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Review Article

Strategies of phage contamination prevention in industry

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Abstract

Phages are potential cause of failure of bacteria-driven production processes. In this paper some observations arising from the experience of the author, concerning the strategies of phage prevention, adopted by different industries is described. The exact choice of the strategy is usually driven by the economy, but also by the regulatory aspects of the operation. The less regulatory or economic freedom industry offer, the more attention is payed to the improvement of equipment and process hygiene.

Introduction

Bacteria-driven processes in industry are commonly employed in various fields, from traditional biotechnology, which mostly focuses on production of food and beverages, through modern biotechnology, including production of solvents, green chemistry, vitamins, amino acids etc., to the most refined fields of use, including biopharmaceutical production of highly valuable proteins and other active pharmaceutical ingredients. All of these processes are prone to irregularities caused by contaminants. The most potent contaminants are bacteriophages, however, the level of susceptibility of the process to contamination by phages and to their deleterious effects is strongly dependent on several factors. Although a lot of information about the impact of bacteriophages on production processes driven by bacteria is available, paradoxically, the smallest number of scientific papers is published about the processes where the potential impact of phages is the most severe.

In general, phage contaminations can be divided into two types:

Primary contamination, the source of which usually lays outside of the facility, and the phage got introduced into the facility in relatively low amount, for example by personnel or by contamination of raw materials.

Secondary contamination, the source of which comes from a previously contaminated process, from which a phage (usually in great quantity), was spread within the facility or installation.

Routes of process contamination in case of primary and secondary contaminations are usually different, and prevention usually requires distinct methods to be employed.

How does the complexity of the bacterial community driving the production process influence the potential phage impact?

In general, a rule of thumb says that the more complex the bacterial community, the less prone it is to the disturbance of the process by bacteriophages. The reason for that is that multiple bacterial strains and/or l species involved in the production process can, at least to some extent, substitute for each other in the conversion of a substrate into the desired product. This is the case in facilities such as biogas production facilities or certain fermented food production facilities, which depend on natural, extremely complex microbial communities that already include bacteriophages, and their operation is not dependent on aseptic process development. Instead, bacteriophages may even be considered an important component of the whole production microbiome [1]. The less complex the microbial community gets, the more damage to the process can be caused by phages. Scientific literature discussing the impact of phages is most extensive in case of dairy processes - it is virtually the only industry quite openly discussing problems with

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phages. Industrial biotechnology [2-4] and biopharma [5], very seldom release any information about phages attacking their processes. There are a few possible reasons for that. Firstly, a phage attack, considered a failure, may not be seen as a valuable result to be published. Secondly, a company's interest may be to avoid any information disclosure, which may be crucial in keeping the know-how and in providing a competitive advantage. Another reason may lay in the fear of losing customers, who may be concerned by the failure to provide safety to the process and may question the competence of the whole team. The relative lack of information in scientific journals about phages attacking processes other than dairy production may create a false impression that bacterial-driven processes in industrial biotechnology and biopharma are safe, or at least much safer from phage attacks. This is not true, but each of the industries suffering from phage attacks has different ways of minimizing their occurrence and impact. The strategies of phage prevention are dependent not only on the ability of a phage to kill bacteria in the process, but also on the volume of the production and the ability to use replacement strains in subsequent production in case of a phage attack.

Strategies to prevent or minimize phage impact

Strain rotation and manipulation: Starter culture rotation is commonly employed in diary industry as a standard procedure preventing production irregularities. This industry relies mostly on utilization of multi-species starter cultures with various phage resistances patterns, with the starter cultures changed on a regular basis. This allows for the formation of the product even if phages are not eliminated entirely from the production environment [6-8]. Another aspect of this strategy is the rotation of starter culture in case of a single species use [9]. This strategy can be also applied in case of certain industrial biotechnology setups, but it is much more challenging than in the case of food production. The main reason is the difficulty in obtaining a sufficient number of strains of a desired producer that would show good enough kinetics of product formation and high enough final concentration of the product to ensure the process is profitable. Since this industry usually operates on relatively thin profit margins, the change in performance of a production strain caused by selection of a phage resistant mutant may have a fundamental impact on the process economy. Nevertheless, such attempts are sometimes made. Usually, the approaches include isolation of phages from the vicinity of the facility, exposure of the production strain to those phages, and isolation of resistant mutants. This approach allows for isolation of a panel of production strains, giving hope that at least one production strain will be resistant to an incoming phage. The strategy may not be very effective in the long run if not repeated frequently, as phages present in the environment will evolve over time. Moreover, one of the frequent sources of bacteriophages are raw materials, which are used in industrial biotechnology in bulk, and which often originate in distant and variable destinations, thus covering many more environments and potential phage sources.

In case of biopharmaceutical setups this approach is, in vast majority of cases, impossible. The regulations of cGMP production, necessary to implement in biopharmaceutical production, do not allow for any flexibility in choice of host once the procedures are established and validated. Thus, no matter what the situation, the production has to be conducted using the host susceptible to a phage, endangering the whole production process. Sometimes bacterial strains with engineered phage resistance to selected group of phages is used, e.g. *TonA* mutants of *E. coli* [10], but these manipulations are not effective for phages utilizing different receptors or metabolic pathways. In case of dairy industry use of such strains is more widely used, but to the date this strategy never resulted in production of ultimately resistant strain [11]. In case of biopharmaceutical facilities, major efforts have to be invested in process design, if possible, and in proper choice of equipment. Very important aspects in this case are facility design, facility hygiene, and appropriate procedures.

