Chapter 19

Minimization and Prevention of Phage Infections in Bioprocesses

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Abstract

Phage infections in bacterial bioprocesses constitute one of the most devastating threats to the productivity of the biotechnology facilities. There are several factors, which can decide if an infection would occur, and if it would turn into an outbreak and heavy contamination of the production facility. This issue is discussed on the basis of literature survey and experience of Phage Consultants.

Key words: Phage, Contamination, Fermentation, Bioreactor, Contamination prevention, Phage outbreak, Decontamination

1. Introduction

Bacteriophages are viruses, which are natural components of every community which contains bacteria. The estimated number of phage particles on Earth is about 10^{31} (1). They are in fact the most numerous entities on Earth. However, usually they have relatively narrow host range, which means that only small fraction of all bacteriophages can be dangerous for particular bacterial species. In practice, due to bacteriophage abundance, bioprocesses based on bacterial activities are in constant threat of bacteriophage infections. The infection by bacteriophage in fermentation facility may lead to destruction of bacterial cultures and subsequent contamination of the facility. This may in turn lead to paralysis of facility productivity, and thus to heavy financial loses. Thus the ability to prevent phage infection and to recover fast, if any infection already occurred, is a key element in all types of fermentation facilities which utilize bacteria for production. However, the optimal

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way of contamination prevention and eradication strongly depends on the process and facility itself. In this chapter, literature survey and some of general remarks derived from work performed by Phage Consultants will be given.

2. Phage Sources

2.1. Facility Location

The fact that bacteriophages usually show relatively narrow host range also has an impact on their distribution in environment. Usually bacteriophages active against particular bacterial species are to be found in these environments which are habitats for the target bacteria. From the bioprocess point of view, it is the main concern when choosing new location for the facility. For example, fermentations based on one of the commonly used host—*Escherichia coli*—will be subject to the most danger when located near water treatment plants, rivers, lakes, and shores contaminated with municipal sewage, and in areas where human or animal manure is used as a natural fertilizer for soil (2, 3). It was also advised to keep them away from bird migration paths (3).

Some bacterial species used in fermentation are so widespread in environment, that it is almost impossible to find a relatively phage-free location, especially in places where infrastructure will be developed to serve as a fermentation facility. Among these bacterial species are mostly bacteria belonging to such genera like, e.g., *Bacillus*, *Clostridium*, or *Pseudomonas*. In such situations whole environment outside the facility is to be considered as a bacteriophage reservoir. In some cases, it is still possible to choose location supporting lower burden of specific bacteria and thus load of bacteriophage which can attack, e.g., by keeping away from farmlands when *Clostridium acetylobutylicum* will be used in fermentations, but this will not assure safety. Additionally, placing fermentation facility in remote location may affect the logistics of supplies and products, and thus it can impact the profit margin of the fermentation.

Use of the relatively rare bacterial species, preferably those from very rare bacterial genera, provides process safety advantage. This reduces the possibility that bacteriophage reservoir will be located near the facility, and the chances that phages will be introduced into the fermentation. Obvious disadvantage of such hosts is that they may not be very suitable for genetic engineering, and thus construction of production strains may not be feasible or economical.

2.2. Raw Materials, Equipment, and Bioprocess

Bacteriophages can infect the process using different entry ways. Sometimes it is not easy or even impossible to find out which measure of protection failed. The most common sources are those that are relatively hard to control. One of the major sources is air used to aerate fermentation or to keep fermentation overpressurized.

The main reason for that may be the presence of filtration membrane failures or the inability of membrane filters to fully stop particles of bacteriophage sizes. The performance of air filters may be dependent on the air moisture. Also, the ability of air filters to stop bacteriophage particles may be seriously affected during improper sterilization procedures or excessive usage of filter and resulting deterioration of filter condition (3).

Another possible source of bacteriophage in fermentation is raw materials. All measures of precaution would be worthless if media was not properly sterilized. As this task seems to be very easy in case of small volume fermentors, it is sometimes not simple to properly treat the media for hundreds- or thousands-cubic meters fermentors. Thus, the proper choice of sterilization method and equipment is crucial for successful phage contamination prevention. In case of large bioreactors sterilization of reactor itself may prove to be relatively difficult, especially when fermentors cannot be overpressurized and thus sterilization conditions used in autoclave cannot be achieved. In such situations combination of effective cleaning method, proper construction of fermentor, and adequate sterilization may considerably reduce the risk.

