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Bacteriophage Infections in the Industrial Acetone Butanol (AB) Fermentation Process

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Abstract

The reported incidence and effects of bacteriophage infections occurring in the industrial acetone butanol (AB) fermentation processes operated in the USA, Japan, and Puerto Rico during the earlier part of the twentieth century is reviewed. The growth characteristics and solvent-producing ability of a lysogenic strain of *Clostridium madisonii* isolated from a phage infection in Puerto Rico was determined in molasses fermentation medium. The host strain harbours a large lysogenic phage belonging to the *Siphoviridae* and the growth rate of the lysogenic strain was found to be slower than the non-lysogenic parent strain and exhibited reduced solvent production. The history of phage infections that occurred in the South African AB process is documented along with the various remedial actions that were taken to restore production. A more detailed account of the last phage infection that occurred in 1980 involving a small pseudo-lysogenic phage belonging to the *Podoviridae* is given. This phage infected *Clostridium beijerinckii* P260 and a number of closely related industrial strains. Factory-scale fermentations contaminated by this phage were compared with equivalent laboratory-scale control fermentations. The effect of the phage infection in the full-scale and laboratory-scale fermentations were monitored. Results obtained in laboratory-based studies included an assessment of the effect of the multiplicity of infection and the timing of phage infection. The general effects and symptoms of phage infections in the industrial AB fermentation are reviewed including gross changes in the fermentation and changes in cell morphology. Common techniques used for the diagnosis of phage infections and approaches for controlling phage contamination in the AB fermentation are discussed. Prevention strategies included good factory hygiene, sterilisation, decontamination and disinfection, and the use of resistant strains immunised against specific phages.

Introduction

Infections by bacteriophages (phages) have been reported to be a major problem in acetone butanol (AB) fermentations and occurred wherever the industrial fermentation process was operated (Hastings, 1971; Walton and Martin, 1979). However, despite the importance of this industrial fermentation process during the first part of this century, there is little detailed information relating to specific phage infections contained in the published scientific literature.

The first reference to a phage infection in the AB fermentation process relates to problems encountered at the Commercial Solvents Corporation (CSC) plant located in Terre Haute in the USA (Gabriel, 1928). This plant had been built during the First World War for the production of acetone for munitions. After the war the plant and the patent rights to the AB fermentation were purchased and it was continued as a commercial enterprise.

In 1923 problems were experienced with sluggish maize mash fermentations that utilised *Clostridium acetobutylicum* and solvent yields at the plant were reported to have been cut by half for almost a year (Gabriel, 1928). The causes of these problems, which had a major impact on the expanding fermentation industry, were eventually established to be due to a phage infection. The severity of the infection appears to have been responsible in part for the decision to build an entirely new plant 200 miles away at Peoria (Hastings, 1971). The repercussions caused by this phage infection resulted in extensive research being undertaken by the research department at CSC into ways of combating such problems, should they re-occur. These investigations led to the patenting of procedures to produce industrial strains immunised against phage infection (Legg, 1928; Legg and Walton, 1938). The CSC company continued as the sole producer of solvents by the fermentation process until 1935 when the patent rights lapsed and a number of companies established similar fermentation processes around the world. This expansion in the fermentation industry coincided with a switch from maize to molasses as the preferred fermentation substrate. Later reports refer briefly to problems associated with susceptibility to phage infection encountered with CSC's newly developed molasses-fermenting strain patented under the name of *Clostridium saccharoacetobutylicum* NRRL B591 (McCutchan and Hickey, 1954). This strain has now been reclassified as *Clostridium beijerinckii* (Keis *et al*., 1995; Johnson *et al*., 1997).

A series of phage infections were also reported to have affected the AB fermentation process that operated in Japan from the 1940's until the 1960's (Ogata and Hongo, 1979). In 1943 the occurrence of abnormal AB fermentations occurred at several factories operating the process in Japan and the phenomenon was referred to as "sleeping sickness". At the time Japan was engaged in fighting the Second World War and there was a demand for large volumes of solvents for the production of munitions and other war material. Research was undertaken to attempt to overcome these abnormal sluggish fermentations but it was only through the work of Kinoshita and his co-workers in 1951 and 1952, as reported by Ogata

