that controls are most needed around high energy operations where aerosolization is likely to occur such as filtering operations, agitator shafts, and sampling ports. In addition, operator work practices can be a major determining factor in the degree of exposure occurring during certain operations.

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Introduction

The rapidly growing field of genetic engineering, and the subsequent ability to produce novel microorganisms, has raised a variety of concerns over unknown risks that may be posed by this new technology.[1] Fermentation is currently restricted to the use of non-pathogenic microorganisms. However, increasing attention has been focused on the potential for immunologic response, after repeated inhalation, to a variety of organic materials (including microorganisms) in both biotechnology and other occupational settings.[2–7] Future use of recombinant DNA (rDNA) technology may produce microorganisms in need of more stringent containment requirements and equally stringent programs in occupational safety and health due to the increased health risks that they may pose to the exposed workers.[8] These perceived health risks are predominantly concerned with viable microorganism exposures. Prudent practice dictates that appropriate controls should be identified and implemented as new production-scale operations are designed and built. In general, a control strategy must emphasize containment within a sealed system through engineering and appropriate work practices. In addition, monitoring to assure system integrity and the use of personal protective equipment are secondary/tertiary components of a complete control system.

The effective containment of the potential hazards in biotechnology industries utilizing rDNA techniques will rely heavily on the equipment design in existing fermentation process technology. The equipment design must include containment factors for the control of emissions from viable microorganisms, biologically active products or intermediates, and processing chemicals such as extraction solvents. An examination of the emissions and controls in existing or conventional fermentation processes (which are in many ways similar to those being developed for genetically engineered microorganisms) can be used to assess current technology effectively and also to evaluate the adequacy of this tech-

nology if it were to be applied to scaled-up rDNA operations. This study provides a baseline of information available for transfer to other fermentation technologies, for both those involved with rDNA or those utilizing conventional technology.

The primary objective of the study was to evaluate and document effective controls in enzyme fermentation processes. The health risks associated with the microorganisms used in the fermentation process were not addressed in this study. Additional objectives include the development of control and research recommendations and the transfer of the documented information to the appropriate individuals in industry, academia, and government. The enzyme industry was selected because it employs fairly typical fermentation process technology, has acceptable workplace sampling and analytical requirements, and has established health risks (enzymes) which require effective emission controls. This paper will focus on the results from microbial air sampling and evaluation of controls at three enzyme production plants which utilized conventional fermentation process technology.[9-11]

Methods

The control measures were evaluated by observation and by collecting environmental air samples for substances involved in enzyme manufacturing processes at three plant locations. Approximately 200 air samples were collected at each plant, using an Andersen two-stage bioaerosol sampler. Each plant used a Bacillus strain (*subtilis* or *licheniformis*) to biosynthetically produce a proteolytic or carbohydrase enzyme. Samples were taken to assess microbial emissions at locations believed to reflect workplace exposures to the process microorganism. Sampling locations were identified based on potential emission sources observed during preliminary surveys. Bioaerosol samples were collected as near as practical to these identified potential emission sites. The sample locations included the laboratory (sites where culture transfers are conducted), inoculum and fermentor tanks (sample ports and agitator shafts), filtering operations, and selected background locations. Background locations were selected so that the influence from unit processes on the ambient microorganism levels were minimized. For example, outside background locations were located at a distance away (upwind, if possible) from buildings which housed the process equipment so that consecutive samples are consistent and the quantity of the process microorganism is negligible. Indoor background locations were located in rooms separate from the emission sources. Microbial sampling was conducted over a four- to five-day period at each plant with sample time ranging in length from 2.5 to 20 minutes at a flowrate of 1 cubic foot per minute (28.3 liters per minute). These sample times were selected based on expected microbial levels (from the exploratory surveys) in order to maintain total colony counts between 20 and 200. This range increases the reliability of the colony counts per plate by reducing the errors associated with inadequate colony numbers or with colony overgrowth. The 50 percent effective cutoff diameter for the top stage of the Andersen bioaerosol sampler is 8.0 μm—larger, nonrespirable particles are collected on the top stage; smaller, respirable particles are collected on the bottom stage. Standard methods agar was used as the sampling media.[12]

