MINIREVIEW

Beijerinck and the bioluminescent bacteria: microbiological experiments in the late 19th and early 20th centuries

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Abstract

Microbiological research in the days before specialized equipment, or even electricity, required a great deal of ingenuity. The revival of 90-year-old bioluminescent bacteria from Beijerinck's laboratory in Delft prompted a review of his work with these microorganisms and revealed their use in simple techniques for the investigation of, among other things, sugar metabolism in yeasts, oxygen generation and uptake and even the survival of microorganisms in liquid hydrogen. He used variant strains of bioluminescent bacteria in an attempt to study heredity and variation in biological systems and described one of the earliest examples of enzyme induction.

Introduction

In January 1888, Mr Enklaar from Deventer brought Martinus Beijerinck a piece of salt pork that glowed in the dark. According to Beijerinck's laboratory journal for 12 January, he found that the flesh, but not the fat, produced light, with some areas being brighter than others. Upon investigation, Beijerinck found a mixture of bacteria that he described as predominantly diplococci (Fig. 1). He tried to isolate the light-producing species, but gave up when none of the three types of colony isolated from the pork generated light. This failure may have been due to the composition of the medium in his plates, which contained pork, gelatine, peptone and sodium carbonate, but no sodium chloride. Clearly, his interest was caught because on 16 January, Beijerinck placed a piece of plaice on an open plate in his cellar. By the 22 of January, the fish was glowing, and he was able to isolate light-producing bacteria using a medium based on fish and sea water.

Who was Martinus Willem Beijerinck?

In 1888, Beijerinck (1851–1931) was the head of the first Dutch industrial microbiology laboratory at the Nederlands Gist en Spiritus Fabriek (NGSF, Netherlands Yeast and Alcohol Factory) in Delft. At the time of his appointment, Beijerinck was a botanist, having written his doctoral thesis on plant galls. His range of interests was so broad (see Table 1) that he was unhappy within the constraints of an industrial setting. Jacques van Marken, the owner of NGSF, used his considerable influence to persuade both the Government and what was then the Delft Polytechnic to appoint Beijerinck as Professor of Microbiology in the Netherlands' first microbiology laboratory not dedicated to medical work. Martinus W. Beijerinck is regarded by many as one of the founding fathers of modern, nonmedical microbiology. He is credited with the first descriptions of a wide range of microorganisms, and was the first to demonstrate that the causative agent of Tobacco Mosaic Disease was a self-replicating filterable particle that he called a 'virus' (Beijerinck, 1898). There have been a number of biographies including van Iterson *et al.* (1940) and Robertson (2003).

When he retired, Beijerinck continued research in a small laboratory in the garden of his house in Gorssel, financed at least in part by Dr Waller of NGSF. Many of the early letters between Beijerinck and his successor, Albert Jan Kluyver,

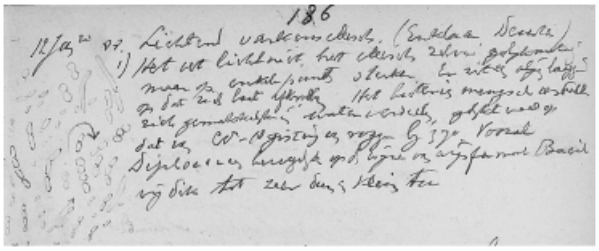

Fig. 1. Extract from Beijerinck's laboratory journal number 6, page 186, showing the drawing that accompanied the first entry describing light production by bacteria. Loosely translated, Beijerinck said that there was light on the flesh, but not the fat, that the bacteria were easily scraped off and suspended in water and that he could see mostly diplococci that moved like *Bacilli*, ranging from large to very small.

Table 1. Topics of publications that appeared during Beijerinck's time in industry (from Robertson, 2003; see volumes 1–3 of Beijerinck's collected publications)