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and Hongo (1979), that the causes of sluggish fermentations were shown to be phage infections. After the war the fermentation process in Japan was switched entirely to the use of molasses as the substrate but fermentations continued to be susceptible to phage attack and sporadic outbreaks continued to occur through the 1950's. In 1959 a number of new strains of solvent-producing clostridia were isolated by Hongo and Nagata which produced high levels of butanol (Hongo *et al.*, 1965a). These clostridial strains were patented under the name *Clostridium saccharoperbutylacetonicum* (Hongo, 1960) and were employed in the industrial fermentation process to produce more favourable butanol ratios. However, during the first year that these new strains were introduced, a series of phage infections occurred that continued to cause problems during the industrial fermentation operation (Hongo *et al.*, 1965b). Conversion to using the *C. saccharoperbutylacetonicum* N1-4 and the N1-504 strains was plagued by 12 phage infections occurring within one year. Three different types of virulent phage were isolated from these factory infections (Hongo and Murata, 1966a; Hongo and Murata, 1966b). Seven variants (HM1, HM2, HM8, HM9, HM10, HM11, and HM12) of a phage with a short tail belonging to the *Podoviridae* were isolated that infected strains belonging to the *C. saccharoperbutylacetonicum* N1-4 subgroup. Four variants (HM3, HM4, HM5, and HM6) of a phage with a contractile-tail belonging to the *Myoviridae* were isolated that also infected strains belonging to the *C. saccharoperbutylacetonicum* N1-4 subgroup. A single variant (HM7) of a larger phage with a long non-contractile tail belonging to the *Siphoviridae* was isolated that infected strains belonging to the *C. saccharoperbutylacetonicum* N1-504 subgroup. A variant of the HM2 phage, designated as HMT that was lysogenic and capable of phage entrapment within spores, was also isolated during the course of these studies (Hongo *et al.*, 1969). The operation of the AB fermentation process was apparently discontinued at the end of 1960 with the problem of phage contamination not fully resolved.

Problems with phage infection were also encountered in an industrial AB fermentation process operated in Puerto Rico in 1943 (McCoy *et al.*, 1944). This infection apparently occurred in a newly established process that utilised a patented strain originally designated as *C. madisonii* 214 (reclassified as *C. beijerinckii*) (Keis *et al.*, 1995) to produce solvents from molasses. McCoy and her co-workers were the first to actually isolate bacteriophages from a contaminated industrial fermentation. Phages isolated from four different infections, designated as A, B, C, and D, were identified but not characterised. Serological studies showed that phages A and B were serologically distinct from phages C and D (McCoy *et al.*, 1944). A series of immunised strains were generated to counter the phage contamination but each new immunised strain tested proved susceptible to a new phage infection. A lysogenic strain designated as *C. madisonii* 4J9 harbouring a large phage was derived from strain 214. The 214 strain had been immunised to phages A, B, and C and was subsequently made resistant to the D phage (Peri, 1948). These phages proved difficult to work with because of the limited technical facilities and rudimentary handling techniques in use at the time.

Solvent-Producing Ability of a Lysogenic Strain in Molasses Fermentations

Cultures of the *C. madisonii* 214 host strain and the 4J9 lysogenic strain were acquired from Professor Palmer Rogers. The 4J9 strain was found to harbour a large lysogenic bacteriophage with an icosahedral head and a long flexible, non-contractile tail belonging to the *Siphoviridae* (Keis, 1992). The genome of this phage is entrapped within the endospores of the host bacterium during spore formation and is capable of withstanding pasteurisation temperatures known to destroy the free phage virions (Wu, 1998). This lysogenic phage has been stably maintained in the host bacterium since it was first isolated in the 1940's.

The phage designated CMX, is capable of infecting a number of industrial strains belonging to the same genomic DNA fingerprint group which includes *C. beijerinckii* NCIMB 8052 and the original *C. madisonii* strain (Keis *et al.*, 1995). The lysogenic 4J9 strain was found to release high titres of the CMX phage spontaneously. In a recent study, the effect that lysogeny had on the performance of the bacterium in molasses fermentation medium was investigated (Wu, 1998). The growth rate of the lysogenic 4J9 strain was found to be considerably slower than the non-lysogenic 214 parent strain from which it was derived as well as the NCIMB 8052 strain. In the various rich culture media that were tested the doubling time on the 4J9 strain was around 42 min compared with 30 min for the non-lysogenic strains. The difference in growth rates was greatly enhanced in chemically-defined minimal media. In addition to the reduction in growth rate, the lysogenic strain also exhibited a reduction in solvent production with total solvent concentrations of around 7.4 g/l being produced on molasses medium containing 5% fermentable sugars that gave solvent yields of 18.6%. The wild type strains produced solvent concentrations ranging between 11.9 to 12.8 g/l and solvent yields ranging between 30 and 32% on the equivalent molasses fermentation medium. A mutant strain from which the CMX phage had been eradicated exhibited a growth rate and solvent production similar to the wild type strains (Wu, 1998).