Analysis of the bioaerosol samples was conducted on-site by a microbiologist. The primary goal of the microbiological analysis was the determination of the number of the production microorganisms (Bacillus strain) in the air at each sampling location. Colony morphology was compared to that of the production strain at the same incubation time and on the same medium. Randomly selected sample populations were streaked onto standard methods agar for isolation and identification. Confirmation of the production strain was made by utilizing standard gram straining techniques and/or a sugar utilization profile analysis.[13] Each step of the microbial identification process by itself is not an absolute indicator of the production strain. However, combining the results of these identification tests afforded the microbiologist the opportunity to produce reliable conclusions concerning the production strain. Sample results are in terms of total colony forming units per cubic meter of air (CFU/m^3). Colony forming units are determined by counting (under magnification) the number of *all* microbial colonies, using standard colony counters, that exist on a sample plate after a 24-hour incubation period at 37°C (98.6°F). The counts were not adjusted for multiple impactions, according to the Andersen Positive Hole Counting Method, due to the inability of the Andersen two-stage to produce a consistent "jet" pattern from which to count "positive holes." In some cases, exact production strain colony counts were unavailable or not verifiable. Sample concentrations around processing equipment were compared to background concentrations to help ascertain the degree of microorganism emissions.

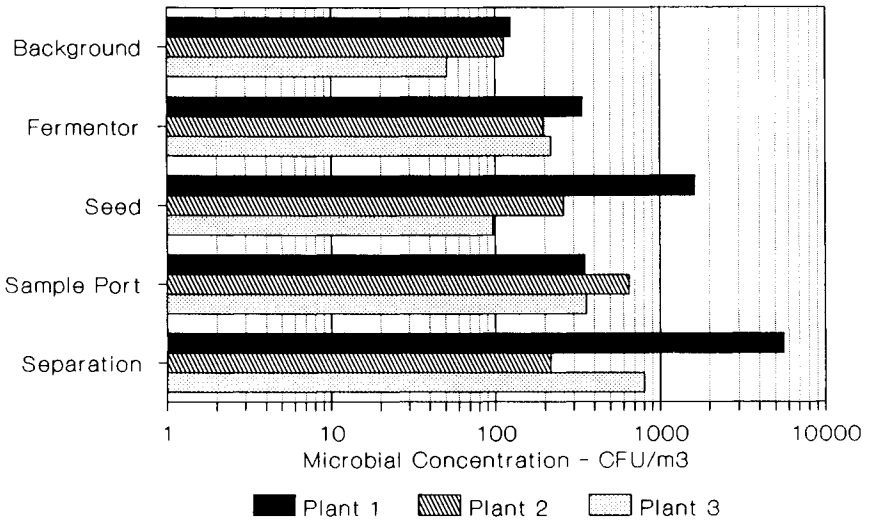
Outdoor background samples were grouped into a single classification. Any effects on outside background samples due to bioaerosol process emissions were assumed to be uniform from sample location to sample location. This assumption is based on outside background samples being located away from the building that housed the process equipment at a distance that would predispose complete mixing of process microorganisms that may have been released into the environment. In fact, analyzed sample plates exhibited a negligible quantity of the process microorganism. Sample sizes between locations were unequal and, at times, numbers of samples were small—ranging from 2 to 56. All results were blank corrected and determined to be log-normally distributed based on an analysis of the residuals.