Sunsets (Were the spectacular sunsets of the time due to dust from Krakatoa?)
Root nodules and their bacteria
Plant galls
Grasses, carrots, gardenias, barley
Algae, protozoa in drinking water, hydrogen peroxide in living organisms
Fermentation, butanol fermentation, *Saccharomyces* associated with beer, *Schizosaccharomyces octosporus*
Lactase, maltase, blue cheese bacteria, kefir
Photobacteria, sulphate reduction
Methods: auxanograms, gelatine plates, Chamberland filters, sampling stratified cultures, microbiochemical analysis

concern items that Beijerinck either forgot to take to his retirement home or took from Delft by mistake. These letters also show that Kluyver periodically sent Beijerinck chemicals and biological samples (e.g. kefyr samples in March, 1922, and activated sludge in 1924) and Beijerinck sent cultures to be deposited in the Delft Collection. After Beijerinck's death, his sister Henriëtte returned material including laboratory equipment, laboratory journals, manuscripts and books to the Microbiology Laboratory in Delft, on the instructions of her brother. This material is now preserved in a small museum within the Department of Biotechnology at Delft University of Technology. The papers, together with those of Professors Kluyver and van Iterson, make up the Archives of the Delft School of Microbiology. Items from the collection can be seen on the museum's website http://www.beijerinck.bt.tudelft.nl.

The recent revival of some of his '*Photobacterium splendidum*' cultures (M.J. Figge, L.A. Robertson, J.C. Ast & P.V. Dunlap, unpublished data) prompted a review of Beijerinck's work on and with bioluminescent bacteria, as described in his publications and laboratory journals. This article uses a survey of Beijerinck's experiments involving bioluminescent bacteria to illustrate biological research at the end of the 19th and beginning of the 20th centuries. In these days of complex and expensive equipment, it is illuminating to see what can be achieved by experiments of great simplicity, careful design, using little equipment and (for much of Beijerinck's career) no electricity.

The discovery of bioluminescence

To set Beijerinck's research in context, the history of bacterial bioluminescence before his research will be briefly reviewed here. For more detail, the reader is referred to Harvey (1957). A 21st-century perspective has been published recently by Haddock *et al.* (2010).

René Antoine Ferchault de Réaumur's description of light-producing slime from a mussel in 1734 was probably the earliest report of bacterial light production (de Réaumur, 1723). In 1821, John Murray (Murray, 1821a, b) speculated that the light given off by dead whiting and mackerel was due to 'parasitic, luminous animiculae, the evolution of light being the effect of the slight increment of temperature produced by the commencement of animal decomposition'. Light production on a human corpse was described, together with the fact that smearing materials scraped from the luminescent area onto another corpse caused the second corpse to glow (Cooper & Cooper, 1838). Under the microscope, the authors observed small globules that they described as 'oily matter' from 'a peculiar state of decomposition'. Heller (1853) attributed the light produced on a range of materials including fish, sausage and human corpses to a 'fungus or plant to which I have given the name *Sarcina noctiluca*' (because this organism was never isolated or identified, it should not be confused with the modern genus of the same name). Pflüger (1875a, b) showed that there were huge numbers of bacteria in the luminescent slime from fish, that a bacteria-free filtrate was also free of luminescence and that he could transfer the luminescence from a marine fish to shellfish and freshwater fish, thereby confirming the link between the bacteria and the light. Beijerinck was certainly aware of this work by 1887, when he attended a lecture on the subject given by J. Forster, Professor of Hygiene at the University of Amsterdam.

Early taxonomy

After Pflüger's publications, researchers began naming various different luminous bacteria including *Micrococcus phosphorescens* (Cohn, 1878), *Micrococcus pflügeri* (Ludwig, 1884) and '*Bacillus phosphorescens*' (Fischer, 1887, 1888). Beijerinck began describing various species of *Photobacterium* from 1889 (Table 2). Bacterial taxonomy before Kluyver & van Niel's (1936) work on combining physiological and morphological characteristics was simple and relied heavily on morphological characteristics combined with a very small set of physiological characteristics (e.g. growth on

Table 2. The names used by M.W. Beijerinck for the various bioluminescent bacteria mentioned in his publications, and their most recently used equivalents