Phage Infections in the South African AB Fermentation Process

Information regarding the phage infections that occurred in the AB fermentation process operated in South Africa from 1936 until 1982 has never been documented in the scientific literature. The following information was extracted from reports and company records obtained from the company that ran the fermentation process.

A standard batch AB fermentation process was in operation at the National Chemical Products (NCP) factory in Germiston, South Africa for a period of 46 years. During this time four confirmed phage infections were reported and another two suspected but unconfirmed phage infections occurred.

The first confirmed phage infection to be reported occurred during late 1943 and early 1944 while the company was under government contract to supply strategic war materials. At this time the company was operating a maize fermentation process using the Melle strain that had been supplied by the French company that

had built the AB fermentation plant. The Melle strain was reported to have been derived from the original *C. acetobutylicum* strain developed by Weizmann during the First World War. This phage infection was apparently confirmed by filtration test procedures recommended by CSC in the USA. During the remainder of 1944 the South African AB fermentation process was converted to using molasses as the fermentation substrate and new molasses-fermenting strains were supplied to NCP by CSC in the USA. After initial teething problems the new molasses process continued to operate efficiently until late 1947 when problems were encountered with erratic fermentations with substantially reduced solvent yields. The cause of these sluggish fermentations were suspected to be due to a phage infection but this was never confirmed. As a result of the problems that were experienced, the company's research and development section embarked on trials using phage-immunised cultures provided by Commercial Solvents, Great Britain (CS-GB) that had acquired a share holding in the NCP company. At this time, studies were also conducted with a virulent phage designated F3, the origin of which remains obscure. The NCP company continued with full-scale factory trials until the mid 1950's but the phage-immunised strains supplied by CS-GB were never brought into use as production strains by NCP.

Another phage infection occurred in August 1960 when one of the factory fermenters exploded. Shortly after the explosion, sluggish and erratic fermentations were encountered in most of the production fermenters. The presence of a phage with a contractile tail belonging to the *Myoviridae* was confirmed by electron microscopy but was not characterised further. As a result of this phage infection the entire plant was closed for cleaning and disinfection. Further trials were conducted with various phage-immunised strains supplied by CS-GB but the AB fermentation was apparently successfully restarted using the standard NCP production strains that were in use at that time.

The next confirmed phage infection reported at NCP occurred in May 1976. This infection followed a decision to switch from the use of carbon dioxide to unfiltered nitrogen as the process gas utilised during the fermenter filling operation. A number of the fermenters in the plant were affected and exhibited sluggish fermentations with poor solvent yields. Problems were also experienced with starting up fermentations and cell protoplasts were observed in the molasses mash. The presence of two different phages were confirmed by electron microscopy. One of these was a large phage with a flexible non-contractile tail belonging to the *Siphoviridae* and the other was a smaller phage with a contractile tail sheath belonging to the *Myoviridae*. Although attempts were made to propagate these phages in the laboratory, these were not successful. This phage infection resulted in the closure of the entire plant for a period of ten days while cleaning and disinfection was undertaken. Specific steps that were taken to eradicate the phage included destruction of all of the cultures in the culture laboratory, fumigation of the culture laboratory with formalin vapour, disinfection of all fermenter lines and valves with a caustic treatment followed by formalin and steam treatments. Washing and spraying of the fermenter house with quaternary ammonium compounds and cleaning and caustic treatment of drains was also carried out.

A further suspected infection occurred a year later in May 1977. The symptoms that were encountered were a reduction in normal solvent yields and slightly prolonged fermentation times. A subculture of the P261 production strain that was in use at the time was suspected of being lysogenic. Although some evidence to support this was obtained, this was never firmly established. The production strain in use at the time was replaced but the problems with erratic and decreased solvent yields continued well into 1978.

One further confirmed phage infection occurred at the NCP plant in 1980. The symptoms produced by this infection were reduced solvent yields and extended fermentation times. Although solvent concentrations and yields were quite considerably reduced the yields were not catastrophically low. Initial testing of the seed culture stages, pre-fermenters, plant fermenters, and surrounding areas in the fermenter house revealed that phage contamination was widespread. Approximately 60% of the seed culture stages, pre-fermenters, and plant fermenters and around 10% of the samples taken from the surrounding environment tested positive on plate assays. As the symptoms of the infection were less severe than those encountered with previous infections a decision was made to continue to operate the fermentation process while steps were taken to eradicate the infection. All infected cultures were eradicated and replaced by a new phage-free production strain, fermenters and lines were steam cleaned, and improved culture practices and plant hygiene was introduced. Apparently, as a consequence of the steps taken, the fermentation process returned to normal over a period of a few weeks.