Analysis of the relationships between unit processes of differing plants was conducted to assess the effectiveness of the engineering controls. Tukey's test for all main-effect (sample location) means was employed to determine any statistically significant differences between unit processes. This statistical test

TABLE I Tukey's Multiple Range Test Applied To Process Locations

Location	Plant	N[A]	Geometric Mean (CFU/m^3)[B]	Geometric STD	Tukey's Test[C]
Background	1	56	124	7.6	A
	2	20	113	3.7	A
	3	7	51	1.4	A
Fermentor Agitator	1	20	339	3.1	A
	2	38	196	2.3	A
	3	28	219	2.0	A
Seed Agitator	1	20	1634	1.5	A
	2	24	260	1.6	B
	3	30	97	2.3	C
Sample Port	1	11	350	3.4	A
	2	6	648	1.6	A
	3	8	358	1.5	A
Separation	1	28	5626	2.5	A
	2	45	216	2.1	B
	3	32	798	1.9	C

[A]N indicates the number of samples per location.
[B]Means are based on Total Colony Counts.
[C]A location which has the same Tukey's grouping between plants does not differ significantly. Tests for differences between locations are not shown.



Note: Total Colony Counts

FIGURE 1. Graphical summary of sampling results at specific process emission points.

is based on a studentized range for comparison of pairs that controls the maximum experimental error rate.[14,15] Causes of significant differences were subjectively identified based on the researchers' knowledge of the process equipment and observation of the engineering controls.

Results/Discussion

Variations in the microbial concentrations from plant-to-plant could be attributed to the type of process equipment utilized, the work practices of the operators, and the microorganism used in production. The results of the microbial sampling at various sites are summarized in Table I and graphically presented in Figure 1. Concentrations were highest around filtering operations at each of the plants except one. In one plant, geometric mean levels of total viable microorganisms at a solid-liquid separation process (filter press) was 5626 CFU/m^3 (159 CFU/ft^3); the predominant strain was the production microorganism. At a second plant, the geometric mean level of total viable microorganisms at a separation process (rotary vacuum drum filter) equipped with a local exhaust ventilation hood was only as high as 216 CFU/m^3 (6 CFU/ft^3); the production strain was identified in very low numbers on only 10 percent of the sample plates. At a third plant, the geometric mean level of total viable microorganisms at a separation process (centrifuge) was 798 CFU/m^3 (23 CFU/ft^3); the production strain existed in significant numbers.

The significantly lower level of microorganisms around the rotary vacuum drum filter in the second plant (Table I) compared with the levels around the filter press in the first plant seem to be due to the inherently better containment characteristics of rotary vacuum drum filters and to the use of local exhaust ventilation. In addition, operator work practices appeared to be a significant factor in determining the higher microbial level during the filter press operation. The operator was observed removing (with a wooden boat oar) the filter cake that adhered to the filtering elements at the end of the filter press cycle. This was a necessary part of the cycle and a plant authorized procedure. The centrifuge at the third plant would be expected to produce higher microbial emission levels compared to the filter press at the first plant, due to the high velocity rotations of the centrifuge. However, due to the effective process enclosure and the use of local exhaust ventilation, the microbial emissions that were detected around the centrifuge were at significantly lower levels than at the filter press.

In a few cases, increased microorganism concentrations were associated with specific locations around the seed and fermentor tanks. These locations are pictorially identified in Figure 2. The highest levels occurred around ports that are used to manually sample for the microbial broth mixture inside the seed and fermentor tanks (Table I and Figure 1). At one plant, the microbial level increased from 196 CFU/m^3 (sample port closed) to 1668 CFU/m^3 (sample port open). Operator technique during the broth sampling procedure appeared to be the primary determinant of the level of contamination. Operators in all three plants normally purged the sample port with a "blast" of pressurized steam, which resulted in the aerosolization of any microbial contaminants re-