Beijerinck's name	Most recent name
Photobacterium splendidum (Beijerinck, 1900c, 1901b, 1916)	*Vibrio splendidus* [AL] (Baumann *et al*., 1980; Spencer, 1955) (for the van Zutphen isolate)
Photobacterium phosphorescens (Beijerinck, 1889a, b, c, 1891a, b, 1901b, 1916; Beijerinck & Jacobsen, 1908)	*Photobacterium phosphoreum* [AL] (Skerman *et al*., 1980)
Photobacterium indicum (Beijerinck, 1889a, b, 1891b, 1900a, 1901b, 1916; Beijerinck & Jacobsen, 1908) (not to be confused with *P. indicum* Johnson & Weisrock, a *Hyphomicrobium*-like species (Ivanova *et al*., 2004))	*Vibrio indicus* (Breed, 1957a)
Photobacterium luminosum (Beijerinck, 1889a, b, 1891b, 1900a, 1901b, 1916)	*Vibrio luminosus* (Breed, 1957a)
Photobacterium splendor maris (Beijerinck, 1900a, 1901b, 1916)	Probably *Photobacterium indicum* (Johnston, 1948)
Photobacterium fischeri (Beijerinck, 1889a, 1891b, 1901b, 1916)	*Aliivibrio fischeri* [AL] (Ast *et al*., 2009; Urbanczyk *et al*., 2007)
Photobacterium degenerans Fischer (Beijerinck, 1900a, 1901b)	*Aliivibrio fischeri* [AL] (Ast *et al*., 2009; Urbanczyk *et al*., 2007)
Photobacterium hollandiae/ hollandicum (Beijerinck, 1900a, 1916)	Probably *Photobacterium luminosum* (Johnston, 1948)
Photobacterium phosphoreum (Beijerinck, 1916, 1917)	*Photobacterium phosphoreum* [T, AL] (Skerman *et al*., 1980)
Photobacterium hollandicum parvum (Beijerinck, 1916)	Probably *Photobacterium luminosum* (Johnston, 1948)
Photobacterium tuberculatum (Beijerinck, 1916) (Fischer called it *Photobacterium tuberosum*)	*Photobacterium tuberosum* (Johnston, 1948)
Photobacterium pflügeri (Beijerinck, 1900c)	*Photobacterium phosphoreum* (Johnston, 1948)
Photobacterium fischeri baltica (Beijerinck, 1891b) or *P. balticum*)	Probably *Photobacterium fischeri* (Johnston, 1948)

Those marked AL are on the current Approved List of Prokaryotic Names with Standing in Nomenclature (most easily to be found at http://www.bacterio.cict.fr/). T indicates the type species for the genus.

potato, gelatine liquifaction). Matters were further confused when Chester's (1901) Manual stated that Cohn's and Fischer's species were the same as Beijerinck's *Photobacterium phosphorescens* and simply called all three of them *Bacterium phosphorescens*. Similarly, Ludwig's species and Beijerinck's *Photobacterium pflügeri* were listed together as *Bacterium pflügeri*. Other luminous bacteria were listed under *Microspira*. Beijerinck's attempt to simplify the situation by naming all bioluminescent bacteria *Photobacter* or *Photobacterium* (he used the two names interchangeably) was never fully accepted [see, e.g., the range of bioluminescent species placed in the genera *Bacterium*, *Pseudomonas* and *Vibrio*, among others, in the sixth and seventh editions of Bergey's Manual (Breed, 1948a, b, 1957b)].

Beijerinck's research with bioluminescent bacteria

In addition to research into the distribution, physiology and light production of bioluminescent bacteria (Beijerinck, 1889a, 1891a, b), Beijerinck used the production of light as a simple detector in other metabolic studies. He had a set of microorganisms including yeasts, algae, fungal spores and *Lactococcus* sp., each of which had a specific, easily tested feature (e.g. light production by the bioluminescent bacteria, lactic acid production by the *Lactococcus* sp.), which he used in different combinations, depending on the aim of the experiment. Their use can be seen in many cases as a precursor of modern tests for metabolic activity using fluorescence microscopy, oxygen uptake measurements and other analyses.

Over the course of his career, he isolated a number of different luminescent species and began work on understanding the biochemical nature of bacterial light production. The light-producing bacteria have since been reorganized and renamed several times (e.g. Breed & Lessel, 1954; Spencer, 1955; Reichelt & Baumann, 1973; Dunlap, 2009), and the various species are now distributed over several genera. Some of the species described in Beijerinck's papers (Beijerinck, 1889b, 1890, 1916) on the subject seem to have been lost. For consistency in reviewing his work, and to allow the reader to refer back to the original papers, Beijerinck's names for the various strains and species have been retained in this review. Table 2 shows the names used most recently for the various species. As many of these bacteria are no longer available, inclusion in this table does not imply that a name is currently valid, merely that it is the last known name of that particular 'species' or strain. As will be seen in the discussion about the revival of Beijrinck's *P. splendidum* and the fact that it is not the same as the current type species of *Vibrio splendidus*, if the original strains are not available for comparison, it should not always be assumed that historic cultures are the same as modern strains bearing the same name.