Characterisation of the 1980 Phage Infection at NCP

A small phage, designated CA1 belonging to the *Podoviridae* and consisting of an icosahedral head, a collar, and a very short non-contractile tail with 12 tail appendages was identified by electron microscopy (Reid *et al*., 1983). This phage exhibited an almost identical morphology to the HM2 phage isolated from the Japanese infections of *C. saccharoperbutylacetonicum* and described by Ogata *et al.* (1969). This highly stable phage was isolated and propagated in the laboratory and was shown to be able to form a pseudo-lysogenic relationship with its host bacterium which was subsequently identified as belonging to one of the genomic DNA fingerprint groups of *C. beijerinckii* as identified by Keis *et al.* (1995). The DNA of this phage was found to be capable of entrapment within the spores of the host bacterium. Spores containing the entrapped phage DNA were heat-resistant and were capable of withstanding exposure to 90°C for 5 min, known to destroy free phage. Pseudo-lysogenic cultures established by phage entrapment were capable of spontaneously releasing free phage in culture over extended periods.

At the time of the 1980 phage infection, a number of investigations were carried out on the infected factory fermenters by the research and development section at NCP. Recently, a number of additional laboratory-based experiments have been undertaken with the phage CA1 that was isolated from this infection and the NCP production strains that were in use at that time, to further extend these findings.

Table 1. Comparison of Factory Fermentations[a] with Laboratory Fermentations[b]

	Factory Fermentations	Laboratory Fermentations
Solvent concentration (g/l)	11.3-16.3	14.7-20.7
Average solvent concentration (g/l)	14.3	17.4
Solvent yield (%)	18.8-26.3	23.7-33.3
Average solvent yield (%)	23	28
Fermentation time (h)	32-52	-
Average fermentation time (h)	40	-
Normal fermentation time (h)	~32	-

[a]17 of 24 fermentations tested positive for phage by plate assay
[b]inoculated with a phage-free strain

Table 2. Factory Fermentations with Inocula that Tested Positive or Negative for Phage CA1

	Fermentation inocula	
	Negative for phage CA1	Positive for phage CA1
Average solvent concentration (g/l)	14.8	14
Average solvent yield (%)	23.9	22.7
Average fermentation time (h)	38	40

In one of the studies carried out at the time, 24 full production-scale fermentations on molasses containing 6.2% fermentable sugars were inoculated with strain P261. These factory-scale fermentations were compared with their equivalent laboratory-scale control fermentations inoculated with a phage-free strain of P261. Of the 24 fermentations tested, 17 tested positive for the phage by plate assay. The results of this investigation are shown in Table 1. The average solvent concentration of the factory-scale fermentations was 14.3 g/l compared with 17.4 g/l in the laboratory fermentations and the average yield in the factory fermentations was 23% compared with 28% for the laboratory cultures. The fermentation times of the factory fermentations ranged between 32 and 52 h and averaged 40 h. The average fermentation time for the normal full-scale factory fermentations was around 32 h.

In a second investigation 25 full-scale fermentations were monitored. In this trial 13 of the final stage laboratory cultures used to inoculate these fermentations tested positive for the phage while the remaining 12 tested negative. A summary of the results obtained from this trial are presented in Table 2. From these results it can be seen that the average solvent concentration and yields were slightly higher in fermentations that tested negative and the average fermentation times were slightly shorter.

The results of a laboratory-based study on the effects of infection by the CA1 phage are presented in Table 3. In this experiment four laboratory-scale molasses fermentations containing 6% fermentable sugars were inoculated with strain P261 and then infected with a high-titre lysate of phage CA1. The infected cultures were compared with four uninfected control fermentations inoculated with the same strain. The experiment was repeated with the same number of laboratory-scale fermentations inoculated with strain P270. The results for both strains showed a significant decrease in both the yield and the concentration of the solvents produced.