Water jacket was previously mentioned as a possible source of contamination of the process. The fermentation vessel integrity should be tested regularly and water used in water jacket should be assured to be phage-free (3).

Basically, all parts of bioreactors which come into contact with raw materials or bacterial culture itself are not easy to clean for mechanical reasons, and can be potential sources of phage outbreak. Tubes and valves are frequently culprits as they are hard to clean and are prone to temperature loss during sterilization. Special attention should also be given to sampling ports, as they may provide direct contact between content of bioreactor and outside world. Due to frequent manipulation, sampling ports may be easily contaminated during fermentor operation.

Inoculation is a very critical moment, as it is relatively easy to contaminate the fermentor if the procedure of inoculation or equipment used were not properly designed. Moreover this part of fermentation is most prone to operator errors.

2.3. Personnel

Despite the fact that the location of the facility in some cases can be a key factor in mitigating the risk of phage contamination, properly trained personnel is critical to the success of the bioprocess. Each person is a carrier of bacteriophages active against *E. coli*, and thus each operator is a potential source of phage infection and subsequent phage outbreak in the fermentation facility utilizing this host. As a result even perfectly located and planned facility cannot provide full protection without proper cooperation of employees. Considerable risk can be introduced by personnel by lack of proper precautions when designing the facility, by improper

choice of equipment, by poor design of the process itself (from the point of view of contamination security) including any of preparation, harvesting, and subsequent steps. One thing to keep in mind is that even the most skilled process microbiologists usually had little or no contact with bacteriophages during their courses and practices. They had their first opportunity to learn by trial-and-error method when contamination already occurred, and they have little chance to prevent phages from causing long-lasting and very expensive phage outbreak. From our experience, it is clear that improperly treated phage outbreak may paralyze facility productivity for up to 10 months. Although it is an extreme case recorded by us so far, much shorter periods of phage problems also cause large financial loses.

2.4. Bacterial Strains

To block phage infection, some authors recommend utilization of phage-resistant mutants (2, 3). This strategy is widely used in dairy industrial fermentation; however, in vast majority of cases it is very ineffective. There is a need for constant change of starter cultures used in dairy fermentations. The reason for that is there is almost unlimited variation of receptors utilized by phages to bind bacteria, and there are many counter-defense resistance mechanisms of bacteriophages. Introduction of any resistance mechanism in the host will rapidly result in selection of these phages, which can easily overcome the host resistance. Thus it is most important for long-term process protection, to work out proper work techniques and to use appropriate equipment in properly constructed facility. Production of resistant strains can be only an additional activity, and it cannot be a main approach to prevent a process contamination.

One of the most common myths is the ability of *tonA* mutants to prevent any T1-like phage from infecting the strain. From our practice, it became obvious that the vast majority of T1-like phages isolated from failed fermentation utilize different receptors, thus *tonA* (*fhuA*) mutants are not safe against T1-like phage infection. Moreover, majority of these phages which were sequenced or partially sequenced show much higher homology with Rtp phage than with T1. Rtp phage was also isolated from failed fermentation, and it most probably utilizes LPS as a receptor (4). It seems that the utilization of FhuA-TonA related infection mechanism by T1 and T5 among selected T-series phages was a coincidence, and thus no general conclusions can be made on protection against T1-like phages by *tonA* mutation.

Some bacterial strains show relatively high resistance against phage infections. The resistance mechanisms were reviewed by other authors (e.g., ref. (2) and references therein). They can be attributed to a few changes in bacterial cells like, e.g., cell wall alteration, receptor mutations, restriction modification systems, exclusion systems encoded by prophages, and other mobile genetic elements. Some mechanisms will work quite well on solid surfaces while in

liquid cultures they will be completely ineffective, e.g., spatial separation of phage from bacterial cell by secretion of large amounts of exopolysaccharides. Production of phage-resistant strains may help in prevention of phage infection by the same phage, which was used for selection of strain, or at best case a group of closely related phages. However, this cannot replace all the preventive measures of precaution, as subsequent phage infection by different phages can easily occur and cause outbreak, since the resistance never covers all possible phage types.