Research into the bioluminescent bacteria

As mentioned above, Beijerinck failed to isolate the bacteria causing salt pork to glow, but on 22 January 1888, he

recorded light production on the plaice that he had left on a plate in his cellar for a week. Judging by the accompanying drawing, this light was due to short rods that often appeared in pairs (Fig. 1). On 30 May, he recorded that he had a culture of *Bac. phosphorescens* growing on fishwater-gelatine plates. This was probably his own isolate, as it was not until the end of the month that he referred to '*Bac. phosphorescens* from Fischer' in his laboratory journal. Thereafter, he described work with his own and Fischer's cultures, referring to the latter as 'West Indian', and then as '*Photobacterium indicum*'. Beijerinck then used his auxanographic technique in a series of experiments to discover the conditions or the chemicals that increased or decreased light production. The auxanographic technique involved mixing growing luminous bacteria with 'fish gelatine,' which was allowed to set in Petri dishes. They were then incubated until their light production began to diminish. Drops of whatever was to be tested were then placed on the surface of the gelatine, and the light production was monitored. Figure 2 shows a typical journal entry, and Table 3 gives a translated transcription. Curiously, Beijerinck recorded that adding fungi, bacteria and yeast stimulated light production. However, while '*coli*' stimulated light production, the bacteria from the root nodules of *Vicia faba* did not. As he obtained other isolates, the experiments were extended to include them and he divided the strains into two groups, depending on their nutritional requirements. He also recorded that some substrates yielded an instant response (e.g. glucose). Others (e.g. aspargine; Fig. 2) required hours for the effect to develop.

An analysis of the metabolism behind light production and the growth of *P. pflügeri*, *P. phosphorescens*, *Photobacterium fischeri* and *P. fischeri* var. *baltica*, *P. indicum* and *Photobacterium luminosum* was published in 1890. After an extensive analysis of substrates and environmental factors influencing the bacteria, especially *P. phosphorescens*, *P. indicum* and *P. luminosum*, he speculated about the biological significance of light production and postulated that there might be different reasons for light production. He spent some time investigating the possibility that symbiosis was involved by trying to isolate light-producing bacteria from various bioluminescent invertebrates. He succeeded in only one case, with a jellyfish that had been washed ashore, but concluded that the bacteria were probably feeding off the jellyfish. He also reasoned that symbionts would lose the ability to live freely and could therefore not be the cause of the light produced in seawater and on beaches. Despite the fact that he had shown, in the same paper, the link between respiration and light production, Beijerinck said that it appeared that bacterial light production was likely to be caused accidentally by chemical reactions during metabolism (he suggests similarity to the luminescent chemical lophine; 2,4,5-triphenyl-1H-imidazole, described by Radziszewski, 1877), with no reason to think that the light production was necessary to the bacteria. His final argument to support this hypothesis was the fact that dark forms of the bacteria frequently grew better than the light-producing strains (Beijerinck, 1890). However, he also said that there could be different reasons, depending on the organisms involved.

By August, he had already recorded that some cultures had stopped producing light, a phenomenon he was to spend a great deal of time studying, and that would influence his views on inheritance.

Table 3. The results of an experiment on 13 June to test the effect of various materials on *B. phosphorescens* cultures that had been strongly producing light on 6 June.

No effect	Light level increased	Light level reduced
Cane sugar	Asparagine – after 12 h, strong and long†	Ammonium‡
Ammonium nitrate	Maltose – after 12 h, strong and long†	Tartrate‡
Illegible	Glucose – strong quickly, and short†	
Oil	Glycerine – strong after 12 h	
Inositol	Coli	
Root nodule bacteria	Fungi, yeast, bacteria, strong flour, illegible	
Lactic acid		

*Units not given.
†This presumably refers to the length of time that the culture produced light.
‡This could also be read as ammonium tartrate.
They were growing in fish-gelatine with 1/2* peptone and 3 NaCl (see Fig. 2)

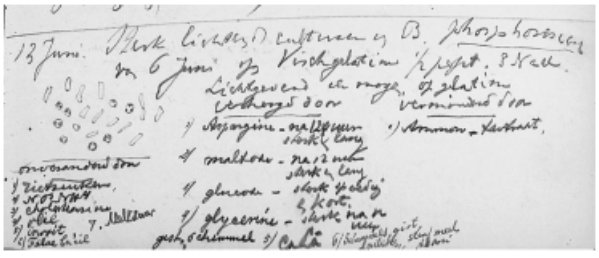

Fig. 2. Extract from Beijerinck's laboratory journal number 6, page 244, showing one of the earliest physiological experiments. For ease of reading, the translation of this tabular entry is given as Table 3.