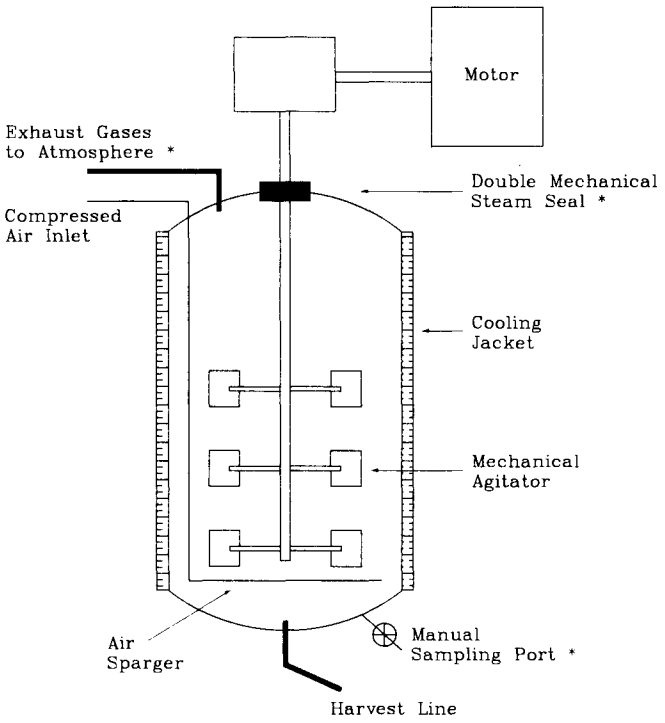


FIGURE 2. Typical fermentor tank.

TABLE II Tukey's Multiple Range Test Applied To Other Locations

Location	Plant	N[A]	Geometric Mean (CFU/m³)[B]	Geometric STD	Tukey's Test[C]
Clean Room	1	12	4	5.1	A
	2	6	4	3.0	A
	3	6	2	1.0	A
Incubation Room	1	6	332	1.1	A
	2	8	173	3.3	AB
	3	2	37	1.8	B
Analytical Lab	1	10	152	1.5	A
	2	6	391	1.7	A
	3	11	9	3.5	B

[A]N indicates the number of samples per location.
[B]Means are based on Total Colony Counts.
[C]A location which has the same Tukey's grouping between plants does not differ significantly. Tests for differences between locations are not shown.

maining in the system. This "blast" of pressurized steam was probably responsible for the inability of the local exhaust ventilation at the sampling port of the third plant to contain microbial emissions. A gentle washing of the interior pipes with steam, producing a liquid condensate which could be collected and disposed of, (this was the stated policy of the plants involved) could prove effective in minimizing the degree of exposure. Microorganism concentrations at the manual sampling port across the three plants were not significantly different. Sampling data concerning the exhaust gases from the seed and fermentor tanks are limited to the first plant due to the inaccessibility of the exhaust gas ducts in the other two plants. However, the data from plant 1 indicates that the exhaust gases, as expected, can be a source of microbial emissions. The water scrubber from plant 1 showed measurable emissions of entrained viable microorganisms.

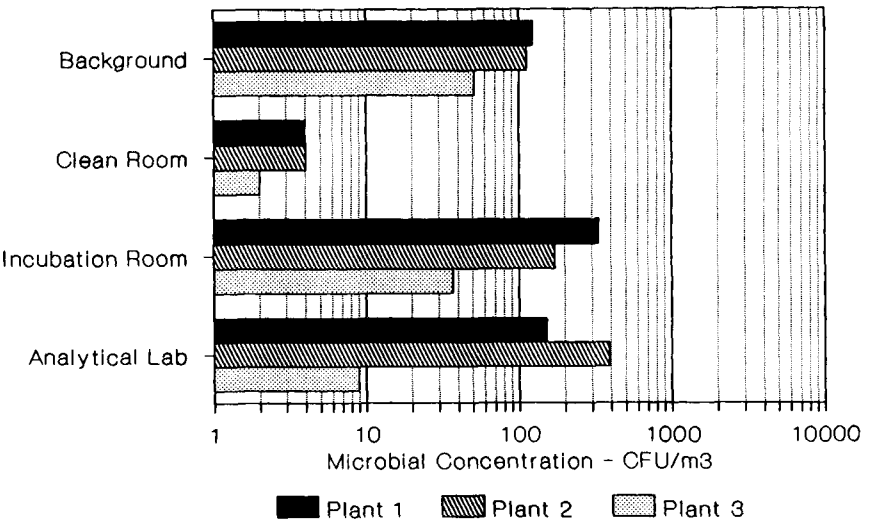
Agitator shafts equipped with double mechanical steam seals appeared to be effective in their ability to contain the microbial culture inside the seed and fermentor tanks, whereas packed seals showed some leakage. Microbial concentrations around the seed agitator shafts were significantly different among plants surveyed, as were the concentration of production organisms around the fermentor agitator shaft (Table I and Figure 1). These differences may be explained by the fact that plant 1 utilizes a packed seal around the seed and fermentor tank agitator shafts, while plants 2 and 3 utilize double mechanical steam seals around all agitator shafts.