Another, quite frequently overlooked issue is the presence of prophages in production strains. Prophages can have very adverse effect on the strain performance. The effects range from occasional lysis of culture due to prophage induction or mutation causing virulence (5), through phage contamination and infection of other bacterial strains (6), to the contamination of fermentation by lysogenic conversion gene products (7). Prophages usually are present in media due to spontaneous induction and release from the host. They can be present in final product from copurification. Properties of some temperate bacteriophages make them quite hard to detect, and relatively easy to spread horizontally (6).

3. Phage Outbreak

3.1. Phage Primary Contamination

Phage outbreak in the facility starts with primary contamination. There are some basic methods, which can prevent it, or make it less likely (e.g., ref. (8)), but they will never prevent all contaminations (9). Phage primary contamination differs from subsequent infections as the source of phage is not located within the facility itself, and the phage may not be adapted yet to the host to give maximal potential phage yield. When it happens, it would be recognized only by the well-prepared personnel, and an outbreak would be prevented only by following well-prepared emergency procedures. However, surprisingly often no emergency procedures are in place, and the personnel have not undergone proper training. Thus primary infection in many cases has the potential to turn into a regular outbreak.

Symptoms of phage primary infection strongly depend on several factors such as virulence of the phage, what stage of fermentation phage contamination occurred, type of fermentation, properties of bacterial strain itself, and even factors like the mechanism of lysis utilized by the phage. Due to the fact that airborne phage can be introduced to the process relatively late, infected culture may not show any obvious signs of phage propagation, and thus fermentation may be considered successful. Also the phage may not be adapted yet to the host, and thus the phage growth may not be fast enough to lyse bacterial culture. However, in most cases the occur-

rence of phage contamination is relatively obvious, especially if phage was introduced early with inoculum or raw materials were not properly sterilized. In such situation phage development starts from the very beginning of fermentation, and thus even relatively unadapted phage could destroy bacterial culture. Sometimes action of bacteriophage can be partially masked by growth of resistant mutants or lysogenized bacterial cells. In such situation bacterial growth may restore after decrease of culture optical density (OD). In general, occurrence of fermentation failure with foaming and OD value drop should be always considered as a possibility of phage lysis, and in vast majority of cases phages are to blame for such failure. Subsequent actions after fermentation failure strongly influence the probability of occurrence of next infection. The most critical thing in phage primary infection is to prevent any phage release from the infected fermentor. As not all primary infections are very obvious to operators, all batches should be handled with care in order to prevent potential phage release. If any material from contaminated culture was released inside the facility, the probability of occurrence of next, secondary contamination would increase dramatically. In general, it is a good practice to keep aliquots of all fermentation samples stored at –80°C; it is very important to store samples from all suspicious fermentations to check later if any fermentation irregularities were caused by phages. In case of regular problems, on the basis of stored samples we could determine if the phage is a resident one, and thus we are dealing with secondary contamination, or the problems are caused by serial primary contaminations. This knowledge is very important, as the method of problem-solving is strictly dependent on phage origin.

The probability of occurrence of primary contamination depends on season. The various dependences were previously suggested by different authors. Primrose (2) suggested that dry weather facilitates phage spreading due to ability of dust particles with adsorbed bacteriophages to be transmitted by wind. Contrary to these observations, Bogosian (3) noticed that wet air makes air filters less effective. Moreover, the infections analyzed by this author occurred most frequently in autumn (October–November) and winter–spring (January–March). This pattern was suggested to be probably dependent on a few possible factors like seasonal increase in phage load in soil and sewage (10), planting and harvesting activities, or bird migrations. We observed additionally that wet monsoon season facilitates phage infection. Due to fact that there are no major bird migrations during wet monsoon, the other previously mentioned hypothesis seems to be more probable.