Heredity

In 1891, Beijerinck (1891a) drew a parallel between the fact that the pathogens that cause diseases such as cholera and typhus lose their virulence after a time and the cessation of

light production by *P. phosphorescens*. He returned to the study of this loss of light repeatedly, even calling dark strains 'degenerans' (Beijerinck, 1900a), in an attempt to understand the inheritance of physiological features.

At the start of the 20th century, long before the discovery of the role of DNA, the mechanisms underlying biological development and inheritance were a matter of great discussion. From Beijerinck's early studies on plant hybridization (Beijerinck, 1884a, b; Zeven, 1970) and galls (Beijerinck, 1877) to his seminal work on nitrogen-fixing root nodules (Beijerinck, 1888), Tobacco mosaic virus (TMV) (Beijerinck, 1898, 1900b) and other microorganisms [Beijerinck (1921–1940) collected works], development and inheritance can be seen as linking themes in his research. The bioluminescent bacteria, with their apparently easy and usually irreversible loss of light production, provided rich material for his studies in this field [for a review, see Theunissen (1996)]. Beijerinck spent a great deal of time trying to understand why some bacteria lost a particular property (in this case bioluminescence) and then gradually lost viability (e.g. *Photobacterium degenerans*, Beijerinck's name for the dark form of *P. fischeri*), while others would grow more rapidly, having lost the ability to produce light (e.g. *Photobacterium luminescens*). The first type of behaviour he called 'degeneration' and the second 'transformation'. Beijerinck also recognized a third type of behaviour, which he called 'variation,' in which most of the culture did not change, but periodically gave rise to a strain that was slightly different. Among the organisms used to illustrate this phenomenon was *P. indicum*, because he had been culturing it constantly since 1887 without loss of light. However, he occasionally found colonies that gave less or no light, and others that grew poorly. In the style of the time, he named these variants *P. indicum* var. *obscurum* and var. *parvum*, respectively, and made the point that most (but not all) could revert to the wild type (Beijerinck, 1900a).

In 1912, in an echo of Charles Darwin, Beijerinck (1912) wrote that growth and variability were linked, and that environmental factors, especially nutrition, could give rise to variants and mutants. He returned to this theme in 1916 (Beijerinck, 1916), this time including *Photobacterium phosphoreum* Cohn (apparently considered to be different from *P. phosphorescens* Fischer), *Photobacterium hollandicum* and *P. splendidum*. He showed that temperature and dissolved oxygen concentration could act as selective pressures, and discussed how they and nutritional factors influenced mutation in *P. splendidum*. Ultimately, he came to the conclusion that enzyme activity lay behind heredity (Beijerinck, 1917), a conclusion also reached by others. Despite the fact that he had a copy of Gregor Mendel's original paper (Vorzimmer, 1968), he does not seem to have tried to apply Mendel's results with peas to his own microbial cultures.

Oxygen and light production

In 1889b, Beijerinck described experiments in which he used H_2O_2 and sodium dithionite to increase and decrease, respectively, the levels of dissolved oxygen in bioluminescent cultures. He used indigo-carmine as a redox indicator. In this way, he was able to demonstrate that light was not produced unless dissolved oxygen was present.

Research using bioluminescent bacteria

As mentioned above, Beijerinck used a small battery of different bacteria (including *P. phosphorescens*), yeasts, fungi and algae in experiments to study unrelated phenomena. After January 1899, *P. splendidum* was added to this battery. Some of these experiments contributed to our fundamental understanding of biology or the development of modern techniques, and their use illustrates the simplicity and effectiveness of Beijerinck's experimental design.