The effect of the multiplicity of infection (m.o.i.) is demonstrated in Table 4. In this laboratory-scale study, strain P260 was inoculated into molasses fermentation medium containing 7.5% fermentable sugars. The cultures were then infected with the CA1 phage at different m.o.i's. From the results obtained it can be seen that a small decrease in the production of solvents occurred as the m.o.i. was increased. In addition to reduced solvent production, the fermentations infected with phage also exhibited a decrease in the ratio of acetone to butanol produced (data not shown).

The results presented in Table 5 illustrate the effect that the timing of phage inoculation had on the subsequent course of the fermentation. In this experiment laboratory-scale molasses fermentations containing 7.4% fermentable sugars inoculated with strain P260 were infected with phage at 2, 5, and 8 h after the start of the fermentation. The fermentations infected early on exhibited a significant decrease in solvent production whereas the fermentation infected after 8 h showed no discernible effect. In these trials the phage-infected fermentations that exhibited a decrease in solvent production also showed a significant decrease in the ratio of acetone to butanol produced.

A number of observations can be made from the 1980 phage infection at NCP. The typical effect obtained when the CA1 phage infects cells growing in an industrial molasses fermentation medium, were a decrease in solvent production with a corresponding decrease in the acetone to butanol ratio, and slightly extended fermentation times. In no instance were more dramatic inhibitory effects observed. These effects differ significantly from those observed in standard laboratory media where complete lysis of the culture can be obtained with high m.o.i's of the CA1 phage. One can speculate that these differences may be related to the different growth conditions, the higher sugar concentration, and the greater viscosity that is

Table 3. Effect of Infection by Phage CA1 on Strains P261 and P270

	P261		P270	
	Uninfected	Infected	Uninfected	Infected
Solvent concentration (g/l)	15.5-16.2	3.7-4.0	16.5-18.0	7.1-8.0
Average solvent concentration (g/l)	15.7	3.8	17.2	7.6
Solvent yield (%)	25.8-27.0	6.2-6.6	27.5-30.0	11.8-13.3
Average solvent yield (%)	26.2	6.3	28.6	12.6

Table 4. Effect of Multiplicity of Infection (m.o.i.) of Phage CA1

	Control	Multiplicity of infection		
	(uninfected)	0.1	1.0	10.0
Solvent concentration (g/l)	23.5	16.7	16.7	15.8
Solvent yield (%)	31.3	22.3	22.3	21.1
Acetone ratio (%)	37	32	33	33

Table 5. Effect of Time of Infection of Phage CA1

	Control	Infected after		
	(uninfected)	2h	5h	8h
Solvent concentration (g/l)	18.2	13.1	16.1	18.6
Solvent yield (%)	24.3	17.5	21.6	24.8
Acetone ratio (%)	41	18	28	38

encountered in molasses medium. It is also possible that the ability of the CA1 phage to enter into a pseudo-lysogenic relationship with the host bacterium could also influence the nature and symptoms of the phage infection. This pseudo-lysogenic state created difficulties in attempting to eradicate infected strains from laboratory and culture facilities. The CA1 phage also appears to only produce significant symptoms when it is present in relatively high numbers during the early stage of the fermentation. The actual cause of this phage infection was never established but the evidence points to the contamination having occurred during the inoculum build-up stages in the culture laboratory. It also seems likely that, although a significant proportion of the factory fermenters tested negative by plate assay at the time, low levels of phage might have been present in some or all of these fermenters.

Symptoms and Diagnosis of Phage Infections in the AB Fermentation

As far as can be ascertained the occurrence of phages capable of infecting industrial solvent-producing strains of *Clostridium* used in the industrial AB fermentation process appear to have been quite strain specific (Beesch, 1952; Ogata and Hongo, 1979). Of the seven phages screened by Keis *et al.* (1995) all had restricted host ranges and were confined to infecting strains belonging to a single genomic DNA fingerprint group.

Although there is only limited information relating to specific phage infections in the scientific literature, there are numerous references to the general effects of phage infections in the AB fermentation in reviews and scientific papers published up until the 1970's (Beesch, 1952; McCutchan and Hickey, 1954; Ryden, 1958; Hastings, 1971; Ogata and Hongo, 1979; Walton and Martin, 1979). These papers refer to common symptoms associated with phage infections in the industrial AB fermentation process. Symptoms appear to have been similar, regardless of whether the infections occurred in maize or molasses fermentations. Typical symptoms included slow or sluggish fermentations with extended fermentation times and reduced solvent yields. A marked reduction in gas evolution was often recorded and the fermentation mash typically remained dark in colour unlike normal fermentations where the substrate becomes progressively lighter during batch fermentation. High acid levels and unused sugars in the fermentation broth were also typically encountered. A number of reports refer to the fact that stalled fermentations often started again after 24 to 48 h with these protracted fermentations giving only moderate to poor solvent yields. Changes in the cell population and morphology are also usually apparent. These may typically include a marked reduction in cell numbers, a loss of motility, and the presence of etched or moth-eaten cells. In some cases, elongated cells or protoplasts may be observed.