The most common bacteriophage type observed by us as a causative agent of *E. coli* fermentation failures is T1-like phages. They were previously reported by several authors (e.g., ref. (11)). It is hard to judge, whether they are the most common, or they simply cause serious problems that require external help to solve

the problems. Their legendary ability to resist desiccation, ability to infect nongrowing hosts, and short development cycles with very high burst sizes make them very dangerous in fermentation environment. The infections which they cause are relatively easy to recognize as they usually cause evident culture OD drop with extensive foaming, unless they occurred in very late stages of fermentation. These symptoms are accompanied with sharp increase in dissolved oxygen due to drop in respiration and increase of gas exchange caused by extensive foaming.

Second most common contaminants found in *E. coli* fermentations are phages belonging to Myoviridae. Their presence in fermentation was also reported previously (e.g., ref. (12)). They seem to be easier to eradicate and to prevent, as majority of them are not very resistant to desiccation. Other types of bacteriophages occur relatively seldom in *E. coli* fermentations. It is possible that some types of contaminations may underwent unnoticed, as not all of them are able to cause evident lysis. Similar pattern seems to be observed in other bacterial species—Siphoviridae and Myoviridae are the most common contaminants detected (13–17)

3.2. Phage Secondary Contamination and Phage Evolution

If phages from primary contamination were not prevented from spreading in facility, or equipment was not efficiently decontaminated, the occurrence of secondary infection would be highly probable. As we observed quite frequently, as well as others reported in the literature (18), phages quite often evolve in fermentation facility to improve their ability to produce higher burst sizes in shorter time, and to improve their ability to survive between infection cycles. The reason for that adaptation is obvious, when one take into consideration, that even a few minutes shorter development period and/or a few percent higher burst size after several infection cycles during one infection would make it possible for more efficient phage strain to produce much bigger amount of progeny phage when compared with the original strain. Probability of any mutation occurs among phage progeny from single contamination is very high, as the final titers of phage may reach as high as 10^{13}/mL (19). The mutations that confer adaptation advantages to the phage may be selected.

The most serious problems in case of secondary contamination is not the gradual adaptation of the phage to the host, it is the fact that the facility is contaminated. Even the smallest spillage may introduce billions of progeny phages to the facility environment, they immediately become dominant specie. If phages are resistant to desiccation, cleaning procedures are ineffective, and continued production despite occasional or frequent failures, phage load inside facility may increase even more considerably, decreasing the chances of successful fermentations.

As a result of the facility contamination, all subsequent fermentation failures are caused by phages which were released during

primary contamination. The fermentation irregularities caused by the same phage should be easier to recognize by operators. It is possible to implement and optimize detection methods, including process monitoring for phage presence.

3.3. Phage Development in Bioprocess

Growth of bacteriophages during fermentation is dependent on several factors. In feeding phase of fed-batch fermentation, phage development may be slower than in batch phase or in batch fermentations due to lower bacterial host resources, which can slow down the development and limit phage burst size. High bacterial density allows for much faster phage development by increasing the adsorption rate, as it minimizes the unproductive stage of phage life cycle when genome is encapsulated. Development of some bacteriophages may be slower if fermentation is conducted at temperature lower than usual for particular host, or when bacterial growth rate is slower (20). Also the type of phage itself may influence development. Phages that are able to grow on starved hosts (e.g., T1, T7) are less prone to slower growth of host cells, while development of other phages can be easily stopped by shutdown of feeding (21). Ability of phages to achieve very high growth rates in bacterial cultures is also dependent on the size of virion, which determines the amount of resources to be consumed to produce single progeny particle, and on host enzymatic machinery and many other factors. As a result, we observe relatively wide range of phage growth rates in fermentation, which span from 0.2 to 4 orders of magnitude per hour. Even the less effective bacteriophages are able to destroy fermentation when introduced early enough. For the most virulent phages, the complete lysis of the culture is achievable in just a few hours, even if introduced to the fermentation at late stages.

3.4. Phage Detection

Phage and prophage detection at first glance looks like relatively uncomplicated task. The most commonly used approach is double layer agar plates (22). However, the performance of this approach is strictly dependent on the skills of the personnel and the phage itself. When properly performed, the test can give outstanding results with excellent sensitivity and relatively rapid results. The time of development of plaques, which are indication of phage presence in this assay, strongly depends on phage itself and it may take from a few hours to 2 days. However, this method can give false negative results for some bacteriophages. Some ways of increase of the efficiency of phage detection using this method are already known, and they are highly recommended to use (23, 24).