Oxygen generation during photosynthesis

In 1772, Joseph Priestly sealed a green shoot of a plant and a burning candle in a glass container. By leaving the container in the light for some time, he showed that something was produced that allowed the candle to burn. In the dark, the candle went out. Returning the container to the light allowed the candle to be relit. Also, mice placed in a container with a plant died when the container was placed in the dark, but survived if the container was illuminated (Priestley, 1773). Jean Sennebier, in 1782, showed that only green plants produced the gas. Etiolated leaves and flowers did not. Carbonic acid was implicated in the reaction (described in Bay, 1931). By 1881, chlorophyll had been named and chloroplasts had been recognized (von Mohl, 1855). Then, in 1882, Engelmann showed that *Proteus* sp. moved towards and accumulated in clumps around illuminated chloroplasts in a strand of *Spyrogyra*. He suggested that the bacteria were moving in response to oxygen production by the chloroplasts in the cell.

In 1901, Beijerinck (1901a) set up three sets of experiments using luminous bacteria to study the production of oxygen during photosynthesis, using their oxygen requirement for light production as his detector.

(1) He tested (and confirmed) Englemann's suggestion by grinding up plant material (e.g. white clover), mixing the paste with distilled water and then filtering the suspension to remove whole cells. He then mixed the green filtrate with a suspension of bioluminescent bacteria in fish broth and sealed the culture bottle. If the bottle was placed in the dark, the light disappeared. If it was then illuminated, oxygen production was sufficient to support bioluminescence for a time, even when darkness was restored to allow the bacterial light production to be seen. He then went on to show that

the filtrate was not enough to 'decompose carbonic acid', and that intact cells were needed.

(**2**) In the second set of experiments, Beijerinck embedded leaves from different seaweeds in fish bouillon gelatine containing a suspension of bioluminescent bacteria and sandwiched the preparation between two sheets of glass. Different plants were activated by light from different parts of the spectrum, producing enough oxygen to allow bioluminescence. Thus, if *Ulva lactuca*, a green seaweed of the *Chlorophyta*, was used, red light worked. Orange light was needed for the red *Porphyra vulgaris*, a member of the *Rhodophyta*.

(**3**) In the final series, Beijerinck used his bioluminescent gels with glass plates to demonstrate the association of the plant stomata with oxygen production. Instead of embedding the leaves in the gel, he laid them on the surface, directly under the glass plate. By selecting leaves with stomata on only one surface, he was able to show that light (and therefore oxygen) production only occurred when the side with stomata was in contact with the gel containing the bioluminescent bacteria.

Effect of freezing biomass with liquid hydrogen

In 1908, Beijerinck & Jacobsen included cultures of *P. phosphorescens* and *P. indicum* in their battery of microorganisms to be used for testing the effect of extreme cold. They appear to have had two aims – to find a way to preserve cultures and to test the possibility of the suggestions of Thomson (1871) and Helmholtz (1876) that viable microorganisms could fall to earth in meteorites.

Cultures were grown in appropriate media and then aliquots in small vials were frozen in liquid H_2 for 15 or 45 min, or 10 or more hours, before being examined. As might be expected, the results varied with the organism. *Photobacterium phosphorescens*, *Lactococcus* sp. and the yeast and fungal spores were not affected by several days of freezing, but *P. indicum* gave less light. The algae and most of the vegetative yeast were dead. Beijerinck concluded that storage at such low temperatures was suitable for some, but not all species, and that microorganisms could indeed survive freezing in meteorites, suggesting that cosmic panspermia cannot be ruled out. This idea gained fresh support in the 20th and 21st centuries (e.g. Hoyle & Wickramasinghe, 1999; Wainwright, 2003). Of course, we now know that protectants such as dimethyl sulphide and glycerol can extend the survival of microorganisms at very low temperatures, and freezing in safer liquid gases such as N_2 has become routine.