Symptoms associated with low levels of contamination by a phage can often prove difficult to diagnose. Typically, fermentation times are extended and some decrease in yield is observed. With heavy phage contamination, fermentations often stop completely and the majority of cells may lyse within hours. The effects that might be observed in any specific phage infection are dependent on a number of factors. These include type of phage involved, the stage of the fermentation at which the infection occurs, the number of phage particles relative to the number of cells, the composition of the fermentation substrate, and the physical and chemical conditions in the fermenters. Infection with the same phage need not necessarily always produce the same symptoms.

Infections that occur during the build-up of the seed cultures are likely to cause widespread infection in all fermenters. Contamination of starter cultures can also cause difficulties in starting up industrial-scale fermentation processes. Phage infections occurring at later stages in the fermentation can have less obvious and widespread effects. Contamination by virulent phages with a short latent period and large burst size can produce very rapid and severe symptoms, however, these may be attenuated to some extent by the properties of the industrial substrates used. Lysogenic and pseudo-lysogenic phages can on the other hand often produce less obvious but more protracted symptoms and can be extremely difficult to eradicate from infected fermentation plants.

Before the development of more sophisticated techniques the diagnosis of phage infections in the industrial AB fermentation was assessed primarily by the ability of the infective particle to pass through a standard bacteriological filter while retaining activity. With the development of liquid and plate phage assay techniques, the transmission of phage activity to uninfected cultures and plaque transfer on bacterial lawns became standard practice with confirmation obtained by electron microscopy.

The Control of Phage Infections in the AB Fermentation

The standard batch AB fermentations were run as mono-cultures under sterile conditions. This required the sterilisation of the fermentation substrate, water, and any make-up gas. The design of the plant was important, minimising opportunities for contamination to occur with effective procedures for steam sterilisation of the fermenters and lines, coupled with facilities for acid or alkali treatment being important. In addition, standard prevention strategies that included good factory hygiene were widely practiced. When required common liquid and gaseous disinfectants were used in the plant to control

contamination. The standard practice when a phage infection did occur was to attempt to eliminate all of the contaminated material, followed by cleaning and sterilisation. This usually necessitated the closure of the whole plant while the decontamination process was undertaken.

A number of approaches to treating phage-infected fermentation broths have been reported in the literature (Ogata and Hongo, 1979). These consisted of chemical treatment that included the addition of chelating agents to remove divalent cations known to be essential for infection with some phages, and the use of non-ionic detergents and antibiotics. However, no effective means of salvaging phage-contaminated fermentation broths were ever developed.

The most successful and widespread approach to overcoming the effects of a phage infection was to maintain a battery of strains or species that were resistant to a range of different phages (Beesch, 1952; McCutchan and Hickey, 1954; Ryden, 1958; Hastings, 1971; Walton and Martin, 1979). The selection and isolation of phage-resistant mutants or variants that retained solvent-producing capabilities was referred to as phage immunisation. The process of producing immunised strains was normally achieved through serial transfer of surviving cells. Usually between 10 and 18 selection cycles were used with exposure of spores to 90-100°C for 3 to 5 min. In a process patented by McCoy the number of selection cycles required was shortened by growing the cells in a tryptone, liver extract glucose culture medium that produced high titres of phage (McCoy, 1946). It proved possible to immunise a single strain against a number of different phages but in most cases immunised strains did not produced solvent yields equivalent to those obtained with the original parent strain. In some cases immunised strains exhibited increased ratios of acetone to butanol (Beesch, 1952). One danger of producing resistant strains was the possibility of generating lysogenic cultures. Such lysogens created a potential problem due to the release of phage particles and therefore continued to cause phage infections (Ryden, 1958).

Based on observations from the more than 60 years that the AB fermentation was operated in the Western World it would seem that no matter how good the plant practice and hygiene, phage infections were bound to occur from time to time. It was therefore important that companies operating the fermentation process developed strategies to minimise the impact of such infections. It can be predicted that the ubiquitous and troublesome occurrence of phage contamination could have potentially serious implications for the operation of industrial-scale continuous AB fermentation processes that are run for protracted periods of time.

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