Majority of alternative rapid detection methods are possible only for well-characterized phages, or at least they require proper preparations, since they utilize antibodies against phage (25) or amplification of phage nucleic acid (26). These methods are useful, when a facility suffers a recurrent infection by the same phage.

As it is the most common scenario, implementation of these methods should be always considered when facility suffers from phage-based production irregularities.

Prophages, often present in production strains, represent a different challenge than virulent phages. First of all, they are much harder to detect. Due to their life cycle, the lysogenic strain may behave perfectly normal, while producing vast amounts of phage particles. They are dangerous to other strains, which do not carry the same prophage. This is an important issue in facilities such as tollers that handle different strains in fermentation. One challenge in detection of prophages is the fact that there are many of them which cannot be easily induced using agents activating SOS response like, e.g., P2. Thus it is not a good practice in prophage detection to rely solely on mitomycin C or UV as induction agents in prophage detection. Moreover, when induced, sometimes temperate phages do not form plaques on double agar layer plates (23). So far, it is hard to predict, even on the basis of complete sequence, if given phage is active or will become active, and with some exceptions, what will be the triggering factor, which would cause induction of a prophage. This sometimes limits the possibility to cure a strain from prophages. To make things more complicated, some prophages offer benefits for the host, which can highly influence strain performance in fermentation (27–29). Thus, the decision on the deletion of prophage sometimes may cause unforeseen effects.

3.5. Phage Eradication

In general, the ability to clean up contamination and successfully eradicate phages is strongly dependent on facility construction and equipment installed. This issue should be considered at the stage of facility blueprints, but in practice it is usually neglected and makes future problems much more expensive and time-consuming to deal with.

First of all, possible phage contamination issues originated from future facility location, the facility plan, and instrumentation should be consulted with experts in phage eradication and prevention in order to prevent some obvious mistakes (9). The most common mistakes are connected with ventilation systems, location of various parts of facility, and lack of control elements to prevent phage spreading in facility. The location and construction of fermentation waste treatment stations, burden water reusage and treatment stations, and sewage systems are the most common sources of reinfection, even when bacteriophages seemed to be eradicated from the facility.

One most common approach in phage decontamination is the use of various disinfectants to clean up the facility and to kill bacteriophages. One must note that not all disinfectants are effective against phages, and various phages show varied susceptibility to a given disinfectant. Effectiveness of different disinfectants may be modified by types of contamination and various properties of

matrix to be disinfected such as type of surface, composition of media, presence of aggregates, solid and/or hydrophobic particles, etc. In general, disinfectants that were tested and effective on bacteriophage or adenoviruses should be chosen.

Another common approach in phage decontamination is heat treatment of equipment. It is important to remember that temperature is not the only important factor in heat treatment. One key element in sterilization is the energy transfer, which is most effective in water solutions, less effective in steam, and least effective in arid air. When dry heat is used for any equipment sterilization, extensive temperature and prolonged time should be used in order to achieve satisfying results. In case of liquids, temperatures as high as 80–90°C can be effective in phage eradication; however, the time of necessary exposure varies from phage to phage (30).

All attempts of phage eradication should be concluded with proper tests to detect possible phage presence despite sterilization efforts. Sampling should be focused on, but not limited to, areas where raw materials and bacterial cultures are prepared, and where biomass transfer to and from reactor/seed reactor(s) normally takes place. Regular testing programs should be implemented in order to detect problems before they become disasters. Another very important aspect, as also recommended by others (e.g., ref. (2)), is the development of emergency procedure that should be implemented immediately after contamination detection. The procedure should be triggered by phage detection or by observation of certain fermentation irregularities like drop in OD, extensive foaming, etc. At the moment, there is possibility to construct rapid, semiautomatic detection methods based on nucleic acid amplification; however, their usefulness may be limited to recurrent infections. Despite this fact, they can provide very effective help in eradication of phage from the facility.

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