Biochemical studies

(**1**) *Photobacterium phosphorescens* cannot use lactose, but can grow and produce light on glucose and galactose. By adding lactose to a mixture of fish bouillon and *P. phosphorescens* cells in gelatine, Beijerinck used this observation to study the behaviour of *Saccharomyces kefyr* (now known as *Kluyveromyces marxianus* var. *marxianus*) and *Saccharomyces tyrocola*, yeasts that he had isolated from kefyr grains (Beijerinck, 1889c). The gelatine plates were allowed to grow for a few days, until the light intensity began to decline because of nutrient limitation. At this point, yeast suspension was added to part of the surface of the gelatine. Within a few hours, the light intensity around the colonies had strongly increased. Beijerinck concluded that lactose fermentation was preceded by hydrolysis of the disaccharides to monosaccharides and that this occurred outside the cells, thereby making the sugars available to the bioluminescent bacteria. He then used the same method with a cell-free filtrate from *S. kefyr* cultures instead of live cells to confirm that the enzyme was excreted. Finally, he showed that an ethanol precipitate from the filtrate was also able to hydrolyze sucrose (Beijerinck, 1889d). This precipitate has been preserved in the small museum in the Department of Biotechnology at Delft University of Technology. The off-white powder, in a small bottle labelled 'Lactase, 4 December 1899,' has been investigated and found to contain active enzyme (Rouwenhorst *et al.*, 1989).

(**2**) *Urease*. Some bioluminescent bacteria can use urea, while others cannot. Beijerinck (1901b, 1902) used *P. luminosum*, *P. indicum*, *P. splendidum* and *Photobacterium splendor maris*, all of which can use urea, and *P. phosphorescens* and *P. fischeri*, which cannot, as part of a study to demonstrate the presence and activity of urease. He showed that the bacteria, if not grown in the presence of urea, could not use it immediately. As they required different amounts of time to begin growing, he correctly surmised that the time was needed to produce the enzyme. This seems to be one of the earliest records of enzyme induction.

Photobacterium splendidum

This story began with the revival of cultures of *P. splendidum* from the 1920s and it is therefore worth taking a closer look at their history in case other researchers are encouraged to attempt the revival of similarly old cultures. Conclusively establishing their provenance was one of the most difficult points. In 1998, the Netherlands Culture Collection of Bacteria (NCCB) was formed by the merger of the Delft University of Technology Bacterial Collection (founded by M.W. Beijerinck) with that of Phabagen in Utrecht. The NCCB still includes a number of cultures in sealed glass tubes originating from before World War II. Among them were tubes labelled *P. splendidum* and *P. phosphorescens*, dating from 1924 and 1925. Because they lack the ability to form spores or cysts, it was generally assumed that Gram-negative microorganisms could not be starved for > 3 months and remain viable. It therefore seemed unlikely that

these old cultures would still be alive, but the decision was taken to confirm that they were dead. Three tubes from different dates were selected, together with a fourth one, which contained a culture which had been opened, grown and sealed into a new tube in 1979. All four eventually grew. The successful culture of these strains, together with subsequent phylogenetic studies, will be described elsewhere (M.J. Figge, L.A. Robertson, J.C. Ast & P.V. Dunlap, submitted for publication).

The first record of *P. splendidum* in Beijerinck's laboratory journals is on an undated page between pages labelled 'end January 1899' and '8 May 1899' and says 'splendidum at 37 °C grew well but no light'. Since the isolate was named, he had clearly had it for some time. In a 1929 letter to A.J. Kluyver, he commented that *P. splendidum* was only ever found in seawater between August and mid-October when the sea was warmest; hence, it could not have been isolated any later than the summer of 1898. It is possible that it was one of three isolates obtained from sea water off Wassenaar on 3 July 1897, one of which was named as *P. luminosum*. The other two isolates are mentioned without names in the laboratory journal from that date. Other isolations that year were made from the water off Scheveningen.

Photobacterium splendidum is mentioned for the last time in one of the laboratory journals at the end of November 1920 when Beijerinck was comparing the effect of different sugars on the level of light production. However, it is clear from his correspondence with Kluyver in 1929 that Beijerinck was still working with light-producing bacteria at that time, although the experimental records have not survived. Beijerinck wrote that his former assistant, den Dooren de Jong, had sent him some *P. splendidum* cultures that had 'degenerated' some years before, and then continued:

> 'Should you ever have a student who is interested in this, may I then ask you to have him isolate *splendidum* again, preferably at different times between July and September. He can then get to know the *fischeri* (from fish) and the *luminosum*. There are many varieties on our coast – mostly small, weakly liquefying ('liquifying' refers to gelatine liquification) varieties, sometimes strongly liquefying, strongly luminescent with green (and not white or blue) or yellowish light; very common in fish from the Baltic, also with non-liquefying varieties and with colonies that smell strongly of aniseed. *Luminosum* is by far the commonest but worthless.'