Although the design of the processing equipment is an important factor determining the effective containment of production microorganisms, a debilitated strain can also reduce the viable microbial levels around processing equipment. Plant 1 exhibited higher concentrations of viable microorganisms around a majority of the process sites sampled compared to comparable sites at plants 2 and 3. This higher concentration of viable microbes could be partially related to the sporogenic nature of the production strain and the ability of this strain to adapt to conditions outside of the fermentation process. Plants 2 and 3 used asporogenic strains of Bacilli in their process operations. Plant 2 utilized a strain of Bacillus that appeared to exhibit an extremely low tolerance to conditions outside of the fermentation process. The confounding effect of these variables is not known. However, the bioaerosol concentrations did correlate well with visual observations of the apparent quality and effectiveness of the engineering controls that were in place.

Variations of the microbial concentrations between plants for other sampling locations were small compared to those near the processing equipment. These locations included the clean room, incubation room, and analytical laboratory. No statistical differences were detected between plant clean rooms (Table II and Figure 3), and the geometric means did not exceed 5 CFU/m³ (0.1 CFU/ft³). Significant differences were detected between plant incubation rooms and analytical laboratories, but these differences involved total microbial colonies and not production strain organisms. The amount of the production strain among these differing plant locations was negligible. Work practices of the technicians in these locations appeared to be the major determining factor affecting the degree of potential microbial exposure. For example, pipetting of any solution by mouth, which was commonly observed, is contrary to safety procedures in any laboratory and should be avoided.

Conclusions/Recommendations

Controls are most needed around certain high energy operations including filters or centrifuges, agitator shafts, and manual sampling ports. These operations are often not amenable to complete sealing, enclosure, or isolation. Generally, where total contain-



Note: Total Colony Counts

FIGURE 3. Graphical summary of sampling results around laboratory operations.

ment of a potential emission source involving non-pathogens is not a feasible alternative, local exhaust ventilation can be an effective means in controlling emission sources. If potentially harmful organisms are involved, a strictly reliable containment system is the recommended control strategy. This may involve the selection of a processing scheme (e.g., not using manual filter presses) which is consistent with such containment. This study primarily focused on the release of viable process microorganisms. If the primary interest is related to health risks due to immune response reactions to process microorganisms, other sampling techniques may be required to detect non-viable cells in addition to viable cells.

Exhaust gases from the inoculum and fermentor tanks are another major emission source of production microorganisms and should be controlled with an effective filtering system. Water scrubbers may not be completely effective for controlling bioaerosol emissions from the exhaust gases. The geometric mean of samples collected from a water scrubber at one plant was 345 CFU/m^3 with approximately half of the cultured microorganisms being the process strain. This was statistically higher than background concentrations.

Work practices of the operators can also be a determining factor in the degree of exposure, as in the case of the operator extracting a broth sample from the sampling port. Work practices are most reliable when used in combination with effective engineering measures such as isolation or automation. Microbial exposures at filtering operations can be greatly reduced by limiting operator interaction with those processes or, if this is not possible, the observance of proper and safe work practices.

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