Process design and choice of equipment

Other aspects of phage prevention are process design and choice of equipment used. This line of defense is mostly used in prevention of primary contamination and, in case contamination occurs, in containment of the contaminated material inside the production line until it can be properly inactivated. To some extent, process design and equipment choice can help in prevention of secondary contamination. Usually, these aspects considered more carefully in case of industrial biotechnology and biopharmaceutical setups. Higher level of protection of aseptic conditions significantly lowers the risk of a phage penetrating the process. As some solutions are more difficult to implement in large-scale production typical for industrial biotech, the level of protection obtained by improvements in process design and choice of equipment is higher in typical biopharmaceutical setups. However, in industrial biotechnology this aspect is carefully considered prior to setting up the final production scale. The prevention of phage, penetration into, usually bulky, processes is very often difficult in facilities relatively poorly isolated from the outside environment. In general, process design with respect to phage contamination prevention focuses on killing or removal of all potentially contaminating phages in raw materials delivered to the process, and in prevention of introduction of phages during all manipulations done by the personnel. Major concerns are in this case proper sterilization or sanitization of media and bioreactors, as well as air filtration. The latter poses the biggest challenge and is also the most difficult to control [12,13].

In case of fermented food production and dairy industry, removal of all phages from raw materials is often impossible [6,8], so the phages will be propagated despite the best design of process or choice of equipment. However, in this situation, especially when complex starter cultures are used, the collapse of the process is usually caused by the accumulation of different phages in the facility and inability to clean them and not by an unfortunate cumulation of various phages in one raw material batch.

In case of biopharmaceutical facilities, process design and choice of equipment to help protecting from phage contamination is much easier due to considerably lower

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volume of the process compared to other processes like food or industrial biotechnology. Relatively high profit margin of biopharmaceutical processes allows for the use of more effective and expensive approaches, such as extensive cleaning and sterilization procedures as well as frequent changes of consumables such as filters. On the other hand, any (even minor) bacterial contamination, if present, may render the whole batch useless for active pharmaceutical ingredient use, and may additionally trigger the expensive process irregularity investigation required by the authorities [14].

The proper choice of equipment, especially in downstream processing, together with facility hygiene are the decisive factors preventing or promoting phage spread into and within a facility. In general, the more open or semi-open steps in the process during bacterial propagation, harvest and downstream processing, the more risk in spreading phage within the facility, and the higher demand for proper facility hygiene in order to prevent phages from infecting subsequent processes.

Facility hygiene

Facility hygiene is an important aspect of prevention of both primary and secondary phage contaminations. It is most crucial in case of cleaning up a contamination, which has a potential to spread from the production line into the facility. Contaminated bacterial culture may contain as many as 10^{12} or even close to 10^{13} phages/ml, thus a release of even a relatively small volume of the phage lysate may result in a massive contamination of the facility [13].

There are various approaches for keeping a facility clean, however, the most surprising observation is that a vast majority of facilities utilize cleaning agents and disinfectants with no proven action against bacteriophages. To make things worse, many of the facilities do not use a single effective anti-phage agent, focusing more on the reduction of bacterial load in the facility environment. Additionally, declared viricidal efficiency of disinfectants is based most frequently on studies on enveloped viruses, and thus they may be misleading. In fact, there is only a very limited array of effective disinfectants capable of inactivation of a wide spectrum of bacteriophages. Among the most effective are strong oxidizing agents, but these in turn may be problematic to use on some surfaces (Marcin Los, unpublished observations).

Nevertheless, facility hygiene is usually the easiest to design in an effective way in small, high-end facilities characteristic for biopharmaceutical setups. The ability to keep a facility clean and to be able to remove phage contamination efficiently strongly depends on the initial facility design as well as the material and personnel flow. Together with the process design and the choice of equipment used, facility hygiene presents the most important aspect of phage contamination prevention and troubleshooting.

Summary

Different industries suffering from phage contamination adopted different strategies of handling potential problems arising from the deleterious effects of phages on bacterial-driven processes (Table 1).

Over the decades of operations, each industry optimized some aspects of bacteriophage problem prevention, while other aspects remained relatively poorly improved. Implemented improvements allowed the industries to operate in a profitable manner, however, in some cases there is room for improvement, which could help to better prevent phage contamination in production facilities. Details of selected methods that could be used for this purpose are described in previous reviews, including Primrose 1990, Bogosian 2006, Los et al. 2004, and Los 2012 [12,13,15,16].

Table 1: The relative levels of investment in various aspects of phage prevention in different industries (Marcin Los, unpublished observations).

Type of industry	Strain/ starter culture rotation	Process design/ equipment selection	Facility hygiene
Food production/diary fermentations	High	Low	Low
Industrial biotechnology	Low to moderate	Moderate	Low to moderate
Biopharma	None	High	High

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