It is not clear what subsequently happened to the cultures. According to the old records of the Delft Collection, *P. splendidum* was first deposited in the Collection in 1922, only months after Beijerinck left Delft. Beijerinck's description of his preparation of cultures for storage is very simple. For cultures of luminous bacteria, he prepared 'fish water' by boiling fish (type and quantity not specified) in 'clean sea water', adding 10% chalk and then distributing this mixture into culture tubes. After inoculation, the bacteria were grown overnight in these tubes, which were then heat-sealed and stored in the collection. This description precisely fits the cultures that have been revived. They date from 1924 and 1925 and are not signed. New isolates were deposited by de Graaf in 1938 and Ijlstra in 1948. When a request was made by R. Spencer for the Beijerinck strain in 1954, he was told that it was no longer available, and he was given a strain deposited by van Zutphen in 1953 (Spencer, 1955; Reichelt *et al.*, 1976), but with the comment from A.J. Kluyver that he could not be sure that this was the same strain as Beijerinck's. This is the *P. splendidum* strain that was transferred by Spencer (1955) to the genus *Vibrio* as the neotype strain of the new species, *V. splendidus*.

In 1979, one of the sealed *P. splendidum* tubes (dated 19 March 1925) was opened and revived by the Curator of the Collection. The resulting culture was compared with others in the Collection including the de Graaf (now NCCB 38017), Ylstra (now NCCB 48036) and van Zutphen (now NCCB 53037) isolates mentioned above. Using physiological tests on these and other bioluminescent cultures, it was concluded that strain NCCB 53037, the neotype strain of *V. splendidus*, was significantly different from the other strains (J. van der Toorn, unpublished data).

Conclusion

The end of the 19th century is sometimes described as the 'Golden Age of Microbiology'. However, microbiological research at that time required a great deal of ingenuity to devise ways of detecting or measuring biological reactions. Microbial taxonomy was in its infancy and analytical methods were primitive or nonexistent. Thus, Beijerinck's use of different microorganisms as detectors was brilliant. Storage methods were also primitive and it is no surprise that many of Beijerinck's isolates have been lost or reclassified. However, the fact that it has been possible to grow *P. splendidum* cultures after decades under widely varying storage conditions suggests that perhaps we should reconsider current attitudes and try to cultivate other apparently moribund cultures. An unexpected benefit has been that recreating some of Beijerinck's experiments has proved popular with undergraduate students, who find that it gives them a fresh perspective on microbiology.

Something else that was brought home to the authors by this survey of Beijerinck's work is the enormous impact that a single discovery can sometimes have in the world of research. Beijerinck and others spent a great deal of time trying to find ways to understand development, heredity and biological change. However, as with his TMV research, the technology was not available to allow Beijerinck to take his work on heredity to its proper conclusion.

One puzzle remains. Martinus Beijerinck was a master of extremely careful observation. Why then did he say that light of different colours ('from red to blue') was produced by different bacteria from the North Sea (Beijerinck, 1900c)? Modern bacterial bioluminescence studies generally involve species that produce similar blue to blue-green light. However, Ruby & Nealson (1977) reported the production of yellowish light by a strain of *P. fischeri* in the lower part of its growth temperature range. At higher temperatures, the light produced was much bluer. Strains producing this yellow-shifted colour of luminescence have since been found; the yellow-shifted strains are now classified as Aliivibrio 'sifiae' (Ast *et al.*, 2009). Thus far, modern attempts have failed to isolate different-coloured light-producing bacteria from the same area of beach and sea at Scheveningen sampled by Beijerinck. This is possibly because the Hague's wastewater is now treated before discharge, thereby considerably reducing the nutrients available to free-living bacteria and altering the composition of the microbial community in the seawater.

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Statement

All laboratory journals, letters and other unpublished material are in the Archives of the Delft School of Microbiology at the Department of Biotechnology at Delft University of Technology (http://www.beijerinck.bt.tudelft.nl). Translations, where they occur, by Lesley Robertson.

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[Since the publications of M.W. Beijerinck are most easily found in the volumes of his Collected Works (which can be downloaded as pdf files from the Internet Archive http://www.archive.org), their volume and page number in that series have been given in parentheses after the reference to his original publication. Papers were frequently published in more than one language, so if the one selected for the Collected Works is unknown to a reader, an extended search may be rewarding. Chester's Manual is also available from this site